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Storage Stability and Improvement of

Intermediate Moisture Foods

CONTRACT NAS 9-12560
PHASE IV

Final Report
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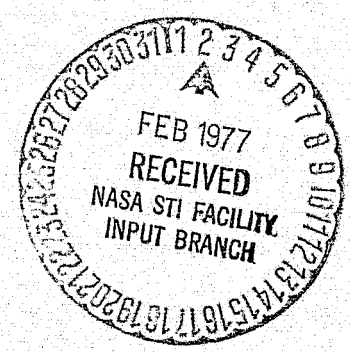
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ABSTRACT

In this contract work was continued on the testing and improvement of shelf life of intermediate moisture foods. Nonenzymatic browning is a major deteriorative reaction during storage leading to loss of nutritional value. Shelf life tests must be used to estimate the rate of browning however, controlling the reducing sugar levels below 23:1 molar ratio to amines, slows the rate. In addition, liquid glycols surpress browning. The protozoan Tetrahymena pyriformis W can be used to estimate nutrition losses during browning. At high temperature (80-120°C) as is used in processing IMF vitamin C destruction shifts to a zero order mechanism. To protect against rancidity BHA and BHT are the most effective antioxidants. In shelf life testing however, 45°C should be the maximum temperature used.

Work was also continued on water binding agents. The five isotherms of thirteen humectants were determined. These are useful in IMF formulation. The results show that neither the method of addition nor sequence of addition affects the a_w lowering ability of these humectants. Further work is being carried out on macromolecular water binding agents.

All these results were used to formulate shelf stable IMF processed cheese foods with at least four months shelf life.

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SUMMARY AND RECOMMENDATIONS

In Phase IV of this contract work was continued in the various areas of chemical and microbial degradation of intermediate moisture foods (IMF). In addition further work was done on water activity (a_w) measurement and control. Specifically it was found that:

1. With respect to nonenzymatic browning –

   a. A protozoan, Tetrahymena pyriformis W (TPW) can be used in a rapid biological assay of protein nutritional loss that occurs in IMF during storage.

   b. The TPW assay shows less degradation than does the chemical DNFB method.

   c. Liquid humectants such as glycerol act as browning inhibitors by shifting the a_w maximum to a lower value thus preserving IMF.

   d. The reducing sugar to available amine ratio should be kept below a 3:1 molar ratio in IMF formulation.

   e. No "a priori" predictions can be made of browning rate based on lysine content of proteins used in IMF formulations.

2. With respect to vitamin C loss at high temperature –

   a. The degradation kinetics change to zero order from first order.

b. The pattern of increased rate of degradation as a_w increases which is found in storage of IMF, is followed. Thus a_w should be kept as low as possible.

3. With respect to antioxidants –

a. The standard accelerated shelf life tests used for fats and oils are not applicable to testing antioxidant effectiveness in IMF.

b. Peroxide determinations are a good indication of oxygen uptake and can be used to get good kinetic data.

c. A plot of log (shelf life) or log (induction time) vs temperature can be used to predict shelf life but may not be a straight line due to changes in deterioration mechanisms.

d. BHA and BHT are far superior to any primary antioxidants for protecting IMF.

e. The chelating agents are poor antioxidants.

f. T\HQ is not a good antioxidant.

4. With respect to a_w determination and predictions –

a. The VPM is the best analytical tool for research purposes.

b. The Abbeon a_w analyzer may serve as a useful quality control tool.

c. The isotherms of humectants published in the literature have been inadequate. New data is presented which will be useful to processors.

d. Neither the sequence or method of addition (dry or solution) affects the a_w lowering ability of humectants for IMF.

5. With respect to IMF processed cheese foods –

a. The results of Phase III can be used to make a stable processed cheese food with at least a four month shelf life.

b. These cheeses are stable to challenges at high levels of inoculums of three molds and two pathogens below an a_w of 0.9.

c. Propylene glycol and K-sorbate are effective anti-microbial agents.

d. The cheeses at lower a_w 0.86 to 0.8 deteriorate more rapidly than at higher a_w with respect to texture, flavor, color and meltability.

6. With respect to water binding –

a. Gelatin gels show unusual behavior at 8-10% solids, indicating a structural change. The same shift occurs at a lower concentration for agar.

b. A method has been developed to estimate the water holding capacity of gels but needs further testing.

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I. INTRODUCTION

A. Study Objectives

The purpose of NASA Contract NAS 9-12560 is to study the mech-
anisms of deterioration of intermediate moisture foods and to improve
their stability through a knowledge of the physical-chemical aspects
of water in relationship to the microbial and chemical deterioration
reactions.

Under the first three phases of this contract methods were developed
and tests were conducted concerning:

1. The effects of glycols on the rates of non-enzymatic browning
   in model systems.

2. The effects of accelerated shelf life tests at up to 45°C on the
   degradation of vitamin C in model systems and in IMF formu-
   lations.

3. The effects of microbial inhibitors and humectants on the
   growth and death of molds and Staphylococcus aureus in IMF
   systems.

4. The effects of water activity (a_w) on the kinetics of death
   of vegetative pathogens in the intermediate moisture a_w
   range.

5. The effectiveness of antioxidants in dry systems humidified
   to the IMF range.

6. The measurement and reliability of water activity measurement.

7. The prediction of a_w lowering ability of various food humectants.

The major objectives of Phase IV of this contract was to expand
on these studies concentrating further on the possible microbial, chem-
ical, and nutritional losses that might occur during accelerated
temperature storage or processing of emergency ration sky-lab IMF
prototype foods and on methods to evaluate the physical chemical
aspects of the binding of water in these products. The specific
studies of this project in this final report involved:

1. Nonenzymatic Browning.

    a. Physical effects of water on browning - a review.

    b. Nutritional loss of lysine during browning of IMF
        model systems.

    c. Effect of glycerol on nonenzymatic browning in an IMF
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2. Effect of high temperature and a_w on kinetics of vitamin C
    loss in the IMF region.

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    for IMF.

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        studies.

4. Evaluation of water activity determination and prediction
    techniques.

    a. Detailed procedure of VPM.

    b. Statistical comparison of several methods of a_w
        determination.

    c. Sorption isotherm data for humectants.

    d. Prediction of and effect of, method of addition of
        humectants on a_w lowering.

5. Shelf life study of intermediate moisture processed cheeses.

6. Prediction of microbial death during drying of a macaroni
    product.

7. Evaluation of water binding properties of macromolecules.

B. <u>Literature Survey</u>

The previous contracts have reviewed the growth of the inter-
mediate moisture food industry and some of the basic aspects of the
microbiology and chemistry of foods with respect to water activity.
The principal investigator has participated in many symposiums related
to a_w and IMF systems. Of note, was the 4th International Congress
held in Spain and the International Congress on "Water Relations of
Foods" in Scotland in 1974. Both the proceedings of these conferences
have been published. The chapters written by the principal investigator
succintly summarize the basic principles of water activity and its
relationship to food stability. The second paper appeared in
<u>Water Relations of Foods</u>, R. Duckworth Editor, Academic Press, NY.

Intermediate moisture foods: chemical and nutrient stability

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ABSTRACT

Intermediate moisture foods (IMF) have become of major economic significance in the pet food industry. They are based on the addition of water-binding agents including sugars and cereal to meat to lower the water activity (a_w) to levels below which pathogens do not grow. The foods are eaten directly without rehydration. Difficulties exist in converting this technology to humans because of chemical and nutrient stability problems. This paper is a review of several studies on the stability of intermediate moisture foods and model systems. It was found that many humectants used in IMF have antimycotic properties and are useful in maintaining a high moisture level. Vitamin C destruction is very rapid and increases as a_w increases due to a drop in aqueous phase viscosity. Rancidity is very rapid; chelating agents and low oxygen packaging are necessary to control it. Non-enzymatic browning leads to loss of lysine and toughening. Processors should avoid high temperatures and use of reducing sugars in their formulation.

INTRODUCTION

Intermediate moisture foods are those foods in which the water content has either been lowered or a water binding agent such as sugar or salt has been added. The amount of water present controls the stability of these foods. The degree to which water controls chemical reactions is based on the availability of the water as measured by its chemical potential. A measure of the chemical potential is the vapor pressure of water in the food relative to that of pure water. This measure is termed a_w or water activity. Figure 1 shows the a_w of a food vs. its moisture content.

The curve in Figure 1 can be divided into three regions; the upper region with an $a_w = 1.0$ which corresponds to all fresh tissue foods such as meat, fish and vegetables; the lower region with an $a_w < 0.5$ which corresponds to dehydrated foods; and the middle region which is the area which intermediate moisture foods come under. IMF are defined as foods with an a_w of between 0.6 to 0.9, moisture content of 15 to 40 g H_2O/100 g solids, they do not require refrigeration and they are stable to microbial growth at room temperature. The fact that the a_w is lowered enough to prevent the growth of pathogens gives them the shelf-stability desired. Troller (1973) has shown that the a_w must be lowered to below 0.86 to prevent the growth of *Staphylococcus sp.*, the most resistant pathogen. Molds can, however, still grow but antimycotics are usually added to these foods.

The IM foods can be eaten directly without rehydration because of the higher moisture content. The foods are also supposedly shelf-stable to chemical deterioration, however, as shown by Rockland (1968), reviewed by Labuza (1970) and shown in Figure 2, many chemical reactions occur in this higher moisture region which can cause rapid deterioration and loss of acceptability.

Labuza (1975a) has recently reviewed the effect of water on chemical reactions in this region. Below the B.E.T. monolayer moisture content (a_w of about 0.2 to 0.3), chemical reactions which usually depend on a liquid phase do not occur. Above the BET, the rates of all chemical reactions increase rapidly as illustrated schematically in Figure 3. Three cases can occur as discussed by Labuza (1975a) depending on the effects of water which include:

1. Water dissolves solids as a_w increases above their solubility point.
2. As moisture increases, the viscosity of the adsorbed phase decreases linear-

ly until high moisture levels (Lee and Labuza, 1974).

3. As moisture increases, the concentration of dissolved species decreases.

In Case I, the rate increases as the phase viscosity decreases because of increased solute mobility. This occurs until dilution of reactant species predominates thereby reducing the reaction rate. In Case III, the viscosity effect predominates along with increased dissolution of reactants as a_w increases. Case II illustrates the situation where the above factors eventually balance each other out at high a_w. As seen in all the cases above, chemical reactions are rapid in the IMF region. These chemical reactions can include hydrolysis (LaJolla et al., 1970), enzymatic action (Acker, 1969), browning (Sharp, 1962), vitamin degradation (Karel and Nickerson, 1964) and rancidity (Labuza et al., 1972). The vitamin degradation studies were done on various dehydrated systems which were hydrated into the IMF a_w range. All of the above studies indicate that intermediate moisture foods can have severe stability problems. Currently this type of food constitutes 30 % of the dog and cat food industry. Obviously if deterioration occurs, either the animals do not deem it to be objectionable or the industry has taken measures to control deterioration. A study of the composition of IM pet foods does not indicate the latter. It is also possible that these foods move through the food channels from processor to consumer rapidly enough so that deterioration is not significant.

Some foods for human consumption can be classified as intermediate moisture foods. Many confectionaries, jams, jellies and honey are in the intermediate moisture a_w range. They are basically sugar products and deterioration is mainly due to drying out or to mold growth. The United States' food industry has put this technology to use in the manufacture of shelf-stable breakfast pastries composed of a baked dough surrounding a jam. These have an a_w of around 0.80-0.85 and are highly acceptable because of their long shelf life at room temperature. They have antimycotics added to prevent mold growth and are packed in foil to prevent moisture loss. Chemical reactions are negligible because of the composition.

Another example of the use of water binding agents and drying is in the manufacture of semi-moisture meats such as summer sausage, cervelat, cured hams, etc. These meats usually are not as low in a_w as the dog foods and therefore must be refrigerated. However, some sausages are stable at room temperature and usually are high in salt, partially dried and have antimycotics added. The dog and cat foods are made to an a_w of about 0.86 by directly combining sugar and cereals with fresh meat by-products in the proper ratio (Burgess, 1962). The 30 % sugar content is too high for the acceptability of meat products for most humans but is acceptable for animals. Humectants other than sugar must be found if intermediate moisture meat products are to be formulated for human consumption.

Humectants are generally chosen on the basis of price and efficiency in water activity lowering. In recent years the price of sucrose and propylene glycol, the main humectants used, have increased because of the world energy crisis and food shortage. In a study in our laboratory (Sloan, 1974), it was found that propylene glycol had the best a_w lowering per dollar when compared to such other humectants as 1,3-butane diol, glycerol, sorbitol, corn syrup and fructose.

The U.S. food industry has recently made another quantum jump in the development of IMF. They have borrowed the American Indian food "Pemmican" which was the main food eaten during winter months and on hunting expeditions. The new developments are "meal bars" which are breakfast or lunch items in bar form supplying 1/3 of all nutrient needs in a form that is shelf-stable and can be eaten directly. Problems with stability, however, have caused several of these products to be taken off the market or to be reformulated to lower a_w's.

The study presented next is a summary of a research project carried out by Labuza and his co-workers on intermediate moisture foods and model systems. Details of the work are reported in the following works: Labuza (1973), Labuza (1974) and Warmbier (1974).

MATERIALS AND METHODS

For the complete details see Labuza (1973, 1974)

Vitamin C Model System Study

Table 1 contains the composition of the model system used to study the effects of water on ascorbic acid destruction. Systems were prepared both by direct mixing (DM) as well as dry-mix humidification (DH) so that the effect of moisture hysteresis could be determined. Ascorbic acid was measured by the 2,6-dichloroindophenol method (Horwitz, 1965).

Casein-Glucose Model System Study for Non-Enzymatic Brwning

Table 1 contains the composition of the model system used to study the rate of non-enzymatic browning in the intermediate moisture range. Both desorption (DM) and adsorption (IIM) systems were made; however, hysteresis did not occur. Browning pigments were measured by the method of Choi et al., 1949. The loss of glucose was measured by the method of Werner et al. (1970) using a glucose oxidase test kit. Available lysine was measured by the DNFB- method of Booth (1971).

Intermediate Moisture Food System

Table 1 also contains the formula for the Intermediate moisture food "Hennican" developed in our laboratories for use in the NASA Skylab and Shuttle program. It is based on the American Indian trail food pemmican. Hennican is prepared by mixing all the ingredients together in a 300 g Farinograph (C. W. Brabender, N.J.) bowl for 5 min. For the microbiological studies, the sorbate was not added and 10^4 CFU/g of A. niger were mixed into the Hennican ($a_w = 0.86$). The various humectants were then added at the levels specified, mixed in and the samples were held at 86 % RH, 23°C. For the stability tests, ascorbic acid and browning were measured as before. Rancidity was determined by the peroxide method (AOAC, Cd 53:8).

Samples were also measured organoleptically by an eight member panel. The food was stored in air at 85 % RH, in a vacuum sealed foil pouch, or in a special oxygen-free pouch system using a N_2/H_2 gas atmosphere on a paladium catalyst pouch (American Can Co., Maraflex 7F Scavenger Web.).

RESULTS AND DISCUSSION

A difficult problem with intermediate moisture foods is determining their micro-biological safety and formulating a product that will be free from microbial decay. If the a_w is below 0.86-0.85 pathogens don't grow; however, mold can be a problem. This first study was done to determine the effectiveness of various glycols, sugars and anti-mycotics added to Hennican which had been challenged with mold (Acott and Labuza, 1974). Table 2 shows the results for the product at two pH's (citric acid used as the acidulant). As seen, under the stress of a lowered a_w most additives severely inhibited mold growth as compared to the control. Also, only a short term storage study of about 2 months would have been necessary except for the systems containing propion-ate and 1,3-butane diol. The addition of acid creates a further stress which insures stability against the mold challenge. Many of the additives tested are not now used in IMF systems. These results show the effectiveness of the various additives and suggest that processors might look at some of these glycols based on their equivalent costs.

With respect to nutrient destruction, vitamin C is one of the least stable vitamins. It is not a problem in pet foods, since the animals don't require it. In citrus foods the pH stabilizes the vitamin. Our studies were done in a model system at pH 6.0 over a wide range of a_w, and in the Hennican at pH 5.7. The half life of ascorbic acid destruction for various systems is summarized in Table 3. As seen, as low a_w increases, the rate of destruction (inverse of half life) increases rapidly and then levels off at high moisture contents. Lee and Labuza (1974) have shown this to be the Case II type reaction, in which the viscosity decreases as moisture increases allows faster mobility and possibly more dissolution of oxygen. At high a_w, the rate levels out since viscosity change is minimal and dilution does not affect the rate constant of a first order reaction. The results also point out the poor stability of the vitamin. In Hennican, as seen in Figure 4, the stability is greater than in the model system, but still is poor at the high a_w (0.86). Based on this, it is obvious why processors have to try to keep the water content as low as possible in IMF if they are to make nutrient claims. The overrun necessary to achieve the desired level after 3 months exceeds the desired product formulation. What is needed is a suitable edible coating for vitamin C which is non-permeable to water.

Two of the most undesirable reactions that occur in dehydrated foods are lipid oxidation and non-enzymatic browning (NEB). As seen in Figure 2, these reactions are accelerated in the intermediate moisture range, Warmbier (1974) has made a study of NEB in a model system to which a liquid humectant has been added at 20 %. The results of the browning rate are shown in Figure 5 for systems prepared by adsorption (IIM) and desorption (DM). As seen, the rate increases first from the BET monolayer ($a_w \cong 0.25$) to a maximum at a_w 0.43. This is probably due to dissolution of the

glucose and decreasing viscosity. Above a_w 0.43, however, the rate drops almost linearly with the moisture content. A similar pattern was found for glucose disappearance as seen in Figure 6, and for lysine loss as seen in Figure 7. It should be noted that the loss of lysine is very rapid. Warmbier and Labuza (1975) have explained these kinetics based on the overall browning mechanism. The drop in rate as a_w increases can be attributed to both dilution of reactions (Case I reaction) and possible feed-back inhibition by water.

The unusual aspect of this study is that the maximum occurred at a fairly low a_w compared to the normal values found for dehydrated foods rehydrated to higher a_w. Usually the maximum occurs at 0.7 to 0.8. This suggests that the liquid humectant, glycerol, is probably acting as a liquid phase to solubilize the reactants down to low a_w. Thus, although glycerol may act as an antimycotic and could serve to increase the aqueous phase viscosity to reduce mobility, it also acts to increase rates of browning. Thus, it should not be used. If it is used, as high an a_w as possible without microbial growth should be used and the food should be protected from water loss.

The glucose and browning data also show that the reaction is rapidly accelerated with temperature with a Q_{10} of about 4 to 5. This suggests that reducing sugars (which are good humectants) either be avoided in the formulation or that any heat processing be done before the humectants is added. Labuza et al. (1974b) have also suggested this same recommendation based on the higher heat resistance of vegetative cells of pathogens. The high Q_{10} can be put to advantage in accelerated shelf-life testing procedures.

The other major area of concern is rancidity due to oxidation of unsaturated lipids. Labuza and co-workers (Labuza et al., 1972; Chou et al., 1973; Chou and Labuza, 1974 and Labuza and Chou, 1974) have studied this extensibely in cellulose linoleate model systems. They found that oxidation rates were much more rapid in the intermediate moisture range than in the dry state. At low trace metal concentration, the rates leveled out as in a Case II reaction; at high trace metals, the reaction was similar to Case I. The metal chelating agents were very effective antioxidants in the high moisture system, BHA and BHT were about 1/2 to 1/3 as effective.

Labuza (1973) in initial studies with Hennican found very poor stability, probably due to the high levels of unsaturated fats from chicken. At 35°C in air the product was rancid in 10 days; when vacuum packaged in foil the shelf-life increased to 50 days. As this is not enough shelf-life, Labuza (1974a) examined the effect of antioxidants and the use of an oxygen-free atmosphere. The results are shown in Table 4. Using BHA at 200 ppm (fat basis) and 1000 ppm EDTA (solids basis) gave adequate protection at 35°C since very little peroxides are produced. The same is true at 45°C. The panelists detected no rancid flavors or odors during the organoleptic tests.

The use of the special pouch gave even further protection with no peroxides being produced. This study also showed that if one reaction is prevented, another becomes important and limits the shelf-life of the product. In this case, browning occurred as shown in Figure 8. Visual ratings of color showed the product to be unacceptable after 35-40 days at 35°C, 10 days at 45°C and about 6 months at 25°C. What is unusual is the slower browning rate in the oxygen-free atmosphere. This has been attributed to the slower destruction of ascorbic acid which can lead to colored compounds. Other results (Labuza, 1974) show that in Hennican the higher the a_w in the IMF range, the greater the stability as in the case with non-enzymatic browning.

CONCLUSIONS

Intermediate moisture foods are a new food technology based on the partial removal of water or the binding of it by humectants. These foods can be made shelf-stable to molds and pathogenic bacteria, but because of their high water content, they can have poor chemical stability. Results in model systems and formulated intermediate moisture foods show a rapid destruction of vitamin C, development of non-enzymatic browning with a subsequent loss of lysine and rancidity development. Through proper formulation, processing and packaging many of these reactions can be prevented.

ACKNOWLEDGEMENTS

TABLE 1

Systems compositions

Non-Enzymatic Browning Model System

Component	Amount
Glucose.	10.0 g
Glycerol.	20.0
Casein.	30.0
Apiezon B oil.	20.0
Microcrystalline cellulose.	20.0
K-sorbate.	0.3
Water.	Per isotherm

Vitamin C Model System Composition

Component	Amount
Oil.	10 g
Glycerol.	40
Mycrocrystalline cellulose.	50
Ascorbic acid.	300 mg
Water (buffer).	Per isotherm

$$pH = 6.0$$

Intermediate Moisture Food Composition

Component	Amount
Raisins.	30 %
Peanuts (dry roasted).	15
Freeze-dried cooked chicken.	15
Non-fat dry milk.	11
Peanut butter.	4
Honey.	2
Water.	23
K-sorbate.	0.3

$$a_w = 0.86$$

TABLE 2

Effectivennes of microbial inhibitors in Hennican, a_w 0.86

Inhibitor	% w/w	Time for 1st Appearance of A. niger* (wks) pH 5.7	pH 4.2
Potassium sorbate**.	0.15	2	ng (no growth)
	0.30	ng	ng
Calcium propionate**.	0.1	2	ng
	0.2	19	ng
	0.3	ng	ng
Benzoic acid**.	0.2	7	ng
	0.3	ng	ng
Methyl paraben*.	0.03	ng	ng
	0.05	ng	ng
	0.10	ng	ng
Propyl paraben*.	0.01	ng	ng
	0.03	ng	ng
	0.04	ng	ng
Parabens Mc/Pro* (2:1).	0.05	ng	ng
	0.10	ng	ng
Pimaricin*.	0.001	1	4.5
	0.002	ng	ng
	0.005	ng	ng
1,3 Butane diol**.	1.0	1	22
	2.0	ng	ng
	4.0	ng	ng
Propylene glycol*.	1.0	ng	ng
	2.0	ng	ng
	4.0	ng	ng
Mannitol*.	1.0	ng	ng
	2.0	ng	ng
Sorbitol*.	1.0	ng	ng
	2.0	ng	ng
Glycerol*.	1.0	ng	ng
	1.0	ng	ng
Control**.		1	4.5

* 6 months storage; ** 9 months storage, 22° C.

TABLE 3

Half-life in days for ascorbic acid destruction in model systems

a_w	23° C DM	23° C DH	35° C DM	35° C DH	40° C DM	40° C DH	45° C DM	45° C DH
0.32.	36.5	49.5	9.0	11.1	5.9	6.9	—	—
	38.9	55.4	11.2	13.3	—	—	3.5	4.2
0.51.	18.2	21.3	5.6	5.6	2.8	3.8	—	—
	22.4	25.2	5.8	6.2	—	—	1.5	1.7
0.67.	7.4	9.8	2.1	3.4	1.2	2.0	—	—
	6.9	11.2	2.9	3.6	—	—	0.9	1.2
0.75.	2.8	4.8	0.8	1.4	0.5	1.0	—	—
	2.8	4.0	0.8	1.3	—	—	0.2	0.6
0.84.	0.9	1.8	0.3	0.6	0.18	0.3	—	—
	1.1	1.9	0.3	0.5	—	—	0.1	0.2
0.88.	0.56	1.2	0.22	0.38	—	—	0.08	0.17
0.93.	0.39	0.83	0.16	0.20	—	—	0.05	0.08

DM = direct mix - desorption.
DH = dry mix and humidifies - adsorption.

TABLE 4

Hennican shelf-life study peroxide value
(meq/Kg fat)

Day	25C Air	25C N_2/H_2	35C Air	35C N_2/H_2	45C Air	45C N_2/H_2
0	N*	N	N	N	N	N
2	0.39	N				
5	0.88		2.08	N	3.12	
10	4.06	N			3.56	
15			3.80	N	3.72	
20	2.62	N	2.27	N	2.48	
25	4.34	N			5.05	
30	4.52	N	2.93	N	4.38	N
35	3.54	N				
40	3.09	N				
45	3.00	N	2.40	N		
50	4.03	N	3.28	N		
60	4.03	N				
65	4.24	N	4.09	N		
70			1.67	N		
75	3.03					
80	3.79		3.31			
85			3.93			
90	3.23					

* N = no detectable peroxides.

Fig. 1.—General sorption isotherm of foods show ; equilibrium % RH (a_w · 100) vs. moisture content.
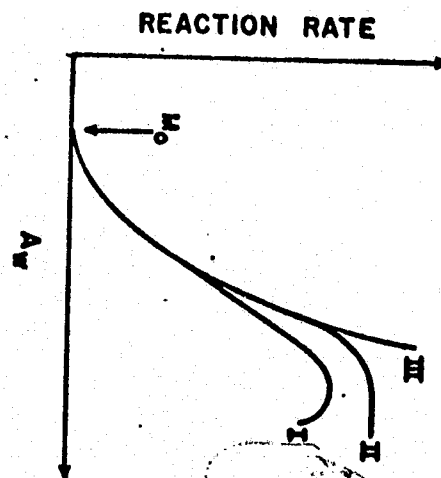
Fig. 3.—Reaction rates of degradation reactions depending on water content as a function of a_w showing (I) dilution, (III) viscosity and (IV) combination effects.
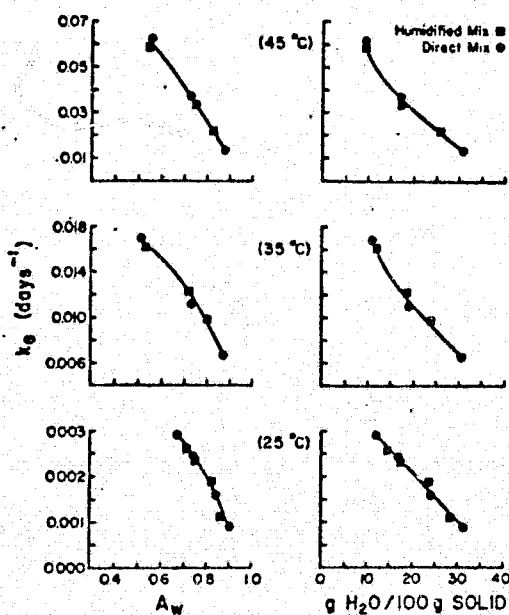
Fig. 6.—First order rate constant for glucose destruction as a function of a_w, moisture content and temperature in a model IMF system.
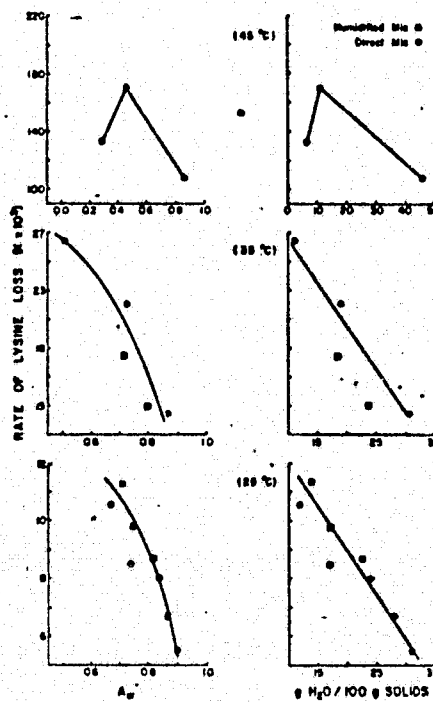
Fig. 7.—First order rate constant for lysine loss as a function of a_w and moisture content in a model IMF system at three temperatures.

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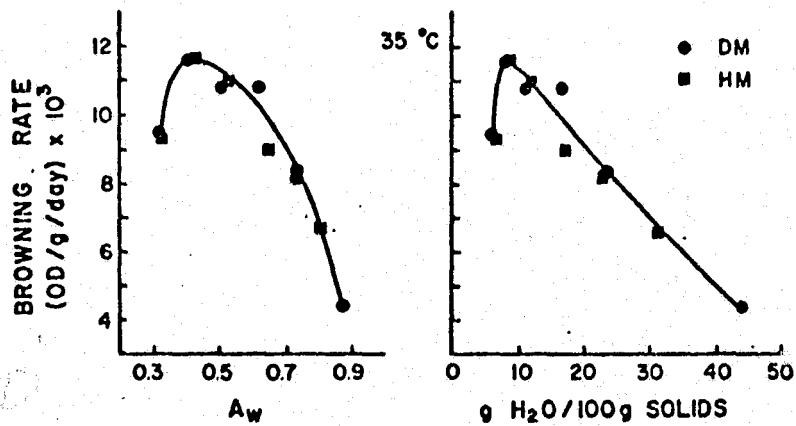
Fig. 5.- Rate of browning as a function of a_w and moisture content at 35°C.
DM = direct mix desorption system; HM = humidified mix adsorption system.
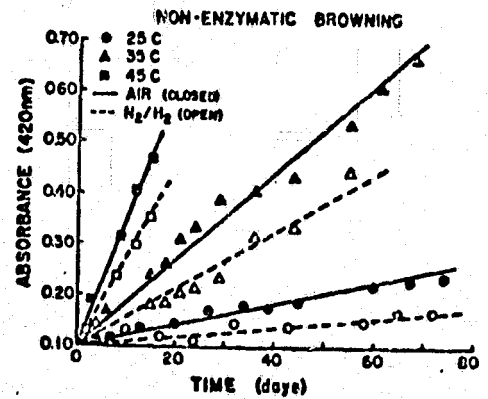
Fig. 8.- Effect of storage temperature and atmosphere on non-enzymatic browning of an intermediate moisture food(----)
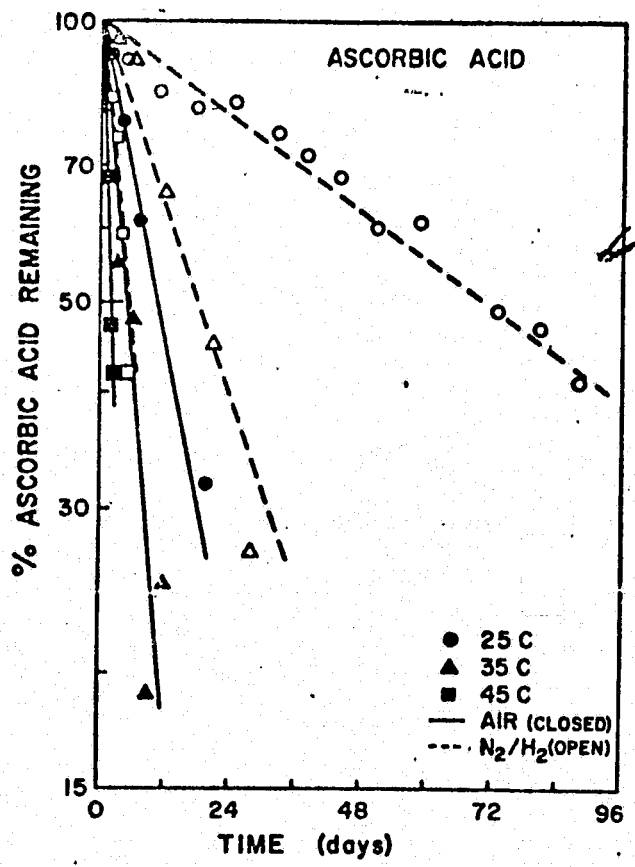
Fig. 4.–Fraction of ascorbic acid remaining as a function of a_w and time for an intermediate moisture food (a_w 0.86 in air and an O_2-free atmosphere).
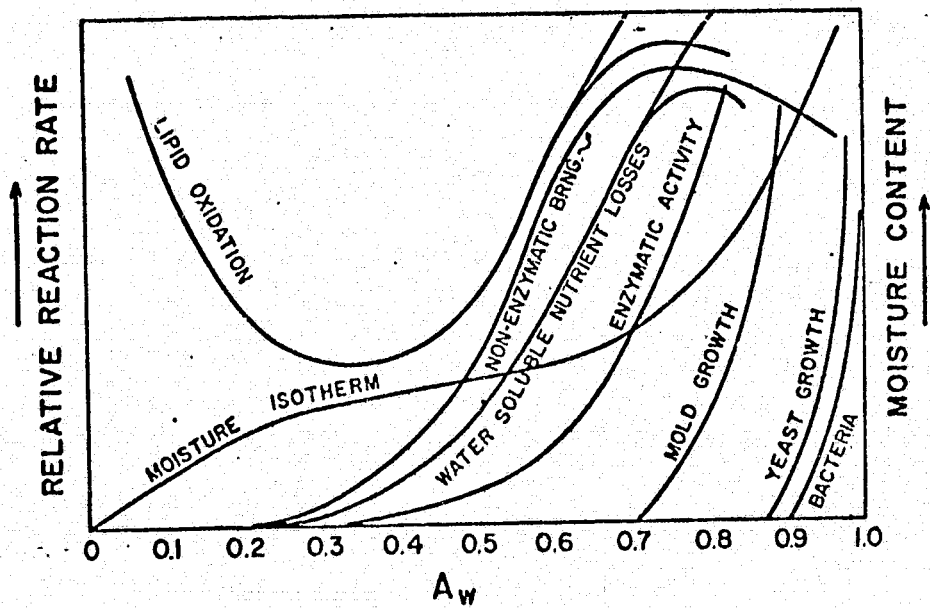
Fig. 2.–Relative rate of degradation of foods as a function of water activity (a_w).

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Interpretation of Sorption Data in Relation to the State of Constituent Water

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I. INTRODUCTION—AN OVERVIEW OF WATER IN FOOD

A. Defining Moisture and Water Activity

Water is a very unusual substance found in nature and is very important from the aspect of its relationship to foods. Not only does it contribute to the texture or structure of a foodstuff, but its interaction with the chemical components present determines the relative storage stability of a food (Labuza, 1971; Rockland, 1969). The degree to which water interacts with components and contributes to food texture is determined by both the amount present as defined by moisture content (g of H_2O/100 g of solids) and its thermodynamic state as defined by the chemical potential in equation (1):

$$\mu_1 = \mu_0 + RT \ln a_1, \tag{1}$$

where μ = chemical potential of water, μ_0 = standard state chemical potential, R = gas constant, T = absolute temperature, a = thermodynamic activity of water.

The latter term (a) or the activity is usually easier to conceptualize and therefore is most commonly used by researchers and processors in the food industry. It can be defined more easily as in equation (2):

$$a_w = a = p/p_0 = \frac{\%\text{ERH}}{100}, \tag{2}$$

where a = activity, a_w = water activity, p = vapor pressure of water in food system, p_0 = vapor pressure of pure water, %ERH = relative humidity at which food neither gains nor loses moisture to the atmosphere.

B. The Water Sorption Isotherm

Dr Gal in the previous chapter has reviewed the methods of determining the properties of moisture content and water activity with respect to the moisture sorption isotherm. Labuza (1974a) has also recently reviewed this area. Fig. 1 shows the moisture isotherm for a general type of food. It can be divided
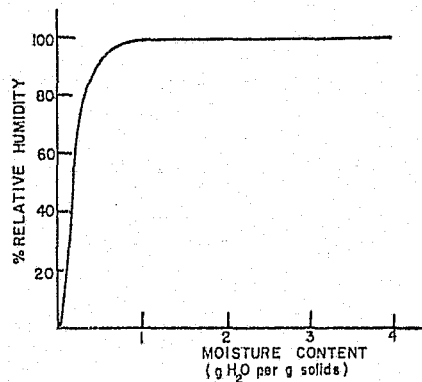
FIG. 1. General isotherm of food—moisure content *vs.* equilibrium relative humidity.

into three areas. The upper part of the curve shows an a_w close to one over a wide range of moisture contents. This area corresponds to fresh tissue foods such as meat, fish, vegetables, fruits, soups, beverages, etc.

The lowest part of the curve, $a_w < 0.5$, corresponds to most dehydrated foods such as milk powder, pasta, cereals, instant coffee, etc. The effect of water on stability and structure is vastly different between these two areas of the isotherm.

The middle portion of the curve is called the intermediate moisture food range. Foods falling in this range are, for example, jams, candies, beef jerky and the newer semi-moist pet foods.

C. Definition of Bound Water

As a starting point, one can consider that the lower the a_w the less available is the water for chemical reaction and deterioration. Many publications have tried to base the degree of boundness by the area or region of the isotherm the water activity falls in. To see this, the isotherm was replotted in Fig. 2 with the axes reversed and the moisture scale expanded. As seen, the isotherm is divided into three regions.

It is considered that in region A water is tightly bound *or* unavailable for reaction. In region C or above, water supposedly exists in capillaries and is relatively free for reaction. Region B then exists as the region in which water

is loosely bound. Unfortunately, these definitions tend to confuse the true nature of the state of water in a food system. This paper will try to point out this state based on the various reactions that have been studied.
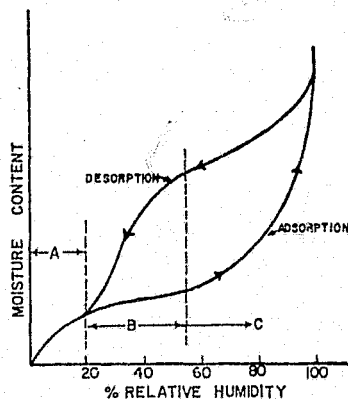
FIG. 2. Type II isotherm showing sorption hysteresis.

D. Hysteresis

It should also be noticed in Fig. 2 that a history effect exists in the state of water. According to equation (2), the thermodynamic state should be constant no matter which way a system is prepared. Wolf *et al.* (1972) among others have shown that an anomaly exists when the food is dried down to a given a_w (desorption) as compared to when it is dried completely and then rehumidified to the same a_w (adsorption). At this a_w, the desorption system will have a greater moisture content. This is called hysteresis. It may not be a true equilibrium condition but with respect to the normal shelf life of foods, it is real and has a significant effect on food stability. We will examine the difference if any, that exists between the state of water in a hysteresis system at the same a_w.

II. PHYSICO–CHEMICAL FACTORS FOR WATER BINDING

A. Solvent Properties

As shown by n.m.r. as well as other spectroscopic techniques (Franks, 1968; Tait and Franks, 1971; Frank, 1970) even in bulk solution some water is bound. Because of the tetrahedral nature of the molecule, it can bond with four other water molecules through hydrogen bonds to build up a polymeric structure. However, the life span of any bond is so short that it is not considered to affect many solution properties. However, water behaves as a high-molecular weight

molecule in terms of vapor pressure or boiling point. This is directly due to the bond structure between the molecules themselves.

The electronic structure of water is also important. Because of the extremely high charge separation as measured by the dielectric constant, it is a good solvent for many species. A combination of these electronic properties and the hydrogen bond structuring has been used as the basis of how water helps to stabilize the structure of macromolecules such as proteins and carbohydrates in solution (Franks, 1968; Berendsen, 1966). In fact, under these conditions, one finds that even though the vapor pressure of water as measured is not significantly reduced, the water is bound to some degree in that it does not readily flow out from between the macromolecules in certain cases. This will be examined for gels later.

The overall properties that must be remembered to examine the influence of water activity on the state of water are:

1. water can dissolve molecules;
2. the molecules can be mobilized in the aqueous–liquid phase;
3. molecules can be concentrated in the water phase until they precipitate out;
4. dissolved molecules can react within the phase;
5. water itself may react;
6. water exists in solution as a polymer and creates and maintains structure.

B. Solute Effects

As molecules are dissolved into a pure water solution, they bind water molecules around them in a hydration sphere. Eventually enough is dissolved to effect the equilibrium evaporation rate of water at the air–liquid interface. If measured, it is found that the vapor pressure is decreased. This can be described by equation (3) which is Raoult's Law:

$$X_{H_2O} = \frac{n_{H_2O}}{n_{H_2O} + n_s} \qquad (3)$$

$$a_w = \gamma X_{H_2O} \qquad (4)$$

where X_{H_2O} = mole fraction of water; n_{H_2O} = moles of water in system; n_s = moles of dissolved species, γ = activity coefficient.

As more solute is dissolved, X_{H_2O} and thus a_w decreases according to equation (4). This interaction must set up some forces to reduce vapor pressure, but may have no effect whatever on reactions taking place in the liquid. It would be expected that for any species dissolved, the decrease in a_w would continue until the solution is saturated and crystallization begins. In fact, this is the basis for the saturated solutions used to create a constant relative humidity. The preparation of this solution can also effect the state of water and create a type of

hysteresis. The lower curve, as seen in Fig. 3, represents the equilibrium mois-
ture content of a crystalline salt (or sugar) as a function of a_w. There is very
little gain in moisture until the solute goes into solution: then water adsorption
becomes large. On the other hand, if water is removed from a solution by
desorption, the upper curve results. What can happen is that the solute solution
will become super-saturated so that it will hold more water at low a_w values
beyond the normal capacity of the surface hydrogen bonds of crystals. This
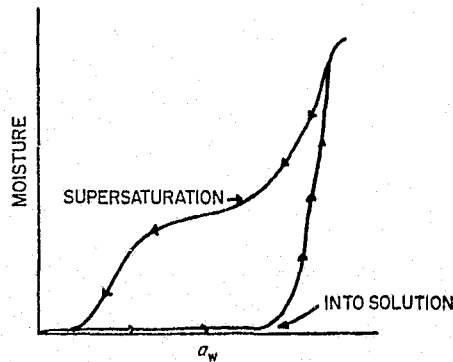
FIG. 3. Sorption hysteresis effect caused by crystalline material in super saturation.

will cease when crystallization finally occurs. This water could be bound
strongly. However, no data really exist as to its state. Wolf *et al.* (1972) have
shown this unusual hysteresis to occur in high carbohydrate foods such as
fruits during desorption–adsorption experiments. Another anomaly in this
area is the problem of water adsorption–desorption of amorphous sugars.
This has been recently reviewed by Karel (1973). Amorphous sugars are in a
metastable state and will hold more water. This water is readily available as a
solvent.

Another factor that is difficult to predict is the interaction of water with the
solute in terms of the degree of non-ideality in a_w lowering. Table 1 summarizes
some values for common solutes. The extra binding for all systems is probably
due to increased ionization of the species and long range effects on water
structure as well as other unknown factors. For example, some polymers
(m.w. 1000–18000) are very effective as cryoprotective agents by lowering the
freezing point beyond the theoretically calculated value. This factor is impor-
tant in frost-hardiness of plants (Levitt, 1966) and protection of cells during
freezing.

C. Structural Effects

In any food system, the three-dimensional structure of the surfaces is also
important in binding water through the lowering of vapor pressure. This is

purely the physical effect of the shape and size of the capillaries in the system. The lowering of a_w is controlled in this case by the Kelvin equation (5):

$$a_w = e^{\frac{-2\gamma \cos \theta v_m}{rRT}} \qquad (5)$$

where γ = surface tension of liquid; θ = contact angle of liquid on capillary wall; R = gas constant; T = °K; r = capillary radius; v_m = molar volume of liquid.

TABLE 1. Molalities of some solutes for various values of a_w at 25°C

a_w	Ideal value	NaCl	CaCl$_2$	Sucrose	Glycerol
0·995	0·281	0·150	0·101	0·272	0·277
0·990	0·566	0·300	0·215	0·534	0·554
0·980	1·13	0·607	0·418	1·03	1·11
0·960	2·31	1·20	0·770	1·92	2·21
0·940	3·54	1·77	1·08	2·72	3·32
0·920	4·83	2·31	1·34	3·48	4·44
0·900	6·17	2·83	1·58	4·11	5·57
0·850	9·80	4·03	2·12	5·98	8·47
0·800	13·9	5·15	2·58	—	11·5
0·750	18·5	—	3·00	—	14·8
0·700	23·8	—	3·40	—	18·3
0·650	30·0	—	3·80	—	22·0

Very little is known of the pore size distribution in foods. Bluestein and Labuza (1972) have shown that most capillaries are of greater than 10 μm size. As water is removed, however, at a_w below 0·5 most of the water is present in capillaries of <100 Å diameter. Using the values of γ and θ for pure water, one finds that significant lowering of the a_w only occurs in these small capillary sizes. At 1000 Å the equilibrium a_w is still 0·99, at 100 Å a_w = 0·91 and at 10 Å a_w = 0·89. It is obvious, however, that this a_w lowering does not occur until water is removed so that a liquid–vapor interface exists and the capillary has enough water to form a meniscus. One might expect a hysteresis effect also to occur from this effect; on desorption the capillary will hold water and decrease its a_w more than would occur on adsorption when not filled. The question that must be asked is how available is the water for reaction at these lowered a_w values.

Other questions that arise are the values of γ and θ in a real food. Labuza and Simon (1969) found that the surface tension is quite low. This would tend to raise the a_w in a filled capillary. No values for the contact angle θ have been found. However, Labuza and Rutman (1968) have shown that if γ is decreased, the amount of hysteresis decreases, bringing the state of water at similar a_w values closer together. This occurs because of the Kelvin equation.

D. Solid Surface Interactions

A strong effect in binding water is the interaction of the water molecule with specific sites on molecules. These may be the weak polar groups available on the surface of a protein or other macromolecule or the strong binding water of hydration of a crystalline solute such as sugar. Table 2 shows the degree of

TABLE 2.   Characteristics of dry polymers

Polymer	Monolayer value m_0 g H_2O/100 g of solids	Q_s surface heat (kcal/mole)	A Mole H_2O bound per mole monomer	B Hydrophilic groups per monomer	Ratio A:B
Agar	9·3	1·83	0·93	2·5	1:2·5
CMC	9·5	1·81	1·46	3*	1:2
Gelatin	7·4	1·68	0·51	0·46†	1:1
Starch	7·5	1·74	0·77	3‡	1:3

* Carboxyl methyl group prevents internal bonding.
† 46% of amino groups have side chains, peptide bonds internally bound.
‡ Usually 2 OH groups internally bound.

binding of water at low a_w for various macromolecules. What is seen is that the degree of hydration varies between the molecules with some of the hydrophilic groups not being hydrated at the monolayer value. This lower degree of hydration is because of internal hydrogen bonding.

The monolayer (m_0) is defined as the value obtained from application of the BET isotherm (Labuza, 1968). At moisture values above this, water condenses essentially as if it were condensing on a water surface. Below the monolayer there is a specific heat of interaction which is large (Q_s in Table 2, ~2 kcal/mol). In fact, values much larger have been found (Hofer and Mohler, 1962; Kapsalis et al., 1968). This high heat of interaction explains the problems of finish drying of foods. The water is bound tightly and extra energy in the form of heat must be applied. This water can be thought of as being in a state in which it is unavailable as a solvent for reactions to occur in. However, it may affect the reactivity of the molecule it is bound to. For reference, the monolayer occurs around the lower inflection of the isotherm and corresponds to region A of the isotherm (a_w 0–0·35).

III. OVERALL STATE OF WATER AS A FUNCTION OF a_w

A. Energy of Bonding

Starting from the dry condition and proceeding up the isotherm, one can examine the state of water. At and below the BET monolayer, water is tightly

bound and is unavailable for reaction. Extra energy must be applied to remove this water. In cases of crystalline salts or carbohydrates, the water can be tightly bound even down to very low values. Wolf *et al.* (1972) found it very difficult to remove this water from high-sugar foods.

Above region A, water now exerts its solution and solvent properties. Duckworth and Smith (1963) found that molecules as large as glucose can be dissolved and mobilized along the surfaces of food macromolecules when just a little more than a monolayer of water was present. This means that many reactions that depend on a liquid–water phase could occur above the monolayer unless the state of water as defined by the lower a_w precludes its participation.

That the water in region B is still bound to some degree to the surface has been shown by Masuzawa and Sterling (1968). It was found that the enthalpy, entropy and thus free energy for removal of water above the monolayer is still larger than that of the heat of vaporization of pure water. The difference was much less, however, than that of water at the monolayer. Thus, it is tied up to some degree in an unavailable state. The water that is available then can dissolve solutes. This should decrease the vapor pressure by Raoult's Law. As capillaries fill, the effect of the Kelvin equation in lowering a_w should also occur. This should take place as the layers of water build up on the monolayer and finally meet at the center of the capillary. It has been a practice to separate the isotherm (Fig. 2) into a multilayer region (B) and a capillary and Raoult's Law region (C). However, it should now be clear that these effects occur over the whole isotherm.

Tait *et al.* (1972) studied the energy of binding of water on starch using n.m.r. techniques over the whole isotherm. As would be expected from the above discussion, the amount of water corresponding to the monolayer was indeed tightly bound so it could not rotate. In addition, the data showed that the monolayer is filled before other layers are built up. Above the monolayer, the water is still rotationally hindered indicating that it is bound to some degree. This condition exists until about region C is entered where the degree of freedom approaches that of bulk water.

Shanbhag *et al.* (1970) and Mousseri *et al.* (1974) have also used n.m.r. to distinguish the degree of binding of water as a function of a_w. They found a change in the n.m.r. responses at high moisture contents (0·2–0·3 g H_2O/g solids) and called this the bound water capacity. Their explanation does not fit in with any of the above factors, but may be a measure of some of the changes in long range forces of water structuring at high a_w. Duckworth (1972) explains this further when he compares n.m.r. and freezing point data. At high a_w (region C) the water is bound to some degree by very weak forces. This water is freezable and results in systems with unfrozen water at levels at about 0·2–0·3 g of H_2O/g of solids using normal freezing ranges. This is remarkably similar to the results of Mousseri *et al.* (1974) quoted above; the only problem

then is in calling it bound water. Duckworth (1972) also found that below the monolayer the water was tightly bound, as did Tait *et al.* (1972).

B. Isotherm Equations

Many researchers have tried to express an equation for the isotherm based on the various states of water. Adamson (1960) and Gregg and Sing (1967) have reviewed the various theories used with respect to the binding of gases. As is concluded, most theoretical isotherm equations have been developed for non-polar gas adsorption on surfaces, not for water. The BET isotherm mentioned before employs the monolayer concept in which the molecule is bound tightly on a homogeneous surface. The equation is quite useful in defining an optimum moisture content for storage stability of foods and is presented here. It may not be totally correct since the food surface interaction is heterogeneous, but the equation is useful.

$$\frac{a}{(1-a)m} = \frac{1}{m_0 c} + \frac{(c-1)a}{m_0 c} \tag{6}$$

where a = water activity; m = moisture content at specific a; m_0 = monolayer value moisture content; c = constant related to energy of bonding.

As seen in equation (6), if the surface energy is very large then the second term drops out and we have the standard Langmuir isotherm (1918) which approaches only a monolayer value. With water this does not occur as more water condenses on the surface of the first monolayer. The effect of solutes present which go into solution would then tend to rapidly increase water holding capacity. The isotherm then becomes a summation of the lower curves for each solute as represented by Fig. 3 added on to the monolayers for the macromolecules.

Other theories developed to explain the shape of the water sorption isotherm do not really apply. Kapsalis *et al.* (1968), for example, have applied the Harkins–Jura equation. This is inapplicable since it only is meaningful for a two-dimensional gas film, which water is certainly not. The use of only the Kelvin equation, assuming only capillary condensation, is also inadequate as is the use of the Henderson equation (Rockland, 1969). Recently Caurie (1970) introduced a new equation based on empirical mathematical manipulation. It should be stated that no equation applies for the whole isotherm because of the varied state of water.

C. Effect of Water State on Reactivity within the Liquid Phase

Given the various above factors which affect the binding state of water to molecules, one should be able to theoretically predict the rate of a reaction as a function of a_w. First it should be clear that at and below the monolayer, water

is not available for reaction. It is tightly bound to polar groups with a significant life time depending on the surface interaction energy. It should not be necessary to remove this water from a food; it would only be necessary to go to the monolayer unless two dry foods are mixed together creating a possibility for exchange. This water, however, may affect the reactivity of a molecule undergoing reaction in a non-aqueous phase. This is the basis for the effect on lipid oxidation as reviewed by Labuza (1971). Trace metal catalysts in the hydrated state are less active as lipid oxidation catalysts.

Above the monolayer, solutes will begin to be dissolved even though they are below the a_w of a saturated solution. For example, Duckworth and Smith (1963) found glucose mobility at a_w values of 0·35, yet a saturated solution of glucose has an a_w of 0·915. Below 0·915 glucose should crystallize out. However, it is possible that a micro-environment exists such that even below crystallization a_w values, water can act to dissolve and mobilize molecules.

Taking a bimolecular reaction (7) as an example, let us examine the effect of increasing the a_w if we assume all the water is available for reaction above a monolayer.

$$\text{Reaction: A + B} \rightarrow \text{Products.} \tag{7}$$
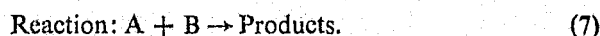
The rate of product formation is:

$$\frac{dP}{d\theta} = k\,(A)\,(B) \tag{8}$$

where k = rate constant.

As more solute A and B are dissolved by the water (if one increases a_w or moisture content) the reaction rate $dP/d\theta$ should increase. This would continue until all available (A) and (B) are in solution and then dilution (concentration of species per cm^3 liquid decreases) would occur reducing the reaction rate.

A second factor of water state should also effect the reactivity. This factor is the mobility of the species within the aqueous adsorbed phase. The properties which would control mobility would be the viscosity of the phase and the ionic interactions of the dissolved species with the surfaces. The latter has not been studied to any degree. The former has been shown to decrease as moisture content increases. Lee and Labuza (1975) used n.m.r. to measure the effect and found a linear decrease in viscosity of the adsorbed phase as moisture increases, as seen in Fig. 4. This factor would tend to cause an increase in the rate of reaction since the rate constant k is inversely related to viscosity as coupled to the mobility of the species.

When coupled with the solution and dilution effects, an increase in a_w could have several different effects as shown in Fig. 5. Case I would be the case where eventual dilution of the bimolecular reaction species causes a decrease in overall reaction rate once high a_w values are reached. Dilution would also
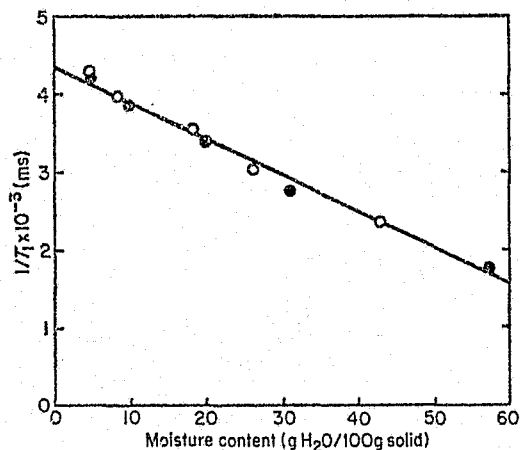
FIG. 4. N.m.r. results showing decrease in phase viscosity as measured by $(T_1)^{-1}$ as a function of moisture content for systems prepared by adsorption (DH, ○) and desorption (DM, ●).

cause some rate decrease, however, with the rate increase that follows the decrease in aqueous phase viscosity, the magnitude of a rate decrease would depend on the balance between the two. Case II is the case of a reaction in which the effects of dilution and viscosity balance out so that at high a_w there is no change in overall reaction rate and it levels out. In Case III, the dilution effect is minimal and the rate increases continuously with an increase in moisture content due to decreased viscosity. A first order reaction can be used to
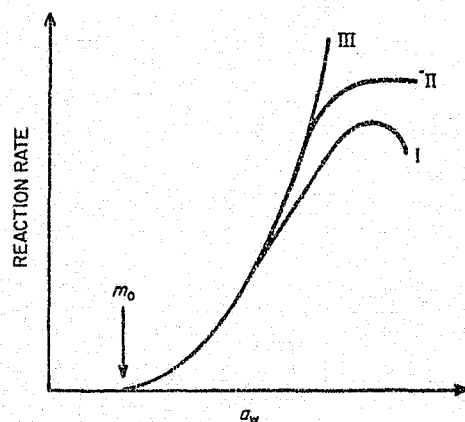
FIG. 5. Effect of increasing a_w on the rate of chemical reactions in the adsorbed water showing three types of effects.

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demonstrate another factor. The rate of product formation for a first order reaction is:

$$\frac{dP}{d\theta} = k(A) = -\frac{dA}{d\theta}. \qquad (9)$$

Upon integration this becomes:

$$\log \frac{A}{A_0} = k\,\theta \qquad (10)$$

If this type of reaction is occurring, any increase in moisture content should not affect the half life if only dilution occurs since the half life is inversely proportional to k. Thus, if k increases with increasing moisture, it is due directly to a viscosity change.

IV. DETERIORATIVE REACTIONS AS CONTROLLED BY WATER STATE

A. Solution Effect

It has been demonstrated by Schoebel *et al.* (1969) that water has the property of solubilization at low a_w. They studied the hydrolysis of sucrose in systems at an a_w of 0·6–0·7, below the a_w of a saturated sugar solution (0·85). It was found that as sucrose reacted, solid sucrose went into solution. Duckworth and Smith (1963) also found solution and mobility of sulfate, glucose and calcium, as well as other ions, just above the monolayer.

B. A Case I Reaction

Although water is bound as defined by a low a_w, as seen, it can dissolve species which means that it also may be available for controlling reactions. Non-enzymatic browning (NEB) is a good example to show these effects. Browning is a reaction between reducing sugars and available amino groups resulting in a brown polymer. Karel and Labuza (1968) showed that sucrose, a non-reducing sugar, could cause NEB if enough acid was present in a low a_w system to cause hydrolysis of the sucrose to glucose and fructose. The rate increased as a_w increased above the monolayer.

Fig. 6 shows the overall effect of an a_w or moisture increase on the browning rate of a dehydrated food. As seen, the rate increases from the monolayer to a maximum and then decreases again, similar to a Case I reaction. This decrease has been attributed to both a dilution effect as well as to product inhibition by water in liquid systems as concluded by Eichner and Karel (1972) since water is a product in the reaction. It can also be seen that at high a_w, the

dilution effect outweighs any increase in rate that reduced viscosity would cause.

More recently, Warmbier (1975) has studied the effect of water in dehydrated model systems over a wide range of a_w values. These systems contained glycerol which could also solubilize glucose and act as a part of the liquid aqueous phase.
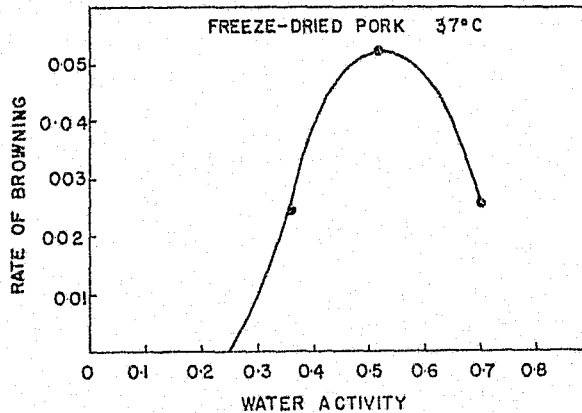
FIG. 6. Rate of browning of dehydrated pork as a function of a_w.

The effect of a_w and moisture on the rate of brown pigment formation is shown in Fig. 7. As seen, the maximum occurs much closer to the monolayer than in dehydrated foods. This is the effect of the extra liquid phase present due to glycerol. After the maximum, the rate decreases in direct relationship to the
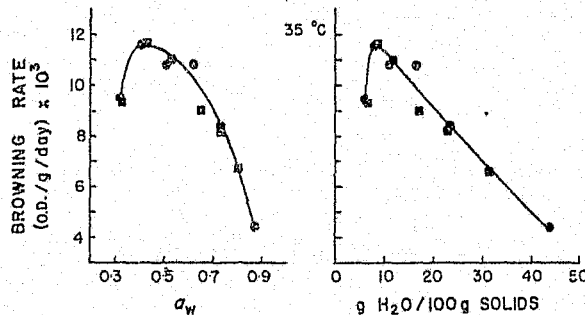
FIG. 7. Browning rate in a glycerol–casein–glucose model system at 35°C as a function of a_w and moisture content. (●) DM desorption system; (■) HM absorption system.

amount of water present. This suggests that dilution is the major factor controlling the rate and that all the water above the monolayer is available to act as a reaction medium. Viscosity is not important. Therefore, the vapor pressure as measured by a_w is not a good measure of the state or availability

with respect to chemical reaction. The rate decreases very rapidly at low a_w where solubility must be limiting, again due to the amount present and not the availability. It is not known whether the rate continues below the mono-layer, however, Acker and Weis (1972) have shown such an effect for enzymatic reactions in a liquid fat at very low a_w.

C. A First Order Reaction—Direct Viscosity Effect

Lee and Labuza (1975) studied the destruction of vitamin C in model systems over a wide range of a_w (0·3 to 0·95). They used two methods of preparation
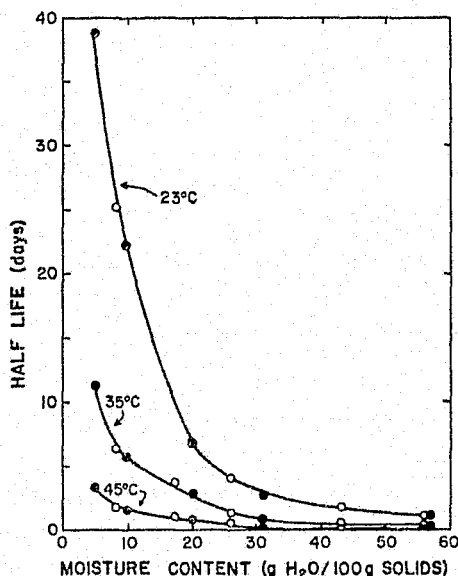
FIG. 8. Effect of increasing moisture content on the half life of ascorbic acid DM = de-sorption system (●); DH = adsorption system (○).

that gave significant hysteresis; a direct mix (DM) or desorption system and a dry-mix humidified (DH) or adsorption system. Representative results are shown in Fig. 8. As seen, at any temperature the DM and DH systems fell on the same curve of decreasing half life (faster reaction rate) with an increase in moisture content. These results further corroborate that the water is available to directly effect a reaction. Vitamin C destruction is first order and according to the kinetics presented before, dilution in this case is balancing out the effect of decreased viscosity (see Case II, Fig. 4) so that above a certain moisture level there is no change in the rate constant. The same vitamin C level was used in all cases so that the same pattern occurs for the reaction rate. This shows how differently water affects a reaction. Thus one must not generalize.

D. Biological Reactions

The previous reactions show that water at an a_w below one, despite its lowered vapor pressure can be treated as a solvent for chemical reaction in the same manner as if it were a bulk solution. With micro-organisms, however, a different situation exists in which the vapor pressure directly affects viability. In general it is found that there is a growth limit below which certain organisms cannot grow. For example, for *Aspergillus niger* (a mould) the lower limit is about a_w 0·76 (Scott, 1957). This is dramatically illustrated in Fig. 9 where growth rate decreases as a_w decreases and then growth ceases. What is not seen is that as a_w is increased to high values, growth rate decreases also due to dilution of the nutrients. This is exactly like a Case I reaction. A hysteresis effect is also shown in Fig. 9. At the same a_w (or vapor pressure) the system with the higher water content (DM or desorption system) has a higher growth rate. One could explain this on the basis of the total volume of liquid present in terms of solutes available in solution but some mechanism must also be occurring that affects the transport across the cell wall. Meryman (personal communication) has stated this to be the same as the case of water structuring agents which are
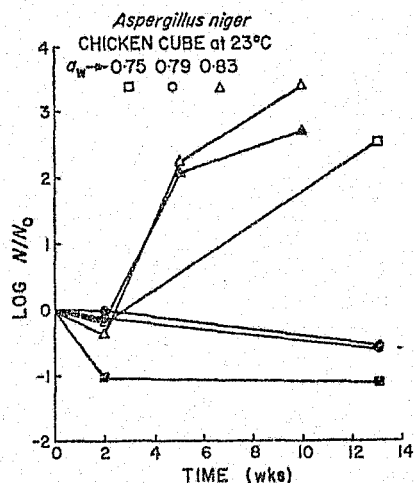
FIG. 9. Viability of *Aspergillus niger* as a function of a_w and sorption hysteresis. DM = desorption system (O, □, △); FDR = adsorption system (⊕, ■, ▲).

cryoprotectants (thereby lowering a_w). It cannot be solely a slowing of enzymatic reactions as these follow the same pattern (at least in those studied) as for the Case III reactions. One would then expect that growth rates would decrease until the monolayer is reached. Of course, the other effect could be a collapse of the cell as it is equilibrated to low a_w, but this is not borne out by recent work (Reeves, 1975; Corry, 1974).

One last effect of a_w on living systems is the effect on death rate. The results are summarized in Fig. 10 based on the work of Murrell and Scott (1966), Hsieh *et al.* (1974) and Labuza (1974*b*). With dry spores, equilibrated to various a_w values, death is at a minimum in the monolayer region. The increase above can be based on chemical reactions, below to a lipid oxidation or free radical mechanism which increases below the monolayer (Labuza, 1971).
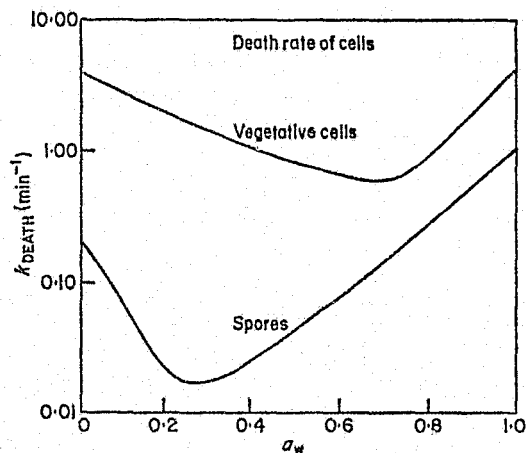
FIG. 10. Death rate of microbial cells as a function of type and a_w.

With vegetative cells, a minimum in the rate of death occurs at high a_w, possibly because of viscosity and concentration effects. This minimum at 0·75 to 0·85 occurs as reaction rates decrease with increasing viscosity. Below the minimum other factors such as cell wall permeability must be changing to increase the death rate.

V. WATER BINDING AT HIGH a_w: FOOD TEXTURES

Water in an agar gel of <0·2% solids or in tissue such as strawberries (95% H_2O) does not readily run out, yet the measured a_w approaches greater than 0·999 (Labuza, 1975). According to the isotherm, water is not bound; yet n.m.r. measurement (Cope, 1969; Hazelwood *et al.*, 1969) suggests a high degree of binding as controlled by long range forces. Hansen (1971) however, disagrees with the interpretation of the n.m.r. data and suggests that the physical barriers present are more important in inhibiting mobility. This is an area which is not clearly understood at all, but deserves much interest especially from the standpoint of controlling food texture.

Labuza (1975) has reviewed this area and suggests that the factors of ionic charges, water binding by added molecules such as salts and sugars and the long range forces of water binding by surfaces all must be accounted for. The

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charges present affect the volume of space available between the surfaces; the other factors structure water enough to act as a solid, yet it has the properties of bulk water vapor pressure. The properties of the surface at low a_w has no relationship to the gel properties.

VI. CONCLUSIONS

One measure of the degree of water binding that has been used is the water sorption isotherm of a food. This isotherm can be divided into a region of a strong association with water molecules to specific sites, called the monolayer region, a region in which the degree of binding decreases as measured by n.m.r. and then a high region in which the water freezes easily and has a vapor pressure equal to that of bulk water.

In examining this water, it is found that even though it is bound, it is available for chemical and biological reactions. Water below the monolayer does not act as normal solvent water. Above the monolayer solutes can be dissolved and react within it. The rate of reaction increases as the amount dissolved increases (i.e. with increasing a_w). The rate also increases with a_w since the mobility of the dissolved species can increase. This is due to reduced viscosity. At high water contents, however, the rates can decrease due to dilution.

At very high a_w, water seems to be physically bound in that it does not leak out of gel systems. This phenomenon is not thoroughly understood and needs further investigation.

Acknowledgements

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II. NONENZYMATIC BROWNING IN IMF FOODS

A. Introduction

The principle investigator participated in a symposium at the 100th anniversary of the American Chemical Society in San Francisco on protein interactions in food. A paper was presented on the effects of water on nonenzymatic browning which is reprinted here to serve as an introduction. It will appear as a book chapter on the symposium which will be published by Marcel Decker.

THE PHYSICAL ASPECTS WITH RESPECT TO WATER AND NON-ENZYMATIC BROWNING

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I. ABSTRACT

Non-enzymatic browning is one of the major problems that occurs during the processing and storage of dehydrated and semi-moist foods. One type of browning is the Maillard reaction between reducing sugars and proteins or free amines. This leads to a darkening of color, protein insolubility with subsequent possible nutrition loss and a bitter off-flavor. The reaction has been extensively researched since the early 1940's. From a storage standpoint, browning rate increases as water activity (a_w) increases up to a maximum where reactant dilution causes a subsequent decrease in rate. Use of liquid humectants in high moisture foods increase phase volume and viscosity, thereby lowering the a_w of the rate maximum. These humectants thus act as inhibitors at high a_w. Sorbitol also decreases the rate by a viscosity effect. Kinetic studies at normal storage conditions show browning to occur by a zero order reaction although the sugar and amine initial reaction occurs by first order. Studies with Tetrahymena show that the initial Schiff's base and reaction products may be partially biologically available although chemical assays show up to 50% loss of protein value. For dehydrated foods the zero order kinetics can be used to predict shelf life under a variable time-temperature-humidity condition.

II. INTRODUCTION

One of the most important reactions that can occur in dehydrated or semi-moist foods is the Maillard non-enzymatic reaction. This results from the reaction of reducing sugars with proteins or

free amine groups and can result in changes in the chemical and physiological properties of proteins and consequently, a change in nutritional value as well.

The influence of the water content on the browning reaction is important to the food industry. Product formulation and processing as well as storage conditions in the case of dehydrated and semi-moist foods, may affect appearance, flavor, and more important, the nutritional quality of the protein. The role of water and the use of various ingredients such as liquid humectants, must be considered in the factors affecting non-enzymatic browning rates and kinetics. An understanding of these factors would be useful in controlling the shelf-life of susceptible products.

III. DEFINITION OF THE STATE OF WATER IN FOODS AND ITS EFFECT ON KINETICS

Water not only contributes to the textural characteristics of a food through its physical state but its interaction with food components is directly related to the chemical reactions that can take place (Labuza, 1971, 1975; and Rockland, 1969).

The term by which the interaction is quantified is called the water activity of the food which is a measure of the relative vapor pressure of water above the food. This is given in equation (1):

$$a_w = P/P_o = \frac{\%RH}{100} \tag{1}$$

a = water activity
P = vapor pressure above food at Temperature T
P_o = vapor pressure of pure water at T
$\%RH$ = equilibrium relative humidity at which the food neither gains nor loses water

Water activity or relative humidity is related to the moisture content of the food through the sorption isotherm as seen in Figure 1. Methods to determine isotherms and measure water activity have been reviewed by Labuza, 1968, 1974, 1975; and Gal, 1975. The lower part of the curve in Figure 1 is the region of dehydrated foods (a_w 0 to 0.5) and the upper part (a_w 0.6 to 0.9) applies to semi-moist products. Most natural tissue foods such as meats, fish, vegetables and fruits have an $a_w \approx 1.0$ and moisture contents greater than 60% water.

To understand the effect of a_w on non-enzymatic browning one must examine the degree of boundness of water. At low a_w water is tightly bound to surface polar sites by chemi-sorption
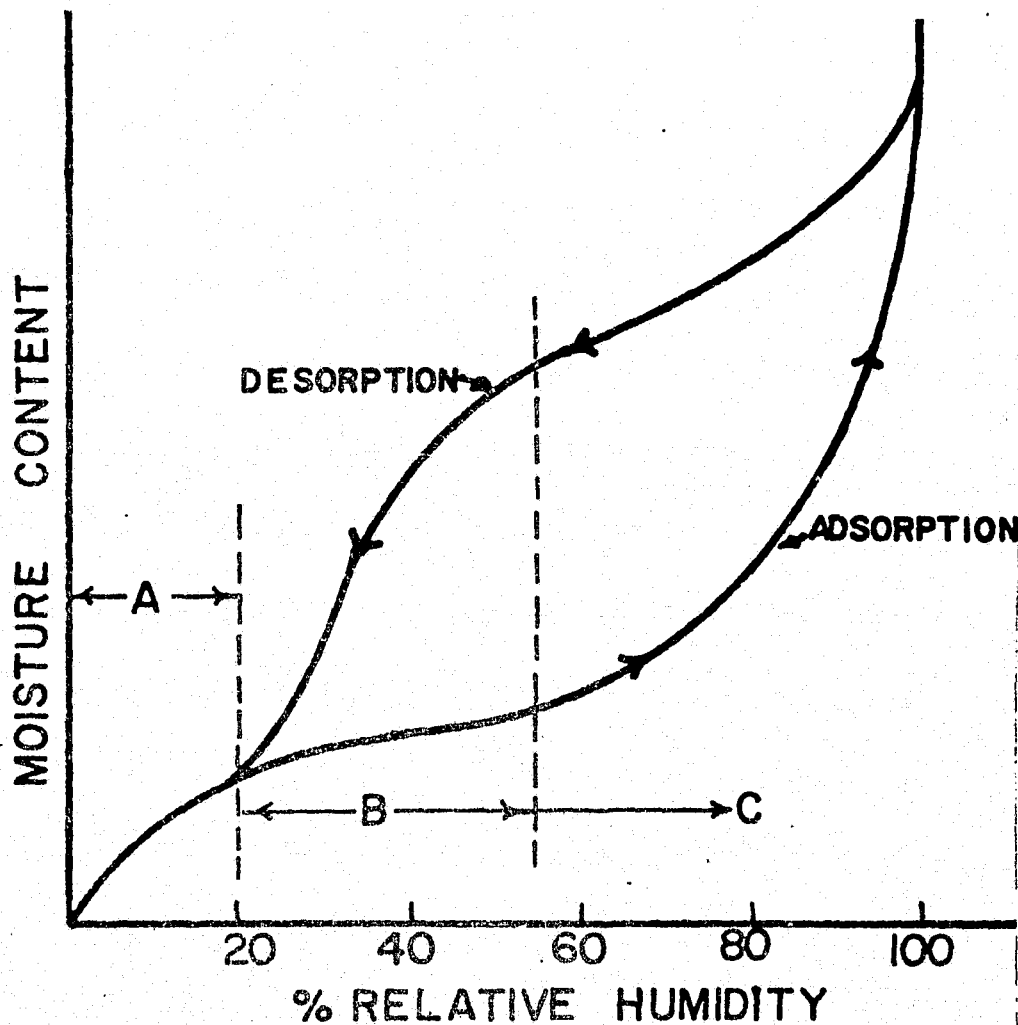
FIGURE 1

Moisture Sorption Isotherm For A Food Product Showing
Sorption Hysteresis
(moisture content vs. % equilibrium relative humidity)

and is generally unavailable for reaction and solution. The upper
limit of this region is called the BET-monolayer value which
occurs at about a_w 0.2 to 0.3 for most foods. This value is the
most stable moisture content for most dehydrated foods (Salwin,
1959).

Above the monolayer the water is held to a varying degree
in multilayers; in capillaries, and possibly entrapped in various
structural components. In addition, dissolved solutes reduce the
freedom of evaporation of water due to colligative properties
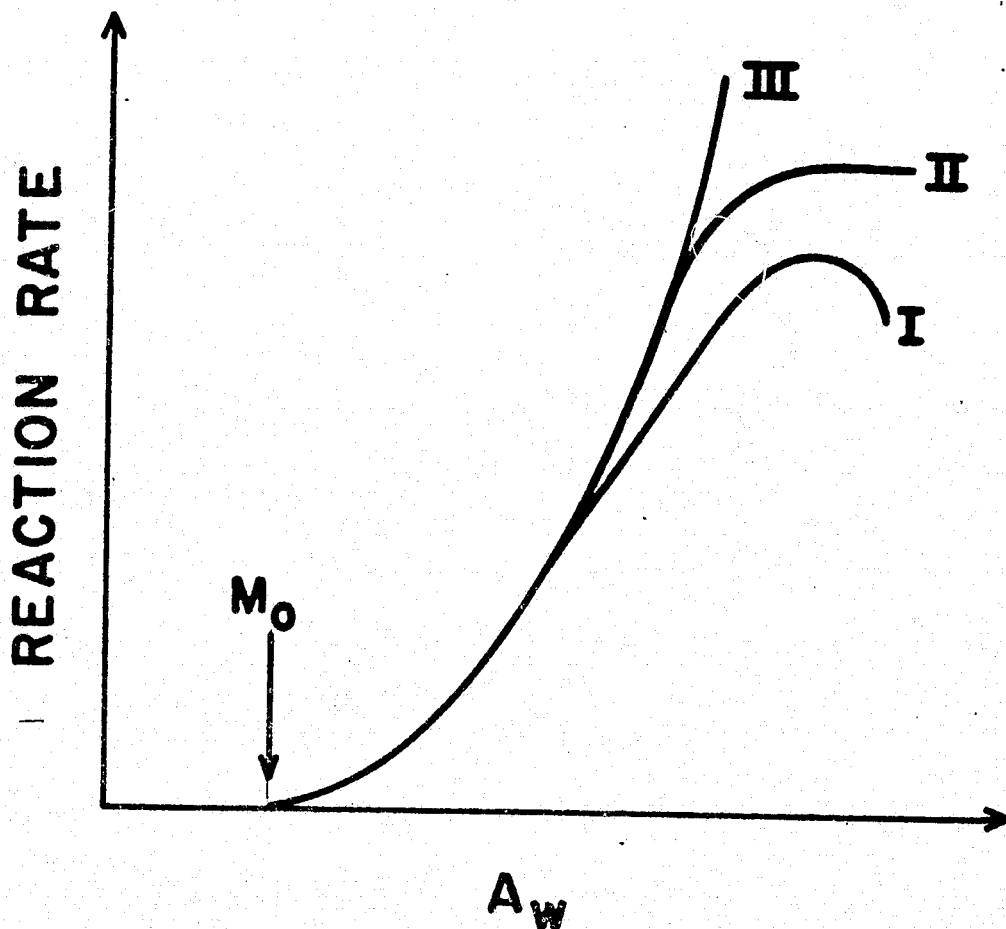
FIGURE 2

Effect of Water Activity on Relative Reaction Rates: I –
Continuous Increase; II – Leveling Off; III – Maxima As In Browning
(M$_0$ is BET monolayer)

as defined by Raoult's Law. All these factors account for a reduc-
tion in the relative vapor pressure of water but do not completely
inhibit its ability to act as a solvent and reaction medium as well
as a reactant itself (Labuza, 1975). Because of this, many deter-
iorative reactions increase exponentially in rate as the a_w
increases above the monolayer. With some reactions however, the
rate may level off at high a_w or even decrease again. This is due
to thermodynamic, dilution and possible viscous effects (Lee and
Labuza, 1975). These effects of a_w on rate are summarized in
Figure 2. Non-enzymatic browning generally follows the pattern
shown by condition I i.e., a rate maximum occurs as a_w increases
and then the rate falls. This is most likely due to three factors.

In browning about three moles of water are produced per mole of carbohydrate used (Eichner and Karel, 1972). Thus water can act through product inhibition by retarding the formation of the initial glycosylamine reaction. However, water may enhance deamination reactions later on in the sequence as shown by Reynolds (1963). In addition, and probably more importantly, dilution of reactive components as water content increases can occur (Eichner, 1975; and Labuza, 1971). Thirdly, the aqueous phase becomes less viscous allowing faster mobility. The first two factors obviously over-compensate for a decreased medium viscosity so the rate decreases after the maximum.

The initial browning reaction which produces brown pigment precursors can be viewed as an overall second order mechanism such as:

$$R + A \rightleftarrows \rightleftarrows RA + H_2O$$

$$\downarrow\uparrow$$
$$\downarrow\uparrow$$

$$\downarrow\uparrow$$
$$\downarrow\uparrow$$
$$A + R*$$
$$\downarrow$$
$$\downarrow \rightarrow \rightarrow \text{ other components}$$

$$\downarrow$$
$$\downarrow$$
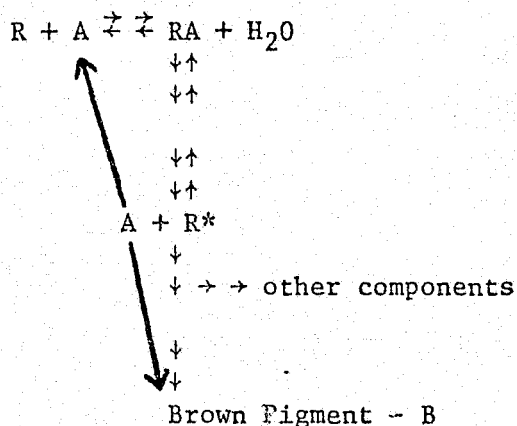
Brown Pigment – B

R = reducing sugar concentration (moles/liter)
A = reactive amine group concentration (moles/liter)
RA = intermediates
R* = reactive reducing compound intermediates
B = brown pigment

The rate of brown pigment formation is related to the formation of R* by:

$$\frac{dR*}{d\Theta} = k_1 (R)^a (A)^b \qquad (2)$$

where: k_1 = overall rate constant, depends on a_w and inversely is proportional to phase viscosity

The exponential factors a and b are usually considered to be equal to one (first order destruction). Thus as a_w increases from the BET value (R) and (A) will initially increase as more reactants can be dissolved above the crystallization point (Sloan and Labuza, 1975).

Once the aqueous phase is saturated and no more reactants can dissolve, concentrations of R and A will decrease continuously as the water content increases. In fact, in the intermediate moisture food (IMF) range for most foods, a change in a_w from 0.7 to 0.9 can mean a doubling to tripling of the water content and thus a concomitant decrease per gram of solids in reactant concentration. If no change in viscosity occurs (i.e., k_1 remains constant), this can mean a four to nine fold decrease in the rate. As noted however, as water content increases the viscosity of the aqueous phase decreases allowing greater reactant mobility. This would increase the value of k since it is inversely proportinal to viscosity. Lee and Labuza, 1974, using NMR T_1 measurements have shown a two fold decrease in phase viscosity going from the BET monolayer to an a_w of 0.9. This should proportionally double the rate. But as previously noted, the concentration effect is greater and the possible local product inhibition of water occurs. Thus a rate maximum occurs with a large drop in the rate in the higher IMF water activity range following the type I reaction pattern. This has been found for many dry foods humidified to different water activities such as seen for dry pork in Figure 3 (Lee and Hannan, 1949; Loncin et al., 1965; Labuza, 1970, 1971; Sharp, 1957; Sharp and Rolfe, 1958; and Hendel et al., 1955).

FREEZE DRIED PORK   37°C
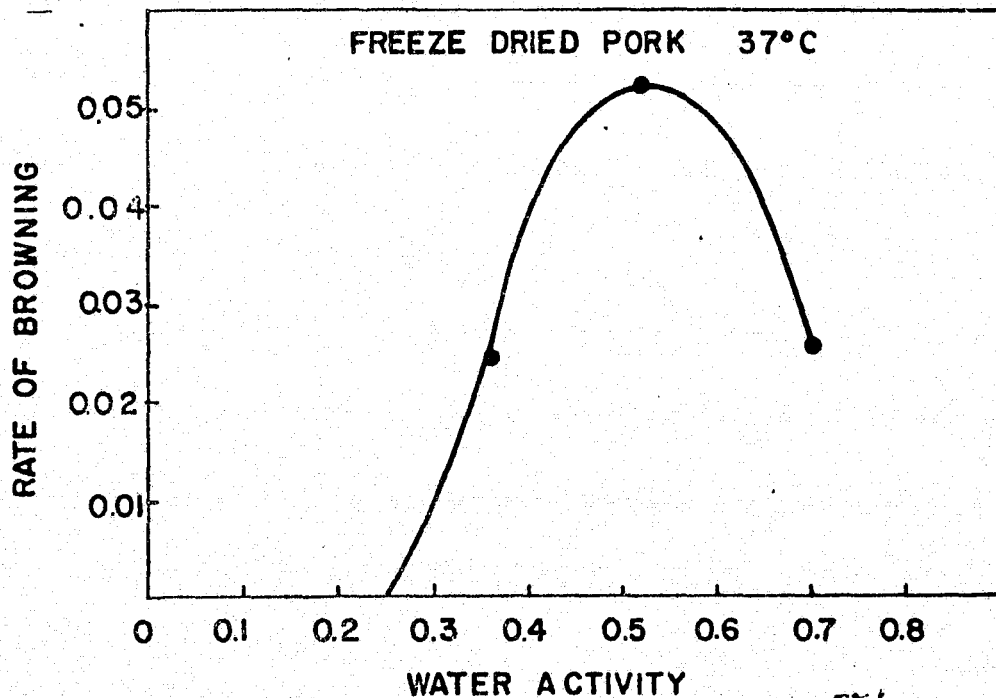
FIGURE 3

Rate of Nonenzymatic Browning as a Function of Water Activity of Pork
(Labuza, 1973)

The browning reaction can occur as an enzymatic process (Eskin, 1971; Greenshields and Macgillivary, 1972), such as that which occurs in fresh cut fruits and vegetables. Tissue damage significantly enhances the rate of enzymatic browning. The injured tissue rapidly darkens upon exposure to air due to the conversion of phenolic compounds to melanins. Polyphenol oxidase, phenolase, o-diphenol oxidase, and tyrosinase are all names given to the enzyme systems that catalyze the hydroxylation and oxidation of phenolic compounds. These compounds subsequently polymerize to form the brown melanoidin pigments which do not involve proteins. Enzymatic browning can be controlled by blanching, pH adjustment, sulfiting or oxygen exclusion.

In general, there are three major pathways for non-enzymatic browning: carmelization, ascorbic acid oxidation, and the Maillard reaction.

Carmelization is that browning reaction in which sugars, in the absence of amino acids or proteins, are heated above their melting point at which monosaccharides are converted into the 1,2-enol form. At high temperature and low pH hydroxymethyl furfural (from hexoses) or furfural (from pentoses) are formed. Under basic conditions, three carbon and/or two carbon fragments are formed. Caramel, a complicated family of pigments, is the end-product of the overall carmelization reaction. Commercially, caramels are prepared by heating concentrated solutions of carbohydrates with ammonia or ammonia salts. Carmelization can be prevented by preventing the exposure of sugars to high temperature.

Ascorbic acid (vitamin C) oxidation is another type of browning reaction. The reaction is catalyzed by low pH and elevated temperatures and converts ascorbic acid to dehydroascorbic acid, which quickly oxidizes to 2,3-diketo-gulonic acid. Ultimately, furfural and carbon dioxide are formed. In total, some 17 decomposition products of ascorbic acid have been identified (Hermann and Andrae, 1963). Polymerization of some or all of the decomposition products produces the typical brown discoloration. Ascorbic acid oxidation is important since some animals, including man, cannot synthesize vitamin C. Prevention of oxidation of ascorbic acid in foods is accomplished by avoiding exposure to high temperatures and, to a limited extent, by excluding oxygen and avoiding neutral pH conditions.

The Maillard reaction is the third type of non-enzymatic browning. It occurs in foods which contain certain reducing sugars, and free amino groups, and/or protein. The Maillard reaction is a major cause of browning developed during the heating or prolonged storage of many dehydrated and intermediate moisture foods resulting in loss of protein biological value.

The Maillard browning reaction was first reported in 1912 by the French chemist Maillard (Maillard, 1912). He observed the formation of brown melanoidin pigments during the heating of a solution of the amino acid glycine and the reducing sugar glucose. Since that time, many researchers have studied the mechanism and control of the Maillard browning reaction. A classic series of experiments was conducted by Lea and his co-workers (Lea and Hannan, 1949, 1950a, 1950b; Lea et al., 1951; Hannan, and Lea, 1952; and Lea and Rhodes, 1952). Subsequently, several in-depth reviews covering the subject have been written (Hodge, 1953, 1967; Lea, 1958; Ellis, 1959; Reynolds, 1963, 1965; and Bender, 1972).

IV. MECHANISM OF THE MAILLARD REACTION

There are three phases to the mechanism of the Maillard browning reaction. Figure 4 represents the overall pathway. Maillard browning as elucidated by Hodge (1953). As one can see from the diagram, the first step involves a condensation reaction between the free amino group and the carboxyl group of a reducing sugar. The amino group can be either a free amino acid or may be attached in a protein such as lysine. The initial product, a Shiff's base, undergoes cyclization to the corresponding. N-substituted glycosylamine. Subsequently acidic conditions catalyze the isomerization of the parent aldose to a ketone. This isomerization reaction is known as the Amadori rearrangement reaction. The end-product of this rearrangement reaction is a 1-amino-1-deoxy-2-ketose derivative. This derivative and its immediate precursors are colorless. Therefore, this initial step of the Maillard browning reaction contributes no brown discoloration as has been verified by Eichner (1975). This first phase of the Maillard reaction is also reversible.

A reducing sugar is required for glycosyl-amine formation. Both mono- and di-saccharide reducing sugars can react, with order of reactivity being aldopentoses greater than aldohexoses greater than disaccharides (Spark, 1969). As seen in Table 1, if a non-reducing disccharide containing a reducing mono-saccharide moiety undergoes hydrolysis, the reducing moiety can participate with the protein in browning reaction (Karel and Labuza, 1968).

Lea et al., (1951) experimentally showed that once the initial glycosyl-amino condensation and Amadori reactions occur, removal of free reducing sugar will not inhibit the development of the brown pigments. Eichner (1975) has shown that in fact, the Amadori rearrangement products will brown at a lower water activity if removed from the system (Figure 5).

MAILLARD BROWNING PATHWAY

| ALDOSE SUGAR | + | AMINO COMPOUND | → ← | N-SUBSTITUTED GLYCOSYLAMINE | + H_2O |

AMADORI REARRANGEMENT

I-AMINO-I-DEOXY-2-KETOSE (1,2-ENOL FORM)

-(1) - 3 H_2O (2) -2H_2O (3)   +α-AMINO ACID   STRECKER DEGRADATION

SCHIFF BASE OF HMF OR FURFURAL

REDUCTONES

FISSION PRODUCTS (ACETOL, PYRUVALDEHYDE DIACETYL, ETC.)

CO_2

-AMINO COMP'D +H_2O -2H | 2H

HMF OR FURFURAL

DEHYRO REDUCTONES

ALDEHYDE

+AMINO COMPOUND   WITH OR WITHOUT AMINO COMP'D   +AMINO COMP'D   +AMINO COMP'D

ALDOLS AND N-FREE POLYMERS

ALDIMINES

+AMINO COMP'   ALDIMINES OR KETIMINES   ALDIMINES

MELANOIDIN S
(BROWN NITROGENOUS POLYMERS)

(1) acid, (2) basic, (3) high temperature conditions

from Hodge, 1953

FIGURE 4

Table 1

Sucrose Hydrolysis and Browning *

Time	a_w	System A ** 0.10		0.31		0.75	
Days	% Hy	OD/g x 100	% Hy	OD/g x 100	% Hy	OD/g x 100	
0	0.9	0.2	0.9	0.2	2.4	0.2
3	1.1	–	4.8	–	11.9	–
7	0.9	0.2	15.9	0.3	21.7	0.7
10	2.9	0.2	14.4	0.4	16.3	0.8
14	2.8	0.2	15.6	1.8	25.8	2.1
17	2.8	0.2	18.1	4.2	40.4	4.8

* Karel and Labuza, 1968
** sucrose, citric acid, egg albumin, water
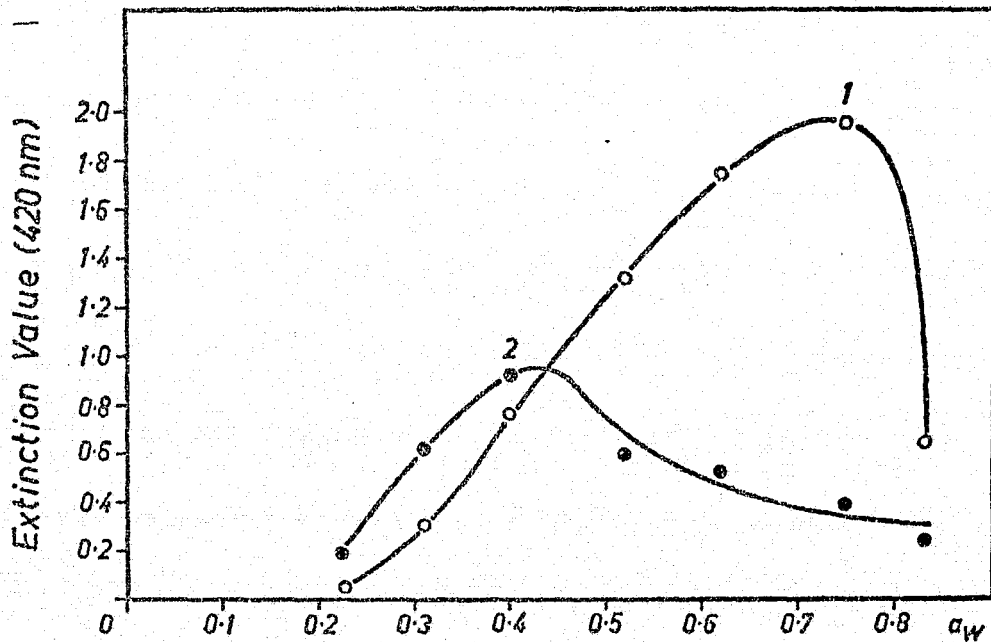
FIGURE 5

Browning rates for a glucose-lysine-cellulose model system (1) as a function of a_w and for the intermediate products extracted from the system humidified to similar a_w's.

Eichner (1975)

Amino groups must be chemically free to participate in the Maillard browning reaction. Primary amines react the most rapidly. Peptide bonded nitrogen and acetylated terminal amino groups do not react. Lea and Rhodes (1952) showed that the rate of the Van Slyke free nitrogen loss, as an indication of initial browning rate, was greater when 2-amino-D-glucose reacted with N-acetylated casein than with regular casein. Color development was approximately the same in both systems. Hence, once the initial glycoslyamine condensation product is formed, excess free amino groups apparently cannot readily enhance the browning rate.

The second or intermediate reaction sequence involves the removal of the amino group(s) from the sugar moiety (except as advocated by Song and Chichester, 1966). This is followed by dehydration and cyclization, fragmentation, or amine condensation. Three general pathways exist for this intermediate reaction sequence. First, under acidic conditions, dehydration and cyclization produces hydroxymethyl furfural (from hexoses) or furfural (from pentoses) with a possible regeneration of the amine. These two products are colorless, but their subsequent oxidation to γ-unsaturated decarboxyl compounds yields yellow colored products (Taher and Cates, 1974). Second, under basic conditions the 2-keto form of the glycosyl parent equilibrates predominantly to the 1,2-enol glycosyl form. Rearrangement to the 2,3-enol form followed by dehydration and oxidation yields reductones and dehydroreductones. The dehydroreductones can combine with α-amino acids yielding carbon dioxide, aldehydes, and amino-keto derivatives. This reaction is commonly referred to as the Strecker degradation (Schonberg and Moubacher, 1952). Third, conditions of elevated temperature can produce fragmentation products from the Amadori product. Three or four carbon aldehydes, alcohols, acids or ketones may be produced. These compounds produce the characteristic flavor and odor associated with heat catalyzed Maillard browning, as, the 2- and 3-methylbutanal and 2-methyl propanal formed in drying of potatoes (Sullivan, et al., 1974).

The third and final phase of the Maillard browning reaction produces the brown melanoidin pigments. Polymerization of the products from the second phase and copolymerization with amino compounds and proteins yields the colored products. The chemistry compounds will react with amines to give polymerized water-soluble brown pigments; furfural compounds will polymerize with amines to give water insoluble brown products (Reynolds, 1965). Heterocyclic amines are thought to be formed to cause the brown color development. These reactions definitely lead to toughening of stored foods as seen in Figure 6 (Labuza, 1973). In this study of an intermediate moisture food product at a_w 0.75 an increase in browning was directly correlated ($R^2 = 0.96$) with an increase
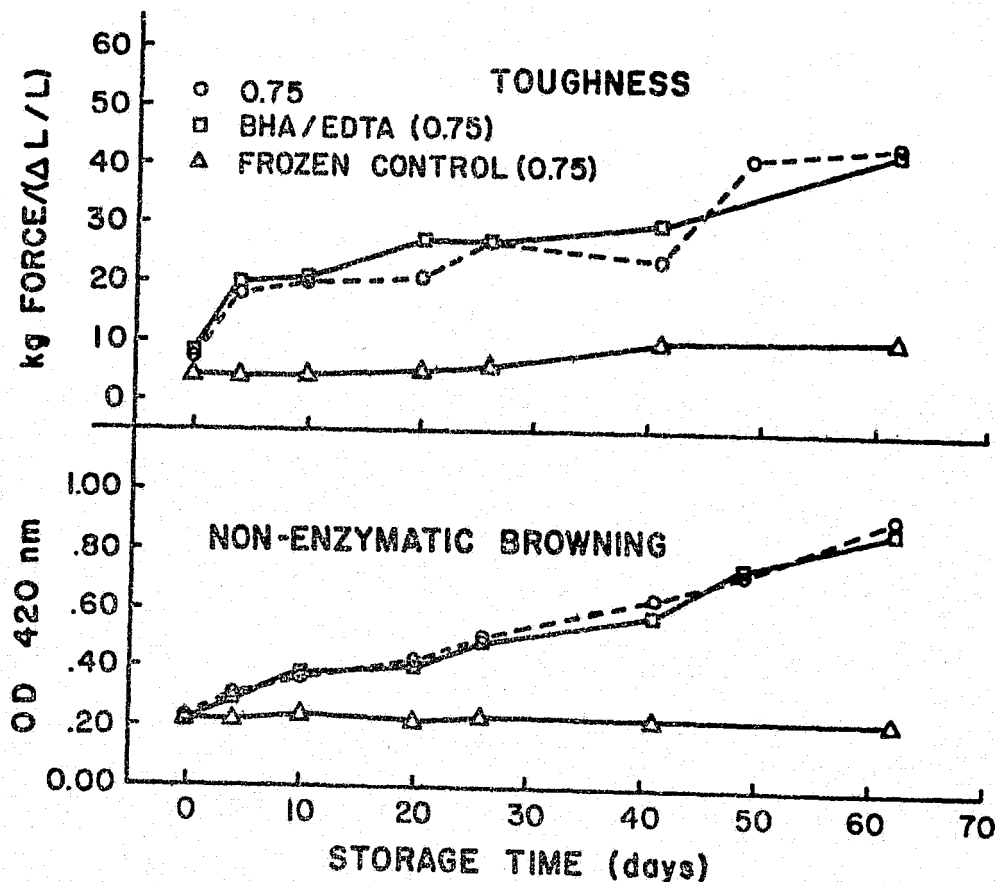
FIGURE 6

Effect of storage at 37°C and a_w 0.75 on browning and toughening of an intermediate moisture food.

in toughness as measured by an Instron. The browning that occurred was not as the result of carbonyl compounds formed from lipid oxidation since the same changes occurred even if protected with antioxidants.

However, in studies with other IMF systems such as chicken die soaked in glycerol to achieve an a_w of 0.7 to 0.8, Labuza (1973) found browning to occur as a result of reactive carbonyls formed during lipid oxidation. In this product no reducing sugars were originally present. As seen in Figure 7, protection of the chicken with either EDTA or BHA reduced the rate of browning. Both act as antioxidants to slow rancidity.

The most obvious indication that Maillard browning has occurred in a food containing both carbohydrate and protein is the accumulation of brown pigments. Choi et al., (1949) and
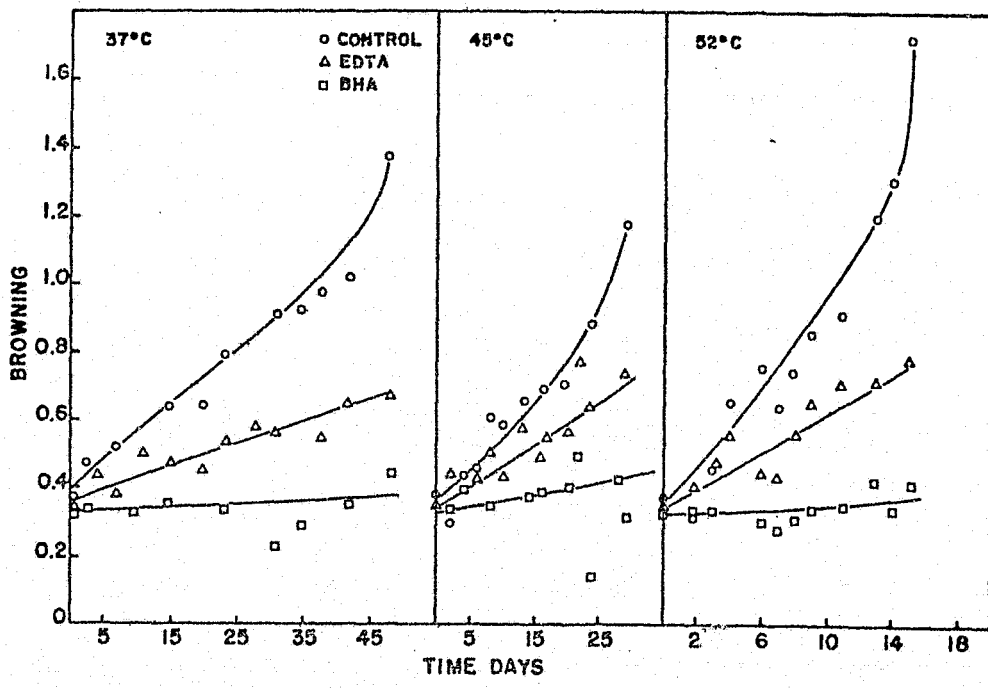
FIGURE 7

Effect of antioxidants and storage at 37°C, a_w 0.8 on browning
in a chicken/glycerol system.

Patton (1955) were the first to recognize the significance of the
brown discoloration in milk products that were subjected to exce-
sive heat during processing or storage. This color change was
particularly noticeable in condensed and dried milk products.
Brown discoloration has, therefore, been used as an indication
of milk products being excessively thermally processed. Aside
from the milk industry, the dry cereal and animal feed industries
well-recognize the undesirable discoloration that can result
in their products.

The semi-moist pet food industry also incurs loss in their
product due to Maillard browning. The dog and cat foods that
are made to an a_w of about 0.85 by directly extruding at 80-140°C
sugars, cereals, and fresh meat by-products (Burgess and Mellentin,
1965). Coloring agents are used to mask the excessive browning
that results from both the initial thermal processing and storage
of these products, but the loss in protein quality cannot be.

The reaction of Maillard browning can be followed by several methods. The first and most obvious method is simple visual observation of the brown color and ranking of the product (McWeeney and Burton, 1963). Lea and Hannan (1949), Tarr (1954) and Arnold (1973) used reflectance readings as an indicator of the degree of browning. Spectrophotometric analysis of enzyme digested extracted filtrates such as the method of Choi et al., (1949) allows for more exact monitoring of the color change as induced specifically by non-enzymatic browning.

The accumulation of hydroxymethyl furfural or furfural has also been used by some investigators as an indicator of the extent of non-enzymatic browning (Reynolds. 1963). However, McWeeney and Burton (1963) concluded that, other than in strongly acidic conditions, HMF is a reaction by-product which accumulates in detectable amounts only because of its relatively low reactivity in browning reactions. Cole (1967) monitored carbon dioxide evolution as an indicator of the extent of Maillard browning. He concluded that although the Strecker degradation may be the main source of carbon dioxide produced during the Maillard reaction, it is not the only pathway by which carbon dioxide may be evolved. Eichner (1975) as well as many others have used carbonyl accumulation as an index. The problem here is that a maximum is reached since the reducing compounds participated in further reactions,

A wide variety of flavor compounds have been used as an index of the Maillard browning reaction. The Strecker degradation reaction is thought to be a source for producing characteristic browning flavors (Reynolds, 1965). Schonberg and Moubacher (1952) have determined that the type of organic group reacting with the amino acids during the Strecker reaction determines what type of end-product will be produced. Reynolds (1965) investigated some of the flavor compounds associated with cooked potatoes, soy sauce, bread and cooked milk and meat and Markova et al., (1972) have studied some of the flavor changes associated with cereal products that were subjected to non-enzymatic browning.

Another very important means of monitoring the non-enzymatic browning reaction is by observing the protein nutritional changes that occur in the food product after processing and storage. This will be discussed in the next section.

V. BIOLOGICAL EFFECTS OF NON-ENZYMATIC BROWNING

Lea and Hannan (1950), Lea (1958), Lea et al., (1958, 1960), Carpenter et al., (1962), Heller et al., (1961), Ben-Gara and Zimmermann (1971), Chichester and Smith (1972), Rao and Rao (1972), and Yanagita et al., (1973) have examined the influence of Maillard

browning on protein nutritional loss. As the free amino-N
groups react, they can become bound to the brown pigments and
cause the nutritional availability of the protein to decrease.
In particular, the N-terminal group of peptides and the ε-amino
groups of lysine react and become nutritionally unavailable.
Folk (1956) and Tu and Eskin (1973) observed a decreased ability
of digestive enzymes, e.g. trypsin, to hydrolyze a lysine contain-
ing system as Maillard browning ensued. As lysine is an essential
amino acid, the availability of lysine is of great nutritional
importance.

Lysine availability is determined by the positioning or
bonding of the ε-amino groups in reference to recognition of
this amino acid by lysine-specific peptidases. Lysine that
is not subject to an otherwise active peptidase is defined as
being unavailable. Lysine may be unavailable naturally within
a food or it may become unavailable during processing and storage
of the food.

Carpenter (with Booth, 1973) reviewed most of the methods
available to determine the loss of lysine availability in foods
subjected to thermal processing and storage. The most common
method employs Sanger's reagent (1-fluoro-2, 4-dinitro-benzene,
FDNB). The methods of Carpenter (1960), Rao et al., (1963a),
Roach et al., (1967), and Booth (1971) all employ the use of
FDNB as the tagging agent for free or available lysine.

In a comparative study between the Carpenter (1960) and the
Roach et al., (1967) methods, Milner and Westgarth (1973) concluded
that, though the two methods give good agreement for available
lysine results, Carpenter's method might be best used for the
analysis of protein foodstuffs whereas cereals and mixed feeds
containing cereals should be analyzed with the method of Roach
et al., (1967). Milner and Westgarth (1973) further concluded
that the method of Roach et al., (1967) is more precise but is
more costly than is Carpenter's (1960) method.

Rao et al., (1963b) claim their method to be superior to
Carpenter's in that better separation of the ε-DNP-lysine from
dinitrophenol and yellow derivatives of the reaction is achieved
through the use of an ion exchange clean-up procedure. However,
use of their ion exchange chromotographic technique does not
really lend itself as a rapid routine procedure for determining
the availability of lysine in foodstuffs.

Kakade and Liener (1969) used the dye 2,3,6-trinitro benzene
sulfonate (TNBS) to tag available lysine. Ousterhout and Wood
(1970) later modified the Kakade and Liener (TNBS) method.
Holsinger et al., (1970) and Hall et al., (1973), however, have

shown the TNBS reaction to give erroneous available lysine results
when glycosylamines and galactosylamines are present. Hurrell
and Carpenter (1974) have also found the TNBS technique to not
fully indicate the extent of the Maillard reaction for measuring
available lysine content in foods subjected to mild (37°C) heat
treatments. This and lesser temperatures would be typical of
warehouse storage conditions for dry foods.

Booth (1971) modified Carpenter's (1960) original FDNB-
lysine method to eliminate arginine interference. Hurrell and
Carpenter (1974) found the FDNB technique to fully reflect the
nutritional damage of food systems in which the Maillard reaction
occurred at mild (37°C) conditions typical of that found in non-
air-conditioned food storage warehouses. Thus, either Booth's
(1971) or Carpenter's (1960) procedure utilizing FDNB as the tag-
ging dye is the current method most commonly used for the routine
analysis of available lysine in foodstuffs.

Warmbier et al., (1976) as seen in Figure 8 followed
FDNB-available lysine as a function of pigment formation in a
casein/glucose model system at 0.5 a_w. As seen, the loss of
lysine occurs much sooner than does pigment formation as would
be expected from the overall reaction, whether this represents
true nutritional loss has not been well established. However,
good correlation has been found between in vitro chemical (e.g.
FDNB) and in vivo nutritional studies for evaluating nutritional
loss in foods subjected to Maillard browning. Mauron and Mottu
(1958) found an excellent correlation (r = 0.99) between in vivo
decrease of protein efficiency and in vitro lysine deterioration
in milk powders.

Milk products are especially susceptible to browning during
drying or heat processing because of the high lactose content and
lysine content in the protein. Henry et al., (1948) determined
that cystine is the first limiting amino acid in milk products
for rat growth. Milk products which had been deteriorated by
browning at 37°C for 60 days gave lower PER's and Biological
Values (BV) than control samples. When lysine was provided in
the rat diets as 1.25% and 2.5% of the protein, growth equivalent
to that on the control diet was observed. Cystine, however, did
not improve the growth response. This emphasized the fact that
the lysine was involved rather than the limiting amino acid,
cystine.

Similar results for lysine supplemented to deteriorated
milk products have been observed by Cook et al., (1951) and Rao
et al., (1963b). Cook et al., (1951) examined PER's of rats fed
casein and lactose which had been treated under mild conditions
(52°C for 24 hr) and under strong conditions (140°C for 3 hr).
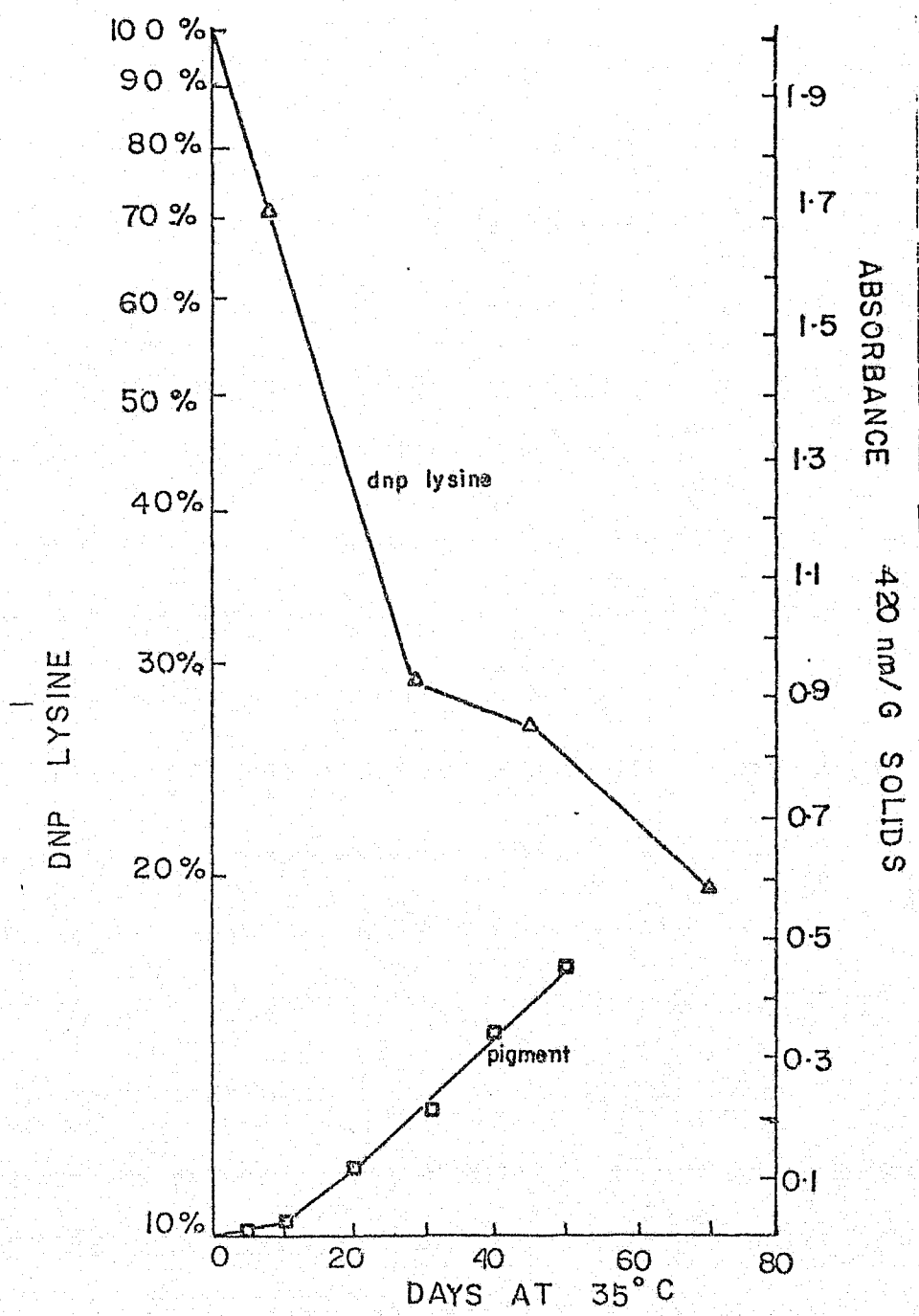
FIGURE 8

Browning pigment production (□) and lysine loss (△) at 35°C in a casein/glucose glycerol model system (a_w 0.5).

Warmbier et al., (1976)

Control samples gave PER's of 2.53, mildly treated samples gave
PER's of 2.29 and the high heat treatment resulted in PER's of
2.18. Lysine supplementation at 0.2% in the similar groups
increased the PER for the mildly treated sample to 2.51 and the
high temperature sample to 2.00. This suggests that amino acids
other than lysine may be involved in severe conditions, whereas
lysine is the main reactant in early non-enzymatic browning.

These studies strongly support the belief that the nutri-
tional loss of protein quality in non-enzymatic browning is due
to a decrease in free lysine groups. Specifically, this has
been shown by in vitro studies to be due to the inability of the
pancreatic enzyme, trypsin, to split the reacted lysine from the
peptide chain (Carpenter and Booth, 1973; Mauron and Mottu, 1958;
Mauron et al., 1955; and Tu and Eskin, 1973). Trypsin activity
in the small intestine of mammals is highly specific for the sub-
strate binding site. The polar groups of the specific amino
acids, lysine and arginine, must be present in the polar forms to
be cleared from the polypeptide linkage. The reacted group
(aldehyde or ketone) attached to the epsilon-amino group prevents
enzymatic cleavage of the lysine-peptide bond linkage. Thus
the effect of decreased enzymatic cleavage lowers protein uti-
-lization by limiting the lysine available for absorption (Finot,
1973; and Goodhart and Shils, 1974).

Hurrell and Carpenter (1974) in fact found very good agree-
ment between in vivo growth studies and in vitro lysine values for
an albumin-glucose system that was stored at $37^{\circ}C$ for 10 days
as seen in Table 2. It is obvious that the FDNB technique gave
better agreement with the in vivo growth studies than did the
TNBS technique.

It has also been suggested that the Amadori reaction compounds
formed in the first phase of reaction are also biologically
unavailable although they are chemically reversible (Adrian, 1974;
and Lea and Hannan, 1950a, 1950b). As was seen in Figure 8,
about a 70% loss in available lysine (by the FDNB modified method)
occurred in a model food system (glucose/casein 3:3) held at a_w
0.5 for 30 days at $35^{\circ}C$. At this point the product was not yet
visually brown. Warren and Labuza (1976) have confirmed that
part of this loss leads to decreased nutritional value using a
protozoan Tetrahymena pyriformis W (TPW) bioassay technique.

The use of TPW in evaluating protein quality has been shown
to correlate well with both rat PER's (Evancho et al., 1976; and
Landers, 1975) and FDNB (Shorrock and Ford, 1973). Warren and
Labuza (1976) used the same model system of Warmbier (1975) and
humidified the product to several a_w's. As can be seen in Figure
9, the loss in protein nutritional value (RNV) by TPW is less than

Table 2

Comparison of Biological and Chemical Methods for Protein Loss

	Albumin Mix & Glucose		Potency Ratio of Stored Sample of
	Unheated	Stored 30 d at 37°C	Unheated Control
Rat Assay			
g wt gain	64.5	18.3	0.28
g wt gain/g food eaten	75.2	25.9	0.34
Chick Assay			
g wt gain	107.4	23.6	0.22
g wt gain/g food eaten	103.3	25.4	0.25
total lysine	86.3	81.6	0.94
FDNB–lysine	50.9	19.6	0.38
TNBS–lysine	53.6	35.9	0.67

From Hurrell and Carpenter, 1974

predicted by the FDNB procedure. However, after 20 days storage in the 0.3 a_w samples where the samples have not changed color significantly (absorbance ∿0.2) the nutritional value has decreased by 50%. At higher a_w (0.7) as seen in Figure 10, the FDNB procedure significantly over-estimates losses in the early stages of browning. This suggests that some of the Amadori rearrangement products are not available nutritionally and also that reaction pathways are changing with a change in the water content.

VI. KINETICS OF MAILLARD BROWNING DURING PROCESSING AND STORAGE

An introduction to the mathematical kinetics of Maillard non-enzymatic browning was shown in section II. several other studies with respect to water and temperature will be reviewed here.
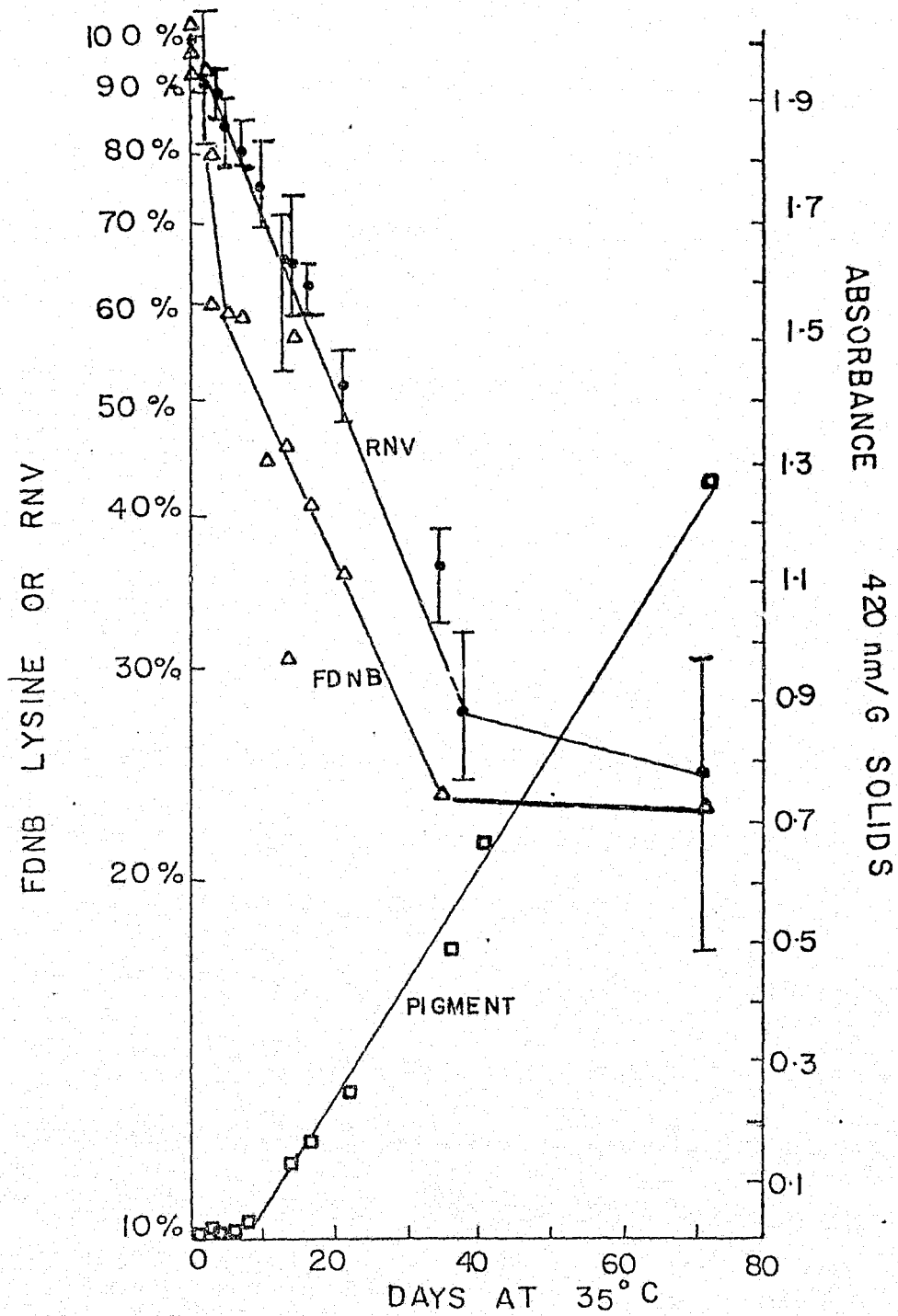
FIGURE 9

Lysine loss (Δ) by FDNB, protein nutritional loss (•) by TPW
bioassay and browning production in a casein/glucose/glycol model
system (a_W 0.3, 35°C).

Warren and Labuza (1976)
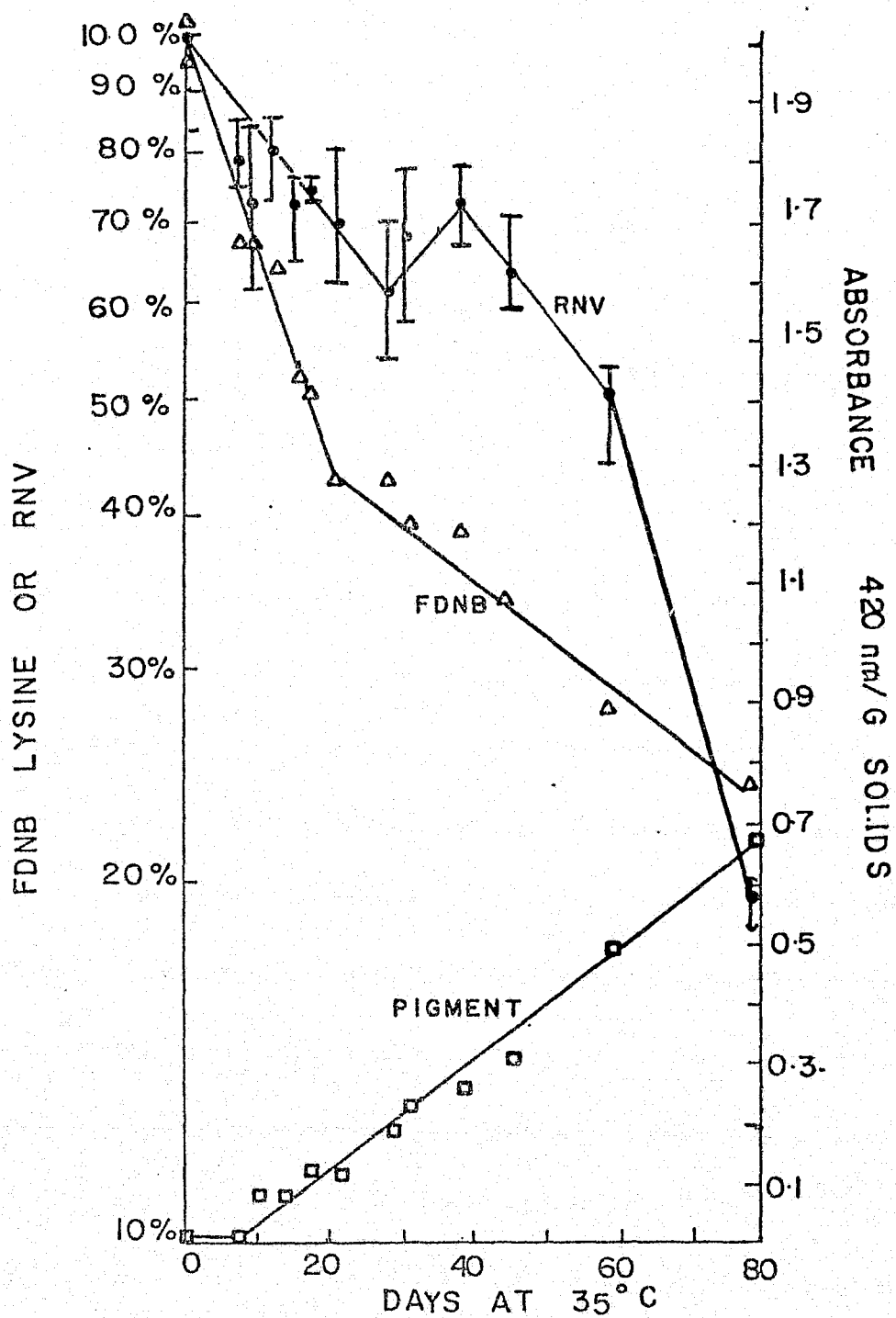
FIGURE 10

Lysine loss (Δ) by FDNB, protein nutritional loss (•) by TPW
bioassay and browning production in a casein/glucose/glycol model
system (a_w 0.7, 35°C).

Warren and Labuza (1976)

Haugaard et al., (1951) studied the browning of an aqueous mixture of D-glucose and glycine at reflux temperature by measuring the absorbance of the reaction mixture at 490 nm. They concluded that the concentration of the brown pigment produced was proportional to the square of the amino acid concentration (A), the concentration of the reducing sugar (R), and the square of the reaction time as shown in equation (3):

$$B = K(A)^2 (R)\theta^2 \qquad (3)$$

where K is the rate constant.

Differentiating this gives:

$$\frac{dB}{d\theta} = K'A^2R\theta \qquad (4)$$

which shows that browning rate increases with time. Eichner (1975) and others have found similar increased browning rate with time in the study of the browning of simple sugar/amino acid systems which undergo rapid browning. It should be noted that Haugaard's mechanism implies that two moles of amine react per mole of reducing sugar which is not as per the mechanism presented earlier.

Accumulation of brown pigments is usually found to be relatively constant with time during the storage of most dry food products. For example, Figure 11 shows the increase in brown pigment in an IMF system (a_w ~0.84) at three temperatures stored in air and an oxygen free atmosphere (Waletzko and Labuza, 1976).

NON-ENZYMATIC BROWNING
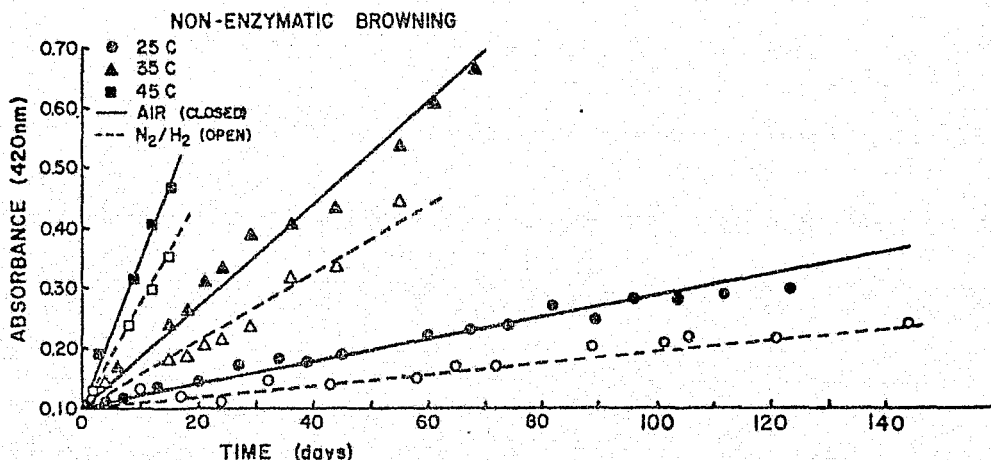
FIGURE 11

Increase in brown pigment as a function of temperature and gas composition for an intermediate moisture food (a_w 0.84)

Waletzko and Labuza (1976)

Similar effects of constant browning occur in complex model systems where the amine is supplied by protein. The data of Warmbier et al., (1976) shown in Figure 12 exemplifies the constant rate of pigment production after a short induction period and the effect of a_w and temperature. This constant rate occurs despite the fact that glucose and lysine of the protein are lost very rapidly by first order reactions as seen in Figures 13 and 14.

Based on these observations at low temperature ($<50^\circ$C) storage of dehydrated and semi-moisture, the rate of production of reactive intermediates must reach a constant value in which the rate of breakdown is equivalent. In addition, some level of R* must be reached before visual browning occurs. Thus,

$$\frac{dB}{d\Theta} = k_B = k_2 \ (R)^*_m \ . \tag{5}$$
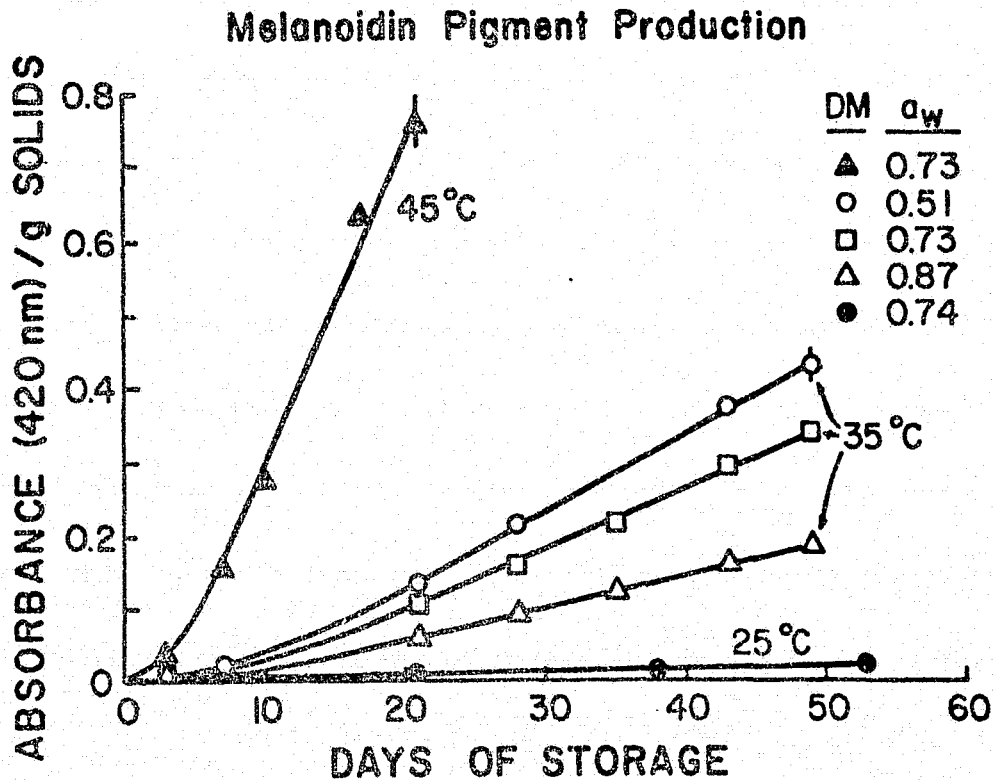
FIGURE 12

Increase in brown pigment for a casein/glucose/glycerol model system as a function of a_w and temperature.

(Warmbier et al., 1976)
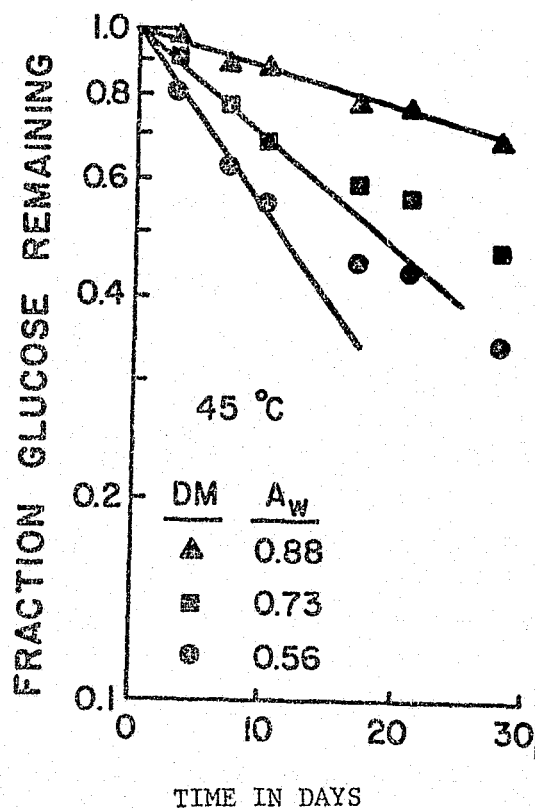
FIGURE 13

First order plot for glucose loss in a casein/glucose/glycerol model system as a function of a_w.

(Warmbier et al., 1976)

where

k_B = zero order browning rate
k_2 = rate constant for (R*) production
$(R*)_m$ = constant level of R* needed for constant brown pigment production

Obviously at higher temperatures such as in the study of Haugaard et al., (1951) or in less complex systems, a change in kinetics occurs to make the reaction rate change with time.

Song et al., (1966) and Song and Chichester (1966, 1967a, 1967b) developed kinetics for the Maillard reaction of glucose-glycine. Their overall browning rate expression is:
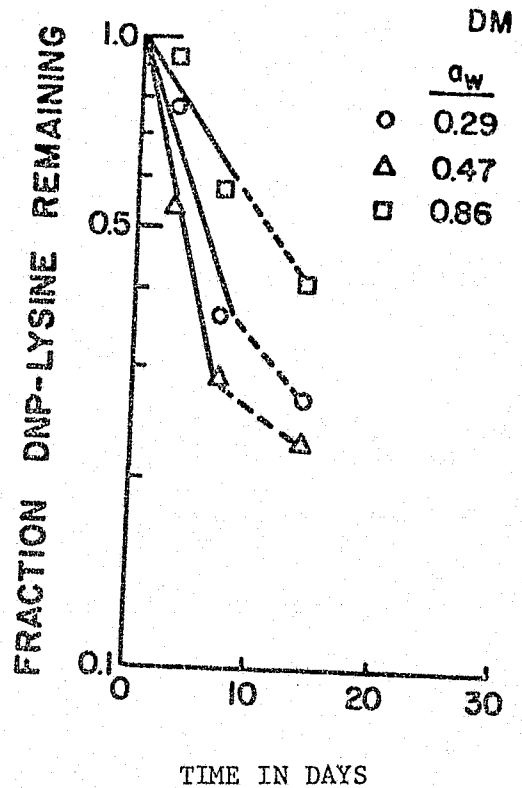
FIGURE 14

First order plot for lysine loss (FDNB) in a casein/glucose/
glycerol model system as a function of a_W.

(Warmbier et al., 1976)

$$d(B)/dt = k_2(k_1(G)k_2'(I))^{\frac{1}{2}} + k_3(g_0 - B)(I - B) \qquad (6)$$

where I represents intermediates, g represents glycine, G represents glucose, B represents the brown melanoidin pigments, and the k's represent constants. The equation is too complex to be readily adaptable to practical use where more complex substrates are present. However, it should be noted that they also found even with a very simple system in the 50-100°C range, the amount of brown pigment followed a linear plot with time after a certain induction period. Their complex equation above accounts for this induction period.

Mizrahi et al., (1970a, 1970b) developed equations to predict browning rates of unsulfited dehydrated cabbage under accelerated storage conditions. Two equations that related browning rate to moisture content were derived by curve fitting:

- 65 -

$$\frac{dB}{d\Theta} = K_1(1 + \sin(-\pi/2 + m\pi/m_x))^n \qquad (7)$$

$$\frac{dB}{d\Theta} = K_2(r_1 + m)/(r_2 + m))^s \qquad (8)$$

where browning is in Klett units/day, m is the moisture content (g H_2O/100g solids), m_x is the moisture content at which browning rate is at a maximum (which was 18.0 in their study with humidified freeze-dried cabbage), and r_1, r_2, K_1, K_2, n and s are constants fit by computer analysis. Equation (7) gave minimum variance for relating browning rate to moisture content for samples stored at 30° and 37°. Equation (8) was best adapted to samples stored at 45° and 52°C. The equations quite accurately predicted the NEB rate of unsulfited dried cabbage from NEB rate data collected under accelerated shelf-life conditions.

Mizrahi et al., (1970a) also found that the apparent activation energy was related to moisture content as given by the following equation (9):

$$E_a = c_1 e^{-c_2 m} \qquad (9)$$

where E_a is Arrhenius activation energy, m is moisture content (g H_2O/100g solids) and c_1 and c_2 are constants. The results are shown in Figure 15.

Eichner (1975) found a similar effect for model systems at low moisture contents. The Q_{10} dropped from 5.6 to 3.9 in going from 1.25 to 3.1% moisture. Figure 16 shows similar data of Hendel et al., (1955) used by Aguilera et al., (1975) in predicting browning during drying of potatoes. As seen, the major change in activation energy occurs below 15% water content ($a_w \approx 0.7$). This verifies that the changes in the pathways of browning mentioned earlier are indeed occurring.

With respect to water activity, as was shown before, the browning rate increases to a maximum and decreases again. The same factors of water feedback inhibition, reactant concentration and aqueous phase viscosity also affect the rates of loss of the reducing sugars and lysine. The data of Warmbier et al., (1976) for a casein/glucose/glycerol model system illustrates this in Figure 17. The E_a for browning was 33 KCal/mole whereas for the initial steps of glucose and lysine loss was 25 KCal/mole. This shows that the latter phases of the mechanism are rate controlling which allows build-up of reaction intermediates. As also noted in this study the rate maximum occurred at a lower a_w (0.5) than found in most dry foods which have been humidified. This is similar to the results of Eichner and Karel (1972) for
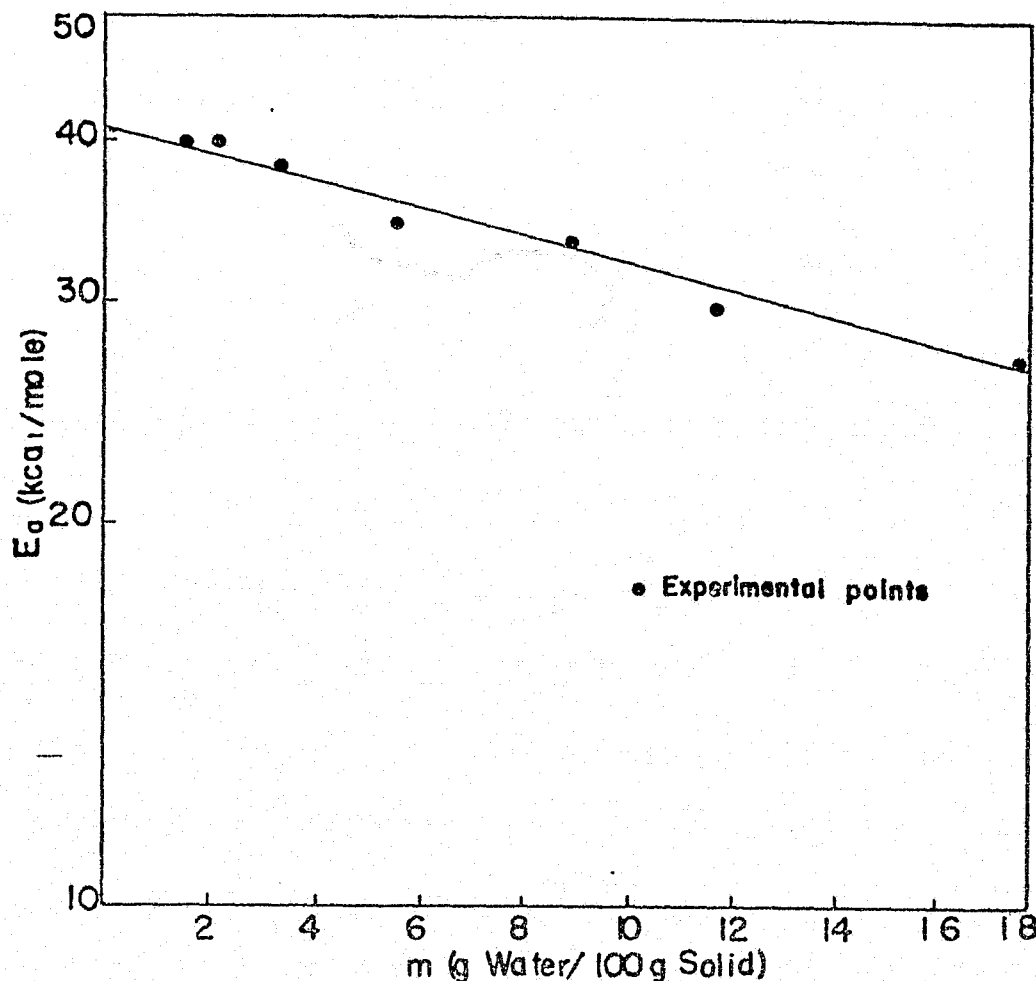
FIGURE 15

Decrease in activation energy E_a for non-enzymatic browning in dehydrated cabbage as a function of water content.

(Mizrahi et al., 1970b)

liquid systems shown in Table 3. They found that with glycerol present the maximum amount of browning occurred at 0.3 to 0.4 a_w as compared to 0.7 to 0.8 for humidified, dry, or semi-moist foods. Addition of gum arabic which increased phase viscosity decreased the amount of browning and increased the a_w at which this occurred.
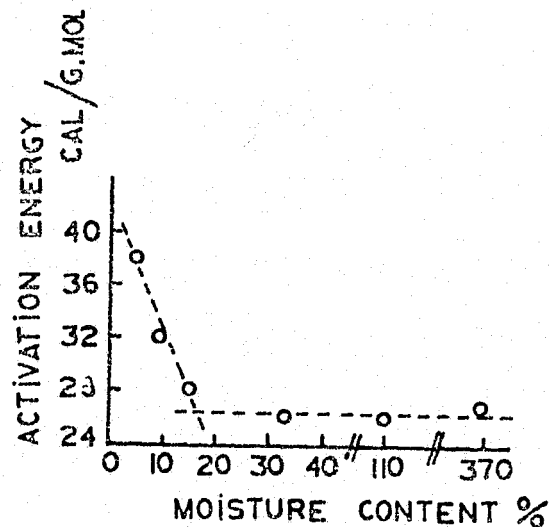
FIGURE 16

Decrease in activation energy for non-enzymatic browning of potatoes undergoing drying as a function of water content.

(Hendel et al., 1955)

Table 3

Browning Value (420 nm) After Storage
3 Days at 37°C*

a_w	Control*	Control With Cellulose	Control with Gum Arabic
0.3	0.08	0.07	—
0.4	0.11	0.11	0.04
0.5	0.09	0.09	0.06
0.6	0.05	0.05	0.05
0.75	0.03	0.03	0.04

* Eichner and Karel (1972)
** glucose/glycine in H_2O-glycerol solutions
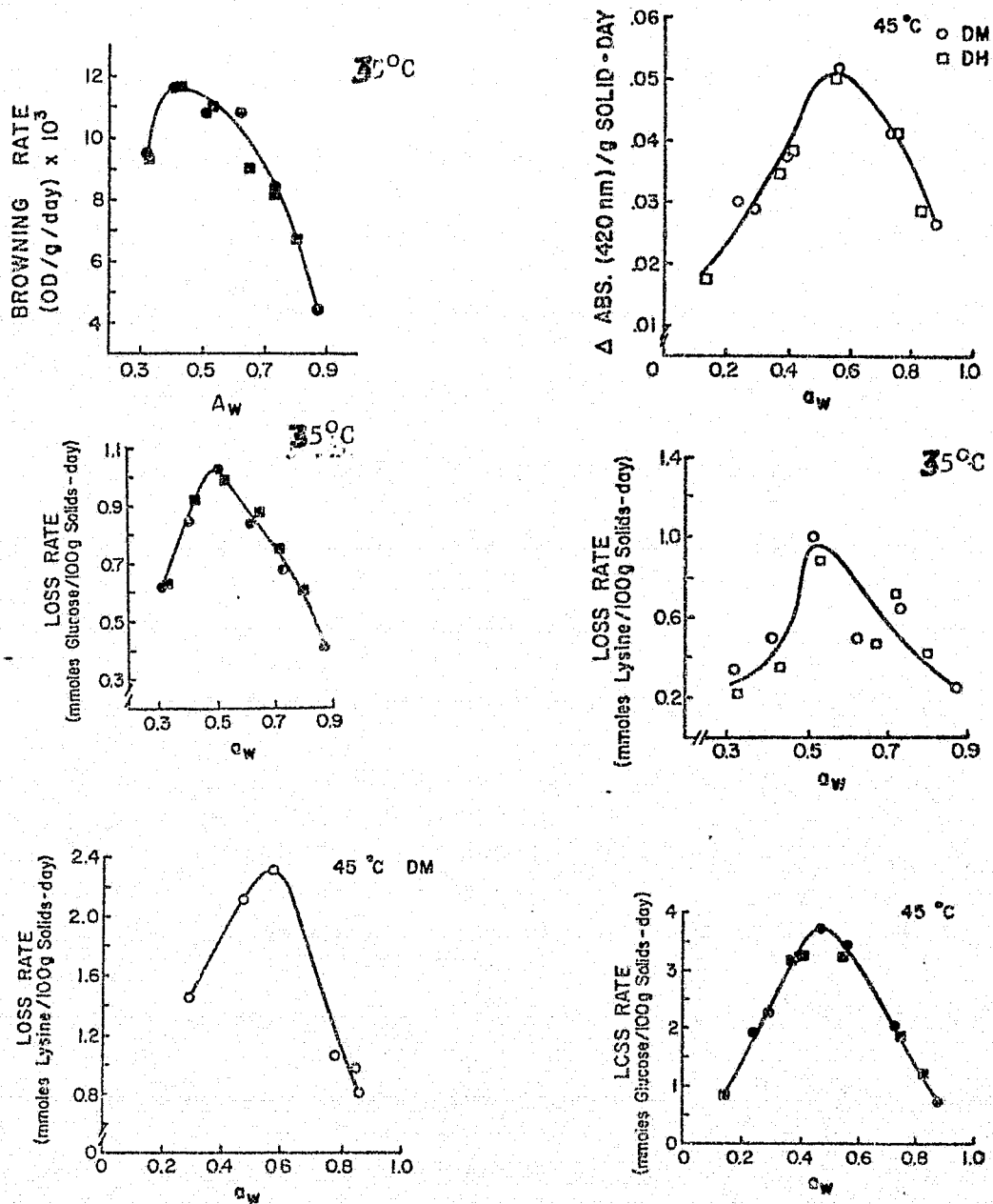
FIGURE 17

Effect on water activity on the rates of browning pigment production, glucose loss and available lysine loss at 45°C in a casein/glucose/glycerol model system.

(Warmbier et al., 1976)

To further verify this, Labuza (1975) worked with solid
model foods to which various humectants were added. As seen in
Figure 18 the control showed a browning maximum at $a_W = 0.82$,
typical of semi-moist or humidified dry foods. The systems with
the liquid humectants (glycerol, butane diol and propylene glycol)
all had a greater rate of browning at lower a_W. This was attri-
buted to the greater phase volume at the lower water activity,
since the liquid glycols are soluble in water and could dissolve
the glucose. Phase volume was not the only factor however. The
differences could also relate to solubilities, oxidation reduction
potentials and pK's, and aqueous phase viscosity. This latter
effect is shown for the sorbitol data. It reduces browning rate
drastically by increasing the solution viscosity. Sorbitol is
a solid and cannot act as a liquid humectant. The use of these
humectants as browning inhibitors has been applied for as a
patent.
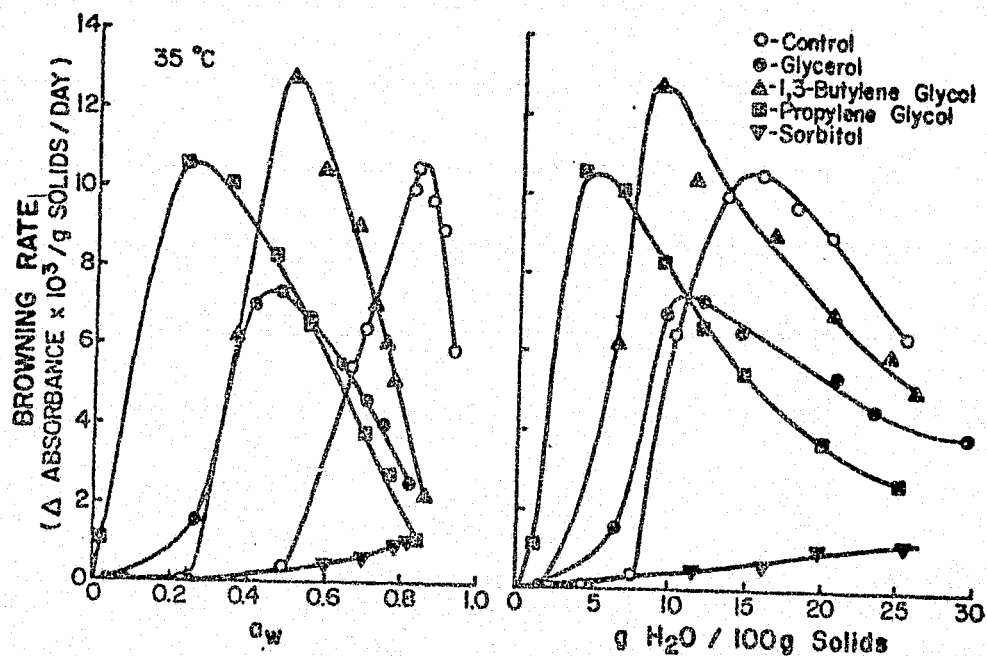
FIGURE 18

Effect of various humectants on browning rate in a casein/glucose
model system as a function of water activity.

(Labuza, 1975)

Many other studies have been done to develop browning rate
equations as a function of system composition and conditions.
Most are specific systems and equipment. For example,
Jokinen et al., (1976) determined available lysine loss due to
browning in samples composed of soybean protein, glucose, sucrose,
potato starch, microcrystalline cellulose and water. The equation
relating available lysine content to sample composition and
thermal processing conditions is:

$$L/L_0 = 0.581 + 0.047 \text{ (pH)} - 0.093 \text{ (G)} - .059 \text{ (T)} - .0068\Theta$$

$$+ .0305 (a_w)^2 + .025 (a_w)(S) + .0331 (S)(s) \tag{10}$$

where L/L_0 is the fraction of available (FDNB) lysine remaining,
G is glucose content, T is temperature, Θ is time, S is sucrose
content, and s is starch content.

This is the first kinetic approach to the Maillard browning
reaction where the nutrient stability (available lysine content)
of a system can be predicted given the system's composition and
thermal parameter. It should be noted that as with food storage
the maximum in browning occurs in the a_w range of 0.65 to 0.7
and that sucrose hydrolysis contributed to browning at the low
pH's. The limitation of the study is that it applies to extrusion
processing where high temperatures (80 - 130°C) are used.

Another aspect of browning is the relative reactivity of
proteins with the reducing sugars. Schnickles et al., (1976)
showed that for IMF systems at 0.68 - 0.78 a_w the browning rate
did not correlate with either total lysine available or time for
50% lysine loss for all the proteins. This is seen in Table 4.

If zero order kinetics apply then the methods of Labuza
et al., (1972) and Labuza (1976) can be used to predict food
product shelf-life undergoing browning for time-temperature-
humidity variable systems. The details for the moisture depen-
dency will not be gone into here, except to show that the moisture
gain with time for a particular food in a certain package can
be predicted by equation 11:

$$\ln \frac{m_e - m_i}{m_e - m} \quad \frac{k}{x} \frac{A}{W_s} \frac{P_o}{\beta} \Theta \tag{11}$$

m_e = moisture content of food in equilibrium with outside atmosphere
(g H_2O/g solids)
m_i = initial moisture content
m = moisture content at time Θ
k/x = permeance of package g H_2O/day m^2 mm Hg
A = area of package
W_s = weight of dry solids

P_o = vapor pressure of pure water mmHg
β = slope of linear portion of isotherm
Θ = time

Table 4

Browning and Lysine Loss In Model Systems

Protein Used	Total Avail Lysine mg/100g solids	Browning Rate (OD/day) x 10^3	Time for 50% Lysine Loss (days)
wheat gluten 10% lysine	3,300	35	< 1
casein	1,416	10.2	22
whey	857	8.5	25
soy (TVP)	762	7.5	7
fish protein conc.	1,283	5.9	19
egg albumin	360	4.9	> 60
wheat gluten	303	0.9	40

Given this and a graph of shelf-life as a function of water content for different temperatures one can use iterative processes to determine shelf-life consumed since:

$$f\text{con} = \frac{(\Theta)}{(\Theta_s)_{Tm}} \tag{12}$$

$f$con = fraction shelf-life consumed
    Θ = time held at some constant moisture (m) and temperature (T)
    Θ_s = total shelf-life at that constant moisture (m) and temperature (T)

The fractions for each interval are summed up to get total consumption. This has been successfully applied to frozen foods where temperature is the only variable (Gutschmidt, 1974).

VII. ACKNOWLEDGEMENTS

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B. Nutritional Losses of Lysine During Nonenzymatic Browning

  1. Introduction

      Lysine and reducing compounds such as glucose can form

Maillard compounds during nonenzymatic browning that may be nutritionally

unavailable for living organisms. The chemical analysis by FDNB

(Carpenter-Booth modification), has been used previously by this lab

to demonstrate loss of available lysine before actual pigment is

observed in nonenzymatic browning.

      Tetrahymena pyriformis W (TPW) is a protozoan requiring

lysine for growth. A procedure requiring only four days and minimal

sample has been developed which is given in detail in Section B2. The

rest of this section reports on the studies of using this method to

assess protein quality loss during browning.

2. TPW Lab Procedure for RNV

(1) Stock Reagents to be Kept on Hand

A. Vitamin stock solution

1. Weigh individual vitamins in Table 1 to the nearest 0.1 mg.

2. Transfer to 200 ml vol flask with hot ($55^{\circ}C$) distilled water.

3. Fill flask about 2/3 with hot water and set on magnetic stirrer until dissolved.

4. Cool and make to volume with distilled water.

5. Dispense 7-10 ml in small screw cap test tubes. Store in freezer in micro lab ($-2^{\circ}C$) up to 3 months.

B. Stock solution B, C, D, and citric

1. Prepare solutions according to Table 2. Refrigerate in reagent bottles.

2. Prepare 0.07 M pH 7.1 phosphate buffer by diluting 35 ml of solution D, to 00 ml distilled water. Adjust pH to 7.1. Autoclave. Use for diluting TPW inoculant after centrifugation.

C. Preserving fluid

1. Prepare according to Table 3.

2. Use 1 ml in screw-cap test tube and 1 ml of TPW assay or inoculant for microscopic counting.

D. Prepare 1.0 and 0.1 N HCl and 1.0 and 0.1 N NaOH for use

    in adjustment of pH in samples.

(2) Preparation for TPW run.

    A. Prior to day of analysis

        1. Transfer 5 ml of TPW stock to fresh maintenance

            broth 3 days prior to time of inoculant.

        2. Sterilize screw-cap plastic centrifuge bottles (100 ml)

            and small glass screw-cap test tubes for use in

            preparation of TPW inoculant.

        3. Analyze sample or control for N content by Kjeldahl

            to determine sample wt equivalent to 100 mg N.

    B. Sample digestion (This may be started day before TPW run.)

        1. Turn water bath to 55°C.

        2. Weigh sample (± 1.0 mg) containing 100 mg N in

            50 ml Erlenmeyer.

        3. Add 20 ml distilled water.

        4. Adjust pH to 1.8 with 1.0 N HCl.

        5. Add 1.0 ml 1% freshly prepared pepsin solution.

            (Prepare for pipetting. For 9 samples dissolve

            0.1 g pepsin in 5 ml H_2O. Add 5 ml 0.1 N HCl.)

        6. Incubate at 55°C in shaking water bath (100 cpm

            agitation rate) for 3 hr.

        7. Remove and cool. If to be stored overnight,

            refrigerate at this point.

8. Adjust to pH 7.1 with NaOH.

9. Quantitatively transfer sample to 50 ml vol flask.

10. Pipette 0.5, 1.0, 1.5, and 2.0 ml sample suspension to labeled 50 ml Erlenmeyers. Frequently agitate to prevent settling. Prepare three assays at each level, corresponding to 1, 2, 3, and 4 mg N/10 ml assay.

11. Adjust volume in each assay flask to 3.0 ml with distilled water. Samples are ready for 2 ml Nucleic Acid - Mineral Solution E.

12. Set aside 5 ml sample suspension for micro-Kjeldahl analysis to ensure 2 mg N/ml sample.

C. Nucleic Acid - Mineral Solution E

1. Weigh nucleic acids (± 1 mg) found in Table 4. (These may be stoppered and refrigerated overnight.) For 36 assays, prepare 100 ml solution E.

2. Dissolve nucleic acid in boiling water with swirling and glass stirring rod.

3. Transfer to vol flask. Cool.

4. Add solutions B, C, D, and citric acid. Swirl well between additions.

5. Adjust volume with water.

6. Pipette 2.0 ml solution E to each 50 ml Erlenmeyer assay flask containing sample.

7. Stopper flask with gauze plug.

8. Autoclave 15 min, 121°C on tray with foil over top to prevent condensation on gauze. Cool.

D. Vitamin solution A

    1. a.) Thaw portion of vitamin stock solution, avoiding exposure to light.

        b.) Dilute vitamin stock to appropriate volume. For 36 flasks, dilute 4.8 ml stock to 50 ml with water.

        c.) Transfer to appropriate glassware for autoclave. Cover with foil. Set aside to autoclave 15 min, $121^{\circ}C$.

    2. Glucose solution

        a.) Weigh glucose ($\pm$ 10 mg) in flask. (For 36 flasks use 7.2 g in 190 ml water.)

        b.) Dissolve glucose in appropriate volume of hot water.

    3. Autoclave vitamin stock dilution and glucose solution in separate container.

    4. Cool.

    5. Aeseptically transfer vitamin stock dilution to glucose solution.

    6. Pipette 5 ml aeseptically to each 50 ml assay flask.

E. TPW inoculant (use aeseptic technique.)

    1. Transfer 50 ml to each centrifuge bottle and balance.

    2. Centrifuge 8 min at 5000 cpm on refrigerated Sorval Centrifuge.

    3. Discard supernatant.

4. Add 2 ml sterile 0.07 $\underline{M}$ pH 7.1 phosphate buffer to cells. Vortex.

5. Transfer cells to sterile test tubes with approximately 3 ml buffer.

6. Count cells in 1 ml.

7. Dilute sufficient cells in appropriate volume phosphate buffer to provide 50 x 10^4 Cells/ml. (Need 0.1 ml/assay plus 1 ml to count).

8. Inoculate sterile 10 ml sample media containing protein sample, 2 ml solution E and 5 ml vitamin solution A. (Vortex TPW cells frequently during inoculation.)

(3) Incubation and Evaluation of Sample

A. Incubate samples at 25 $\pm$ 1^oC in New Brunswick Tabletop Shaker Incubator at 100 cpm (agitation rate).

B. After 4 days (95 to 97 hr) remove samples. Thoroughly mix and transfer 1 ml from each sample to labeled screw-cap test tube containing 1 ml preserving fluid.

C. Determine total TPW cells/ml assay.

1. Using Fuschs-Rosenthal hemacytometer, count 8 alternate mm^2 in microscopic field at 10 x 10 power light microscope. Determine avg/mm^2. This value x 10^4 represents total cells/ml assay.

2. Determine RNV by averaging 3 assays at each protein level and comparing test sample to the control at 3 mg N/10 ml level.

Table 1

Preparation of Vitamin Solution A for TPW

Vitamin Stock

	mg/200 ml
D-calcium pantothenate (* P-2250)	12.5
Nicotinamide (* N-3376)	12.5
Pyridoxine hydrochloride (* P-9755)	125.0
Pyridoxal hydrochloride (* P-9130)	12.5
Pyridoxamin di-hydrochloride (* P-9380)	14.8
Riboflavin (* R-4500)	12.5
Folic acid (* F-7876)	1.25
Thiamin hydrochloride (* T-4625)	125.0
Inositol-meso (** 4071)	12.0
Choline chloride (** 2301)	125.0
p-Aminobenzoic acid (* A-9878)	12.5
D-Biotin (* B-4501)	1.25
DL-Lipoic acid (thioctic acid) (* T-5625)	0.4

  * Sigma Chemical Co., St. Louis, MO

** Calbiochem, LaJolla, CA

Glucose Solution

D-Glucose (Fisher)	3.6 g
Distilled water	95 ml

Dissolve. Autoclave. Cool and aeseptically combine with 2.4 ml vitamin stock diluted to 25 ml. Use 5 ml of Vitamin Solution A per assay.

Table 2

Preparation of Mineral and Buffer Stock Solutions for TPW

Stock Solution B *

	g/200 ml
$MgSO_4 \cdot 7H_2O$	2.8
$Fe(NH_4)_2(SO_4)_2 \cdot H_2O$	1.25
$MnCl_2 \cdot 4H_2O$	.025
$2nCl_2$	.0025

Stock Solution C *

	mg/200 ml
$CaCl_2 \cdot 2H_2O$	600
$CuCl_3 \cdot 6H_2O$	60
$FeCl_3 \cdot 6H_2O$	15

Stock Buffer Solution D *

100 ml Solution D_1 (0.2 M KH_2PO_4 + 0.2 M K_2HPO_4 to give pH 7.1) *

100 ml Solution D_2 (0.2 M pH 7.1 Tris HCl buffer, hydroxymethyl amino methane) *

Citric Acid Solution * (0.02 M)

	g/100 ml
Citric acid, monohydrate	0.42

* Store in refrigerator

Table 3

Preparation of Preserving Solution for TPW

Component	ml
Distilled water	688
Formaldehyde (36%)	160
Stock buffer solution D (see Table 2)	112

Table 4

Preparation of Nucleic Acid - Mineral Sol꜠ion E for TPW

Nucleotide Solution	mg/20 ml
Guanylic acid (sodium salt) (** 3720)	15
Adenosine - 2'(3')-phosphoric acid monohydrate (** 118352)	10
Cytidylic acid (** 2500)	12.5
Uracil (* U-0750)	5

Dissolve nucleotides in 10 ml hot distilled water on day of use.

Add 1 ml each of stock solution B, C, D and citric acid (see Table 2).

Adjust volume to 20 ml with water.

Adjust pH to 7.1 with 1.0 $\underline{N}$ NaOH.

Use 2 ml of this solution E for each assay.

* Sigma Chemical Co., St. Louis, MO

** Calbiochem, LaJolla, CA

3. Comparison of chemically measured available lysine with

relative nutritive value measured by a Tetrahymena

bioassay during early stages of nonenzymatic browning.

a. Abstract

Tetrahymena pyriformis W (TPW) was used as a biologi-

cal assay to study the loss of overall protein quality due to nonenzy-

matic browning. The Relative Nutritional Value (RNV) as determined

by this organism decreases in a model food system as Maillard compounds

are formed during storage at 35°C for 80 days and at three water

activities (0.3, 0.5, and 0.7). These results were compared to the

fluorodinitrobenzene (FDNB) chemical assay. The latter method shows

a greater loss of available lysine than is seen by TPW growth at

early stages of browning. Significant accumulation of brown pigment

production does not occur until after RNV has diminished by 35-50%

and FDNB has decreased by 60-70%. At advanced stages of browning,

however, the TPW test showed equivalent or greater losses of nutritional

value. Considerable variation in the TPW bioassay occurred due to the

problems in the technique but analysis of all stored samples in a

single test allowed comparison to a standard casein control for

measurement of protein quality loss.

b. Introduction

Reaction of sugars with amino acid through Maillard

browning results in compounds that cannot be nutritionally utilized.

The greatest destruction and loss occurs with lysine, which has a free ε - amino group that can react with reducing sugars (Mauron et al., 1955). The early stages of the reaction are reversible and no color develops; however, some results show that the nutritional availability of the amino acid is reduced (Mauron et al., 1955; Adrian, 1974; Carpenter and Booth, 1973).

Acid hydrolysis used in many chemical assay methods for nutritional losses may release bound amino acids which have reacted but are unavailable for biological enzymatic breakdown. Therefore, chemical assay techniques which rely on reaction with the labile amino acid may underestimate true biological loss. It has been shown that one of the assays most used for lysine measurement, the fluorodinitrobenzene (FDNB) procedure, may underestimate protein losses in nonenzymatic browning (Hurrell and Carpenter, 1974; and Mauron et al., 1955). In browning however, early stage reaction compounds of lysine with carbonyls may not react with FDNB but could be available in the gut so that the FDNB procedure could actually overestimate nutritional losses from a biological standpoint.

The nutritional loss that occurs from nonenzymatic browning affects overall protein quality, usually by reducing the amount of available lysine, an essential amino acid for humans. This could be especially important in cereals that are already low in lysine and in foods formulated to meet the protein requirement for a meal, such as shelf stable intermediate moisture food bars

(Labuza, 1976). These typically have a water activity (a_w) in the 0.6 to 0.85 range and contain both protein and reducing sugars.

Lea and Hannan (1949) demonstrated that a_w affected the rate of browning in a casein and glucose system. They found a maximum in browning rate in the a_w range of 0.65 to 0.7 at temperatures from 37°C to 90°C. Warmbier (1975), however, demonstrated that the rate of browning is reduced by the presence of liquid humectants in the high a_w range.

Tetrahymena pyriformis W (TPW) is a protozoan requiring the same essential amino acids as man, including lysine. It has been used over the past twenty years for research on protein quality evaluation (Fernell and Rosen, 1956; Stott et al., 1963; Helms and Rolle, 1970; Rolle and Eggum, 1971; and Srinivas et al., 1975). It has also been used by several workers as a measure of available lysine. In these tests, the sample provides the only supply of lysine in an otherwise nutrient rich media (Stott and Smith, 1966; Boyne et al., 1967; and Shorrock and Ford, 1973). Values obtained in such a TPW assay tend to be slightly lower and yet correlate well with FDNB results, particularly if an enzymatic digestion of the sample aids the proteolytic TPW in digesting the intact proteins (Shorrock and Ford, 1973).

The purpose of this project was to evaluate the use of a TPW assay for the study of loss of overall protein quality due to nonenzymatic browning in an intermediate moisture model system. Values obtained by the chemical FDNB assay for available lysine were compared to the TPW assay.

c. Materials and Methods

Model food systems

A model food system using ANRC reference casein
and glucose in a 3:1 ratio was used. Individual components listed
in Table 1 were mixed in descending order. Water was added to obtain
the desired water activity. The systems were equilibrated over
appropriate saturated salt solutions for three days at room temper-
ature. Three systems were prepared to a_W's of 0.3, 0.5, and 0.7.

Ten gram portions of each system were sealed in
202 x 214 tin cans and dipped in paraffin to prevent moisture changes.
Cans representing zero day samples were held at -35^OC. The remaining
cans were stored at $+35^O$C. Samples were periodically removed for
up to an 80 day period and were also placed at -35^OC. At the end
of storage all samples (12) at each a_W were simultaneously analyzed.

Tetrahymena pyriformis W assay

A microbiological assay using Tetrahymena pyriformis W
(TPW) was used to measure Relative Nutritional Value (RNV). The method
was that used by Stott et al., (1963) and Landers (1975), with an
adaption of the nucleic acids which is listed here.

A portion of the model food system containing 100 mg
nitrogen (as measured by Kjeldahl) was incubated with 20 ml water and
1% pepsin at pH 1.8 for 3 hours (55^OC). The digested sample was

TABLE 1
MODEL FOOD SYSTEM COMPOSITION (0.3, 0.5, 0.7 a_w's)

Component	Grams
Potassium sorbate	0.3
Glucose	10.0
Propylene glycol	20.0
Casein (ANRC reference, Sheffield Chem. Co.)	30.0
Apiezon B-oil (J.G. Biddle Co.)	20.0
Microcrystalline cellulose	20.0
Water	variable for a_w used

cooled, the pH was adjusted to 7.1, and made to a final volume of 50 ml. Triplicate sample suspensions containing 3 mg N (1.5 ml) were added to 1.5 ml water and 2.0 ml of freshly prepared nucleic acid solution. This nucleic acid solution was prepared according to Lander's method (personal communication), except that the final concentrations of phosphate and TRIS buffers (pH 7.1) were at 0.005 $\underline{M}$ and the final concentration of citric acid was 0.001 $\underline{M}$. These conditions were found to maximize Tetrahymena growth.

Sample, water and nucleic acids were autoclaved in 50 ml, gauge stoppered Erlenmeyer flasks for 15 minutes at 121°C to prevent growth of other organisms. Solutions of vitamin stock and glucose were autoclaved separately and aeseptically added to the cooled samples.

Samples were inoculated with 0.1 ml of a three day old broth culture of TPW which had been centrifuged for 8 minutes at 5000 RPM in a Sorvall refrigerated centrifuge. Cells were diluted in 0.07 $\underline{M}$ pH 7.1 phosphate buffer to 5.0 x 10^5 cells/ml.

After 4 days in a 25°C shaker incubator (100 cpm) 1 ml of the assay solution was delivered into 1 ml formaldehyde preserving solution. Cells were then counted in a Fuschs-Rosenthal haemacytometer under a light microscope (300 x 10 power).

RNV was determined as the average total growth of triplicate stored samples per ml assay as a percentage of the total

growth of the zero day sample per ml assay material. The range for the TPW is shown in the results. The standard deviation for the control was ± 18.6% RNV based on values for the control done over a two year period. This includes both the variation in the casein as well as the problems in counting. It is felt that most of the error is in the counting procedure however, as experienced by Evancho et al., (1976).

FDNB Procedure

Available lysine was also determined using the FDNB method of Carpenter and Booth (Booth, 1971). The FDNB tags to free ε - amino groups at pH 8.5. The sample after treatment with FDNB reagent was refluxed in 8.1 $\underline{N}$ HCl for 16 hours, filtered hot, and diluted with water to a known volume. Available lysine was determined by absorbance at 435 nm.

Available lysine per gram of solid system was compared to the original available lysine determined in the zero day duplicate samples. The standard deviation for FDNB measurement was ± 2.2 mg lysine/gram solids which gives a range of ± 11.8% FDNB lysine.

Nonenzymatic browning pigment production

Brown pigments were measured by the method of Choi et al., (1949). Two grams of the model food system were incubated at 45°C for 2 hours with 20 ml phosphate buffer (pH 7.8) and 2.5 ml 10% (w/v) trypsin. Proteins were denatured with 2.0 ml

of 50% (w/w) trichloroacetic acid. Samples were filtered with 0.1 g

celite filter aid and the filtrate optical density was read at 420 nm

against a reagent blank. The OD was reported on a per gram of solids

basis against a reagent blank (precision $\pm$ 0.001, Warmbier, 1975).

d. Results and Discussion

The effect of storage at 37°C on loss of nutritional

value and browning in the three model systems is shown in Figures 1,

2, and 3 respectively. Available lysine loss by FDNB assay and

protein quality loss (RNV) by TPW assay were plotted on a semi-log

scale while browning increase was plotted as a zero order reaction

(Warmbier, et al., 1976). The TPW assay was a comparison of the stored

sample to that of the original control at the 3 mg nitrogen/10 ml assay

level, so it is a relative nutritional value (RNV). In all cases,

loss of lysine occurs as measured by both procedures before signi-

ficant browning occurs, as was found by Lea and Hannan (1949) and

Warmbier et al., (1976).

The early reactions in the Maillard reaction sequence

are reversible and the compounds formed are usually colorless. Table 2

compares TPW, RNV and FDNB values when the absorbance reached 0.02/gram

dry solids. It is obvious that the FDNB procedure overestimates the

loss of lysine as compared to the TPW test procedure by about the same

amount at each a_w. The organism may be able to enzymatically digest

the early reaction products while the FDNB test procedure measures

these as being biologically unavailable. It should be noted, however,

LYSINE LOSS AND PIGMENT DEVELOPMENT
OF 0.3 a_w MODEL FOOD AT 35° C
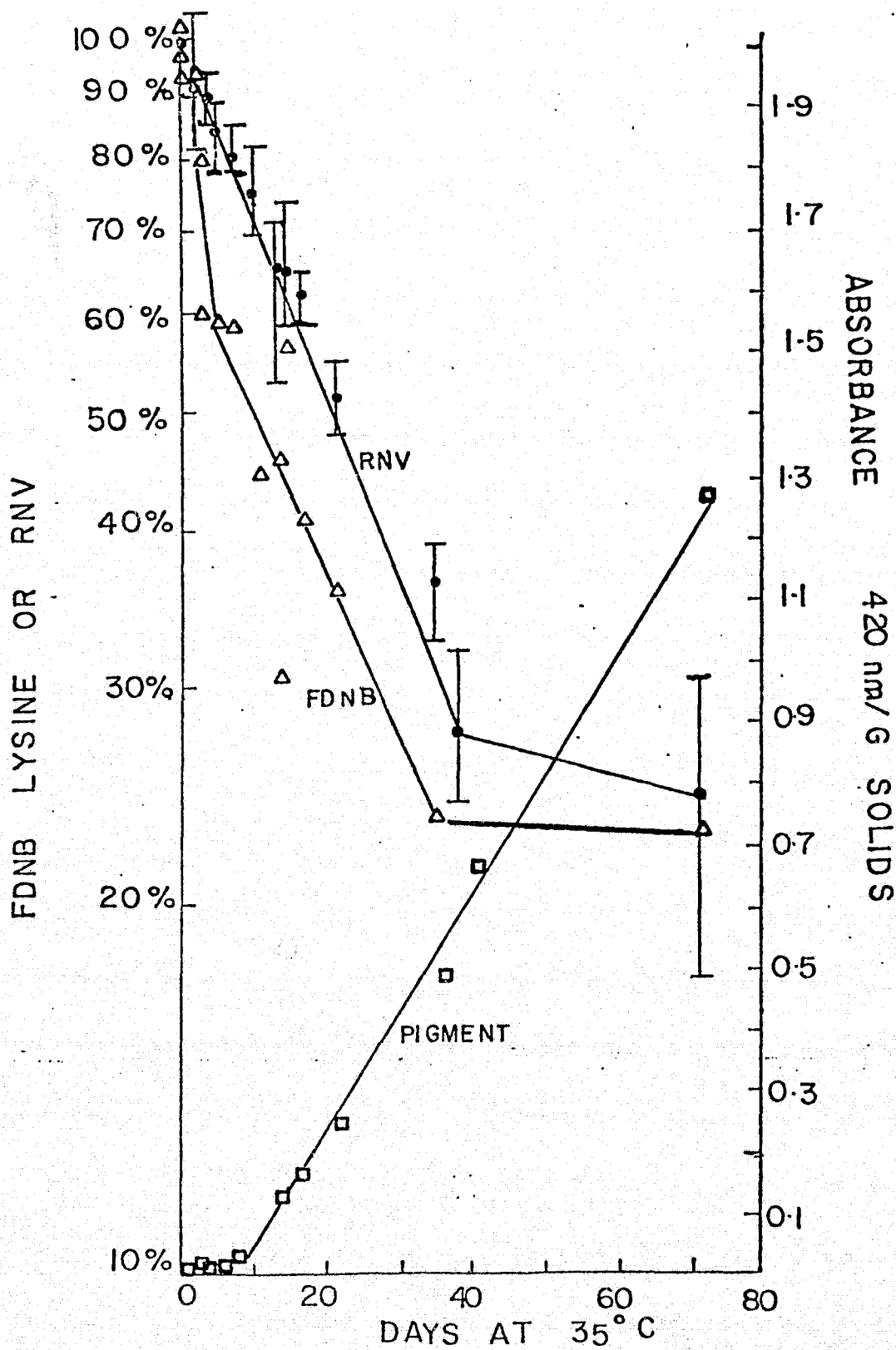
Figure 1

Lysine loss by FDNB protein quality loss by a TPW bioassay (RNV),
and pigment development of 0.3 a_w model food at 35°C.

LYSINE LOSS AND PIGMENT DEVELOPMENT
OF 0.5 a_W MODEL FOOD AT 35° C
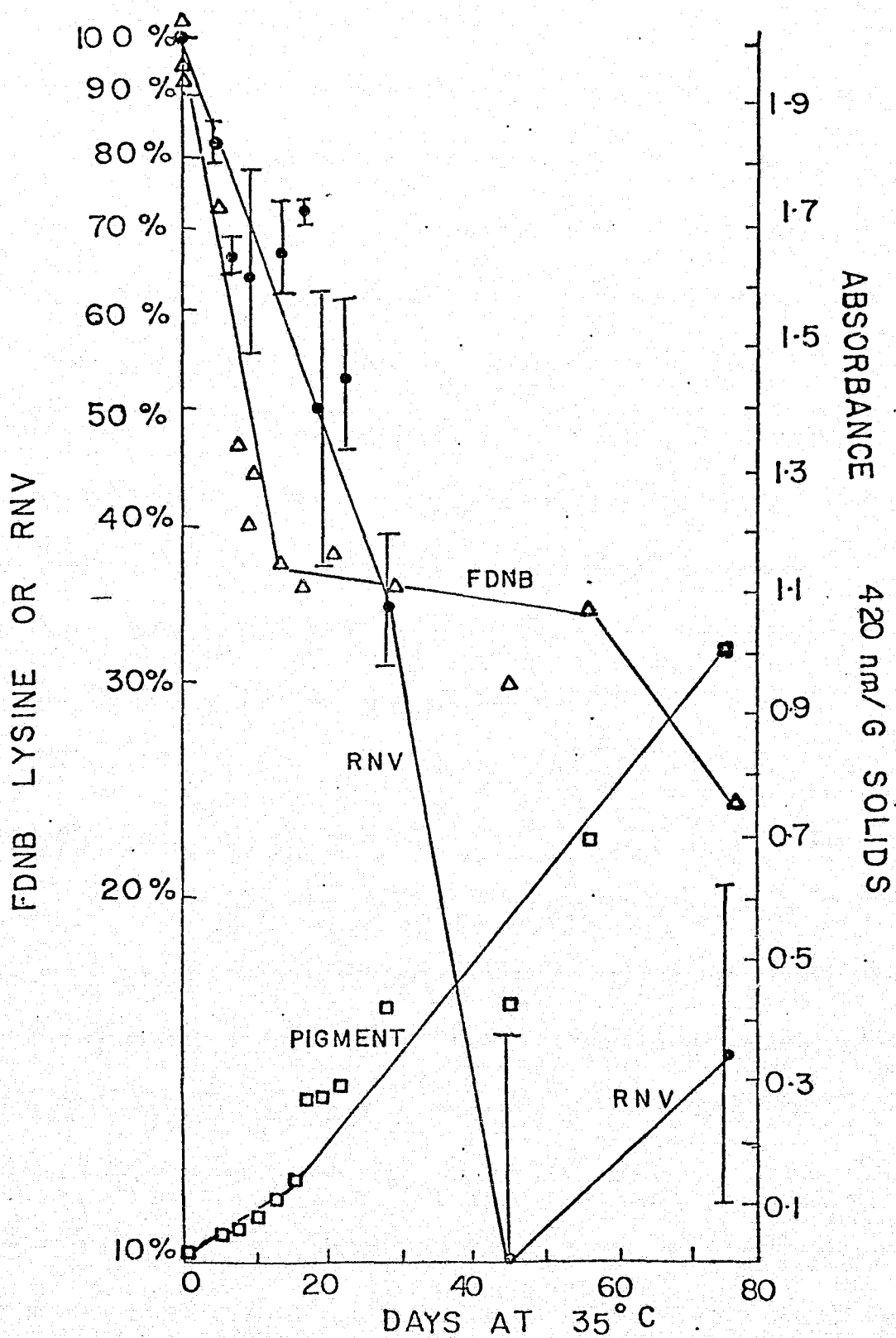
Figure 2

Lysine loss by FDNB protein quality loss by a TPW bioassay (RNV), and pigment development of 0.5 a_W model food at 35°C.

LYSINE LOSS AND PIGMENT DEVELOPMENT
OF 0.7 a_w MODEL FOOD AT 35° C
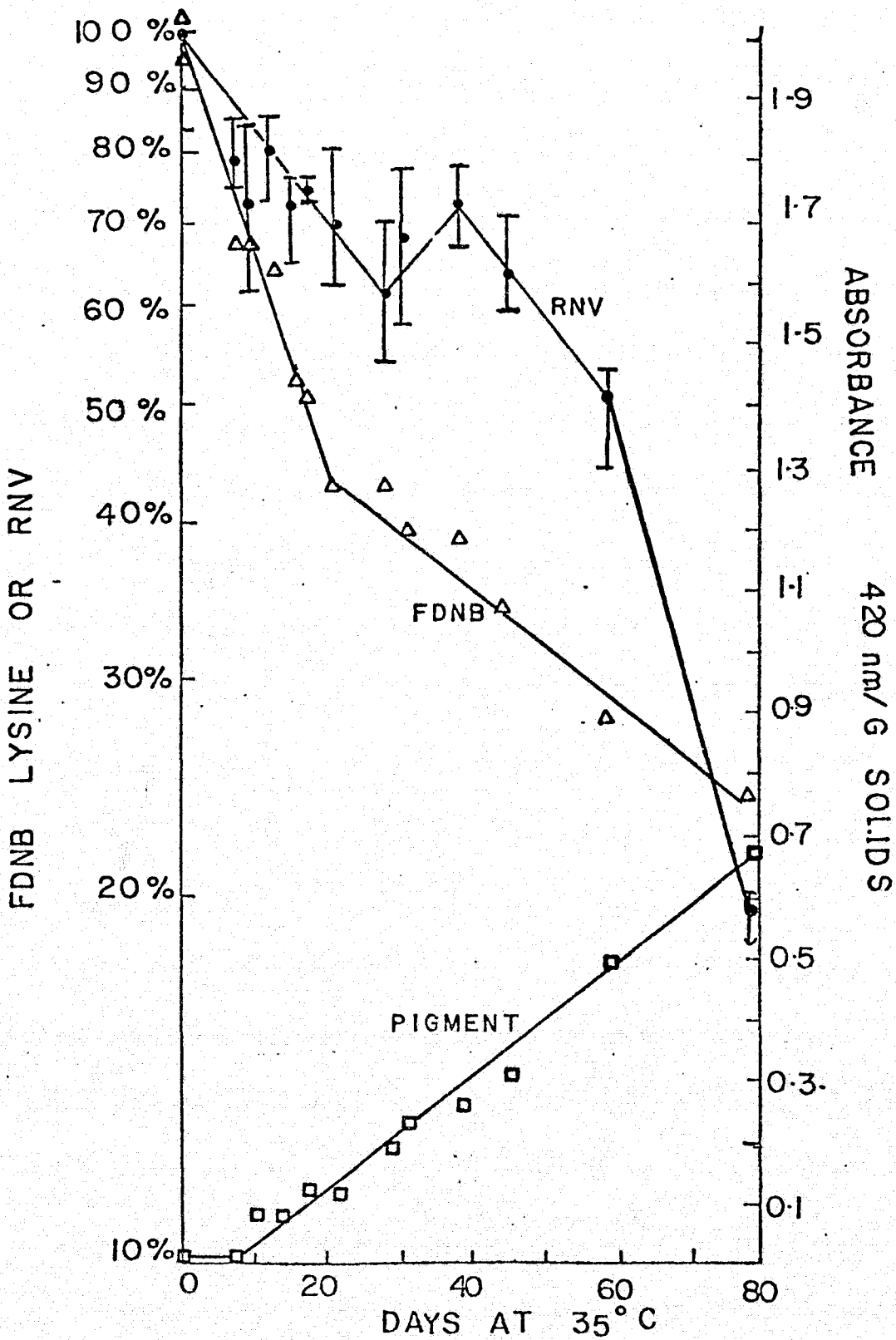
Figure 3

Lysine loss by FDNB protein quality loss by a TPW bioassay (RNV),
and pigment development of 0.7 a_w model food at 35ºC.

TABLE 2
AVERAGE LOSS OF AVAILABLE LYSINE BY FDNB AND TETRAHYMENA RNV AT THE EARLY STAGE OF BROWNING

Model Food System	Days stored at 35°C	Pigment development @420 nm OD per g solids	FDNB % original lysine left	RNV at 3 Mg N by TPW procedure
0.3 a_w	22	0.2	32	48
0.5 a_w	18	0.2	35	50
0.7 a_w	30	0.2	40	65

that the FDNB procedure is specific for available lysine. On the other hand, the TPW procedure measures effect on overall protein quality. Thus, some loss of lysine and other more limiting amino acids may not be detrimental to growth. The early reaction products should be isolated and tested in a limited lysine medium to prove they are available however.

The level of lysine needed to support growth of TPW (as estimated by Rolle, 1975), is sufficient in casein. Thus, total growth capacity should not be affected greatly until lysine becomes the limiting factor. The growth capacity was maximized by choosing the 3 mg nitrogen test level and comparing it to the control to reduce the error in the TPW assay. At lower levels growth is poor and at 4 mg N the assay has high variability (Stott et al., 1963).

The overall results seen in the figures show the browning was more rapid at the lower a_w. This is because of the presence of propylene glycol which is similar to that found by Warmbier et al., (1976). At a_w 0.3 the FDNB procedure showed greater losses over the whole storage time. At the two higher a_w's where the reaction was slower the TPW procedure eventually showed equal or greater protein quality loss. The reasons for this are not clear since one might expect that with greater browning the protein would become less readily digestible to the organism. Thus the lower a_w sample would be expected to show this crossover. On the other hand, it is possible that reaction pathways change with a_w, so that the change in patterns shown

might occur. The data indicate that at stages of advanced browning the TPW test shows equivalent or greater losses in protein quality. The test also indicates the problems that would occur if a similar response is found in rat PER assays. Usually in the latter test, only one system at one storage time and temperature can be evaluated, due to time and labor costs. Thus the value may not be meaningful as to the actual changes that occur during transportation and storage of a food. The TPW test is simple and easy to perform and thus allows comparison of many more test conditions. The problem would be in the evaluation of the reliability of the TPW test. As can be seen, some unexpected fluctuations occur in the TPW assay which cannot be accounted for. This shows that one should not use single point analysis even with reliance on numerous replicates. Rather, one should test more points over a period of time with less replicates, to determine a trend.

Considerable evaluation of the TPW procedure preceded the actual storage study presented here. Variation from trial to trial on total count at a given $\underline{N}$ level was large even with a standard casein control. Strict control of the inoculant level eliminated part of this problem, although biological variation could not be completely eliminated as might be expected. To eliminate the variation, with respect to the casein standard, stored samples at each a_w, were frozen until they could be analyzed in a single TPW test.

Another consideration examined prior to this experiment was the effect of the individual model food components and possible substitute humectants on TPW growth. Each diluted component was added separately to casein reference samples at levels found in Table 1 and also at a final concentration 25 times that level. As seen in Table 3, only the use of the humectant glycerol completely suppressed growth for this organism. Chloroform:methanol (3:1) extraction of the glycerol from a model food system, resulted in normal growth patterns.

These results indicate the care that must be taken if formulated foods are evaluated with Tetrahymena pyriformis W, since they may contain inhibitory substances. It also exemplifies the difficulties that might result from comparison of different food sources to an unrelated reference sample for purposes of protein evaluation.

TABLE 3

EFFECT OF MODEL FOOD SYSTEM COMPONENTS ON TETRAHYMENA RNV

Component	Average RNV 3 mg N/10 ml *
Potassium sorbate	105
Apiezon B oil	102
Microcrystalline cellulose	112
Humectants:	
Propylene glycol	110
Butylene glycol	103
Glycerol	88

* variation was $\pm$ 8 RNV for triplicate samples

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C. <u>Effect of Glycerol on Nonenzymatic Browning in a Solid</u>

    <u>Intermediate Moisture Model Food System</u>

       The following pages contain a copy of the paper which was
published in the Journal of Food Science 41:526 (1976).

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EFFECT OF GLYCEROL ON NONENZYMATIC BROWNING IN A SOLID INTERMEDIATE MOISTURE MODEL FOOD SYSTEM

ABSTRACT

Maillard browning is one of the main chemical reactions causing deterioration and shortening shelf life of intermediate moisture food (IMF) systems. The purpose of this research was to study Maillard browning in an IMF model system containing casein, glucose and the liquid humectant glycerol. The kinetics of pigment production, glucose utilization and loss of DNP-available lysine were studied as a function of temperature, moisture content and water activity. It was found that the factors which control reactant (glucose and available lysine) utilization also control end-product (brown pigment) accumulation. The rate of the Maillard browning pigment production, after an initial induction period, follows zero order kinetics. The initial loss rate of both glucose and available lysine, however, follows first order kinetics. Exceedingly large nutritional (available lysine) losses occur before brown discoloration is appreciable. Slightly greater than one mole of glucose reacts per mole of lysine made unavailable. Based on this, nutritional losses may be relatively easily estimated by monitoring the loss of specific reducing sugars. The Maillard browning reaction proceeds 33 times faster at 45°C than at 25°C, with the maximum rate occurring at $0.4-0.5$ a_w. This is an a_w range considerably lower than the $0.65-0.75$ a_w range usually found for maximum browning in dehydrated foods. The downward shift in the a_w maximum for browning is because glycerol being liquid has water-like properties and increases reactant mobility and/or solubility at a_w's below which most water soluble reactions occur very slowly.

INTRODUCTION

FOOD STABILITY has been shown to be controlled by the moisture content or water activity (a_w) of foods (Bone, 1969; Labuza, 1970, 1975; Lea, 1958). Bacterial degradation of foods can usually be controlled by keeping the water activity of the food at less than a_w 0.9. Yeast and mold growth can be inhibited by maintaining the a_w at less than 0.8 (Bone, 1969). Chemical degradation reactions of foods can usually be controlled by keeping the water activity or moisture content of foods low. Maximum stability is usually thought to occur when the food's moisture content is near the BET (Brunauer et al., 1938) monomolecular moisture layer coverage of foods (Salwin, 1959). As the moisture content increases above the BET coverage, the rate of chemical degradation of foods usually increases. And as the moisture content decreases below the BET coverage, lipid oxidation can again increase to cause rapid degradation of foods.

Water controls the degradative reactions of foods by various means. Osmotic shock, insufficient availability of required nutrients, or a build-up of metabolic end-products that are toxic to the organism are thought to be some of the means by which low moisture contents or a_w limits or inhibits the growth of microorganisms. In addition, the chemical degradative reactions within foods are controlled through the effect of moisture content or a_w on reactant dissolution, mobility and concentration. An increase in moisture content or a_w can increase solute (reactant) solubility and/or mobility and thereby cause the rate of a chemical reaction to increase. Conversely, an increase in moisture content will tend to dilute the concentra-

tion of reactants and thereby decrease chemical reaction rate. If water is a product of the reaction of concern, an increase in moisture content will decrease the reaction rate by mass action, i.e., end-product inhibition. This increase may be small overall, but at the reaction site if diffusion is slow will have a great effect. One or a combination of the above factors may be rate limiting and thereby limit the rate of degradative reactions within a food as the food's moisture content or a_w is increased or decreased.

Maillard browning is a reaction wherein a reducing sugar, such as glucose, combines with a free amino group, such as the ε-amino group of available lysine and thereby decreases the biological availability of the protein. In food products where processing or storage conditions cause an accelerated rate or prolonged duration of the Maillard reaction, a significant loss of food quality may result.

It is generally observed that most foods exhibit a maximum rate of browning near a_w 0.65–0.75. The data of Loncin et al. (1968) shows that for milk powder which was humidified to a moisture content above the BET monolayer, both an undesirable accumulation of brown pigments and a loss of available lysine occurred when the milk powder was held at 40°C for only 10 days. It was observed that the maximum in pigment production occurred near a_w 0.65, an a_w of intermediate moisture foods, with a concomitant loss of 75% of the available lysine. A similar observation is found for most dehydrated foods humidified to an a_w common to intermediate moisture foods. Nonenzymatic browning (NEB) is one of the main degradative reactions that often occurs at a maximum rate in intermediate moisture foods which have an a_w range of 0.60–0.85 (Karel and Labuza, 1969; Labuza, 1970; Loncin et al., 1968).

Very little data are available on the extent of Maillard browning in IMF systems to which a liquid humectant has been added. A liquid humectant can increase the palatability of a food or lower the water activity of the food and thereby increase its microbial stability. Eichner and Karel (1972) studied the extent of Maillard browning in a liquid model system of glycine-glucose which contained the humectant glycerol. They found that under certain conditions the maximum in browning, as measured by pigment production, occurred near a_w 0.4. They concluded that glycerol, through its plasticizing effect, partially restores reactant mobility to increase the browning rate at low moisture contents.

Since solid IMF items, such as meal replacement items, are becoming more common in the marketplace, a study of the rate of the Maillard browning reaction as well as a determination of the loss of nutritional value within these IMF foods is necessary. This current study examines the rate of Maillard browning as it occurs during the storage of a solid IMF model system which contains the liquid humectant glycerol.

MATERIALS & METHODS

TO FACILITATE the collection of browning data, a model food system was used. The composition of the model system is shown in Table

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1. Potassium sorbate was used as an antimicrobial agent. Glucose is the sole source of reducing sugar for the Maillard reaction in this study. Glycerol is used as a liquid humectant to control the water activity and plasticity of the model system. Casein serves as the only source of free amino groups for the Maillard reaction. Apiezon B oil, which is liquid at room temperature, adds plasticity to the product. It is saturated and therefore should not participate in the Maillard reaction. Microcrystalline cellulose is inert to the Maillard reaction and serves as a solid support for the model system. Water is added in varying amounts so the rate of Maillard browning could thereby be studied as a function of water activity or moisture content. The average initial glucose/available lysine molar ratio was 2.9.

The components, as tabulated in Table 1, were mixed together in descending order of appearance in the Table. Water was added by two different methods such that the effect of method of water addition on the rate of Maillard browning could be studied. Direct Mix systems were brought to proper moisture content by mixing in a predetermined amount of liquid water to the nonaqueous ingredients. The Direct Mix samples were then held in vacuo over an appropriate saturated salt solution (Rockland, 1960) for 1 day at room temperature for final moisture equilibration. The Humidified Mix samples were humidified by storing the mixed nonaqueous components in vacuo over an appropriate saturated salt solution for 3–6 days at room temperature until the proper water activity was obtained. Moisture content was measured with a methanol extraction GC technique (Tjhio et al., 1969). Water activity was measured by a vapor pressure manometric technique (Karel and Labuza, 1967; Karel and Nickerson, 1964).

Following the formulation process, including the addition of water, the samples were transferred to 202 × 214 epoxy-lined cans. The cans were sealed and the newly sealed ends were dipped in glyptol to retard moisture loss should the can seal not be perfect. The canned samples were then incubated at 25, 35 or 45°C and periodically analyzed for extent of Maillard browning. If sample analysis could not be done on the desired day, the samples were held at −29°C until the analysis could be performed.

The Maillard browning reaction was monitored by measuring NEB (melanoidin) pigment production, glucose utilization, and loss of available lysine. NEB pigment concentration was measured by the trypsin digest, aqueous extract procedure of Choi et al. (1949) as modified by Labuza (1971). Glucose content was measured with a glucose oxidase Blood Sugar test kit [Bolhringer Mannheim Corp., New York, Cat. No. 15756. Method: adapted from Werner, W., Rey, H.G. and Wielinger, H. (1970). Z. Anal. Chem. 252: 224]. The FDNB method of Booth (1971) was used to measure available lysine content.

RESULTS & DISCUSSION

THE INCREASE of pigment concentration as a function of storage time, temperature and a_w is presented in Figure 1. After an initial induction period, the amount of NEB pigment increases linearly with time for each condition. During the initial induction period, predominantly colorless browning intermediates are being formed. After a sufficient amount of these intermediates have been formed, the rate of pigment production follows zero order kinetics.

Temperature controls both the length of the induction period and the rate of pigment production during the zero order period. At the higher temperature, the rate of NEB pigment production is increased. The average activation energy for pigment production is 32.9 kcal/mole, which gives a Q_{10} of about 6.1 between 25 and 35°C and 5.4 between 35 and 45°C. Thus, the reaction occurs 33 times faster at 45°C than at 25°C. Also, as is shown in Table 2, the induction time is less at the higher temperature.

The 35°C data of Figure 1 also show that browning rates are controlled by water activity. The influence of water activity on browning rate is further shown in Figure 2 for 35 and 45°C. The same pattern was also found at 25°C (Warmbier, 1975). As seen, the maximum rate of NEB pigment production occurs at a_w 0.45–0.55; this is unlike that found in most solid food systems which show a maximum rate near a_w 0.7–0.8. The rate maximum at 35°C occurs at a moisture content of 8.9g H_2O/100g solids. At 45°C, the maximum is at 10g H_2O/100g solids. The calculated BET monolayer for the systems of this study is 8.1g H_2O/100g solids. A dehydrated

Table 1—Model system composition

Component	Grams
K-sorbate	0.3
Glucose	10.0
Glycerol	20.0
Casein	30.0
Apiezon B oil	20.0
Microcrystalline cellulose	20.0
Water	Variable

Table 2—Percent loss of reactants (glucose and available lysine) during the induction time for melanoidin pigment production

Temp (°C)	Avg a_w	Browning induction time (Days)	Glucose % Loss	Lysine % Loss
25	0.86	100	10	60
25	0.72	100	21	65
25	0.67	100	26	66
35	0.87	20	13	20
35	0.51	20	33	60
35	0.32	20	18	12
45	0.84	7	29	37
45	0.39	7	33	69
45	0.14	7	17	71
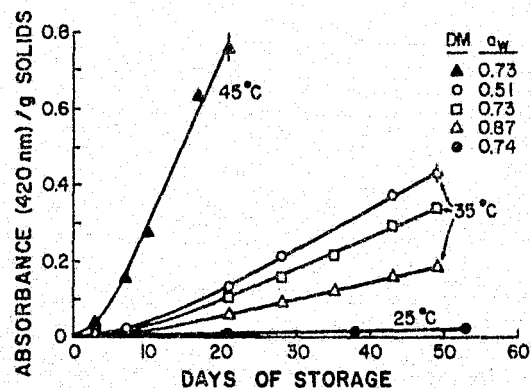
Fig. 1—Browning pigment production as a function of time in model systems as a function of a_w and temperature. DM—direct addition of water to system.
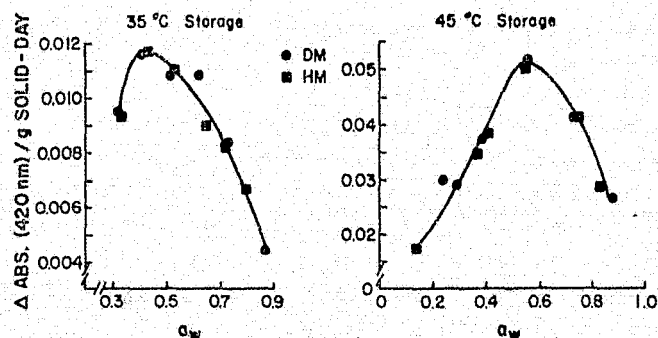
Fig. 2—Rate of browning as a function of a_w at 25 and 35°C. DM—water added directly as liquid; HM—water added by humidification.

food is usually considered to be most stable to chemical reactions if its moisture content is at or near the BET monolayer (Salwin, 1959). In this study, however, with a liquid glycol added a maximum in the rate of degradation occurs close to the BET value.

The above observation is similar to the findings of Eichner and Karel (1972). Their liquid model system containing glycerol had a maximum in browning at a_w 0.41 when stored at 37°C. They concluded that glycerol can increase the rate of browning at low a_w values by increasing reactant mobility and that as a_w increases, the water decreases the browning rate by a mass action effect. This should also be the mechanism in the solid food systems used in this current study. The results of
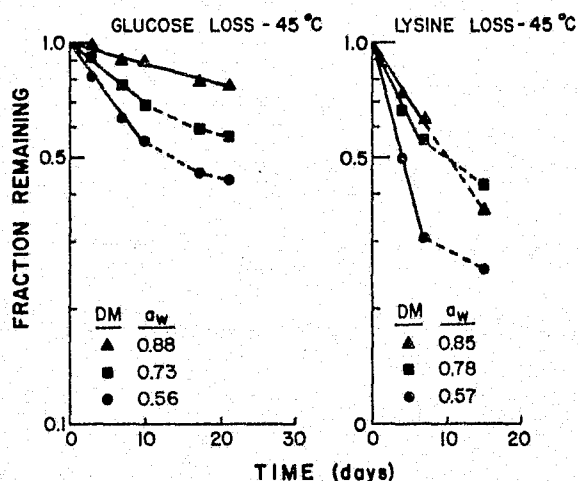
Figure 2 thus indicate that glycerol could cause a decrease in browning if it is added to intermediate moisture foods.

One additional observation to be drawn from Figure 2 is that the method of water addition to the food samples does not appreciably influence the rate of NEB pigment production. At any given water activity or moisture content, samples to which liquid water was added directly (Direct Mix system) browned at the same rate as those samples to which water was added by a vapor humidification process (Humidified Mix system). This suggests that solubilization of the reactants is the same no matter what the method of addition of the water is.

As stated earlier, the induction period must occur before the rate of pigment production follows (constant) zero order kinetics. During this initial storage period when pigment production is not yet appreciable, reactants of the Maillard reaction are utilized to form colorless browning intermediates. Figure 3 shows the loss of both glucose, a reducing sugar, and available lysine at 45°C. The lysine serves as a source of free ε-amino groups for the formation of glycosylamines for the Maillard reaction. The data indicate that initially the destruction rates of both glucose and available lysine follow first order kinetics. A significant amount of glucose and available lysine is destroyed even before pigment production becomes appreciable. As is shown in Table 2, up to one-third of the glucose and, more importantly, as much as 70% of the available lysine is reacted before the rate of pigment production follows zero order kinetics and visual browning appears. Obviously this has serious nutritional implications. A food in which Maillard browning can occur may not have produced pigments at a sufficient rate such that the color of the food becomes objectionably brown during processing or storage. However, the protein nutritional loss within the food, as indicated by decreased available lysine content, may be significant.

As previously indicated in Figure 3, water influences the rate of glucose and available lysine loss. Figures 4 and 5 show the initial (first order) loss rate of glucose and available lysine at 35 and 45°C as a function of water activity. In general, the

Fig. 3—Loss of glucose and lysine vs time as a function of a_w at 45°C. DM—water added directly as liquid.
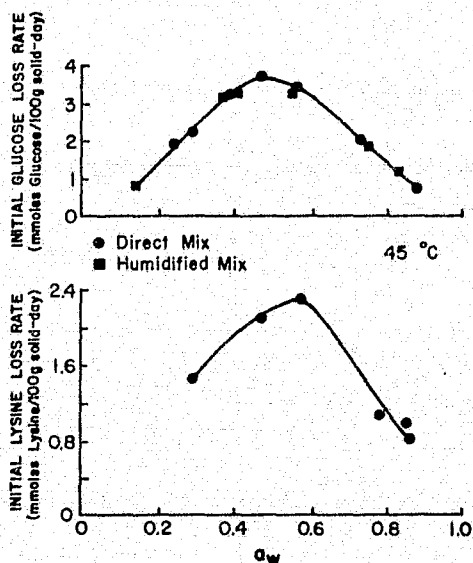
Fig. 4—Initial glucose and lysine loss rates as a function of a_w at 45°C.
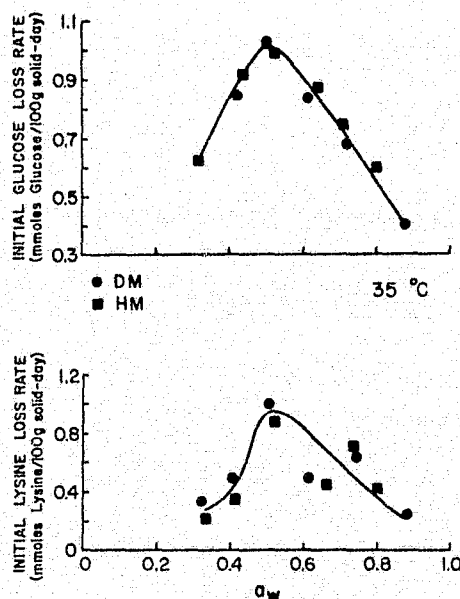
Fig. 5—Initial glucose and lysine loss rates as a function of a_w at 35°C. DM—water added as liquid; HM—water added by humidification.

loss rate of both glucose and available lysine are controlled in the same way by water activity. It can also be observed that the maximum loss rate of these reactants occurs near the same a_w as for the maximum rate of browning.

Loncin et al. (1968) showed that maximum lysine loss in dehydrated foods occurs at an a_w greater than approximately a_w 0.6. Their food systems were very similar to our model systems except a liquid humectant was not added to their systems. Therefore, the downward shift in the a_w maximum for lysine loss and glucose loss must be caused by glycerol in a manner similar to that which controls NEB pigment production as proposed by Eichner and Karel (1972). The practical significance of the addition of glycerol is thus obvious. Glycerol, or perhaps other liquid humectants, when added to meal bars, semi-moist pet foods, or other IMF's, can decrease the rate at which lysine, and perhaps other essential nutrients with free amino groups, becomes unavailable.

One further observation drawn from this study is that at 35 and 45°C, an average of 1.3 moles of glucose initially react for one mole of lysine that becomes unavailable. From a practical standpoint, the monitoring of the loss of glucose or other specific reducing sugars may be used as a relatively rapid and easy method for estimating the nutritional (available lysine) loss of foods suspected of being degraded by the Maillard nonenzymatic browning reaction. This type of method is much more simple than the FDNB procedure which requires much technical training and over 24 hr for the determination of available lysine.

SUMMARY

THREE SIGNIFICANT and practical conclusions can be found from this research. First, the addition of glycerol to intermediate moisture foods can cause the a_w maximum for the Maillard reaction to be shifted downward. Thus, IMF's which contain glycerol, or perhaps other liquid humectants, would have a browning rate that is less than would occur if a liquid humectant were not present. Second, the Maillard reaction can cause significant nutritional losses, e.g. of available lysine, to occur before the food has become appreciably or objectionably brown. Third, it is possible that the protein nutritional loss of foods susceptible to Maillard browning may be relatively easily and quickly estimated by following specific reducing sugar loss during storage.

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D. Nonenzymatic Browning Kinetics in an Intermediat Moisture

Model System: Effect of Glucose to Lysine Ratio

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NONENZYMATIC BROWNING KINETICS
IN AN INTERMEDIATE MOISTURE MODEL SYSTEM:
EFFECT OF GLUCOSE TO LYSINE RATIO

———————— ABSTRACT ————————

An intermediate moisture model food system containing casein, glucose, glycerol, oil, microcrystalline cellulose and water, was used to study nonenzymatic browning. The initial molar ratio of glucose to available lysine was varied from one-half to five. The model food system samples were prepared to 0.52 water activity and were stored in sealed cans at 45°C and analyzed periodically. The browning was followed by determining pigment accumulation, glucose utilization, and loss in FDNB-available lysine. The rate of pigment formation followed zero order kinetics after an initial short induction period. This rate increased linearly as the initial molar ratio of glucose to available lysine increased from one-half to three. Above this ratio the rate did not change. The initial rate of glucose utilization and available lysine loss obeyed first order kinetics, and increased as the initial molar ratio of glucose to available lysine increased from one-half to five. This work shows that in the presence of glycerol, the initial condensation reaction is not the rate controlling step for pigment production.

INTRODUCTION

NONENZYMATIC BROWNING through the Maillard reaction is a major deteriorative reaction in intermediate moisture and humidified dry foods (Burgess and Mellentin, 1965; Choi et al., 1949; Labuza, 1972; Lea, 1958; Loncin et al., 1968; Patton, 1955; and Warmbier et al., 1976). Identification of those physical chemical factors which control the browning reaction would be useful in establishing methods of prevention. Several in-depth studies have elucidated some of these factors (Ellis, 1959; Hodge, 1953; Reynolds, 1963 and 1965). In general, the rate or extent of browning increases with increasing pH and temperature during processing or storage of foods. In addition, the rate increases as moisture content or water activity increases up to a maximum at a_w 0.6–0.8 and then decreases again (Labuza, 1970; Lea, 1958; Sharp, 1957). The presence of humectants such as propylene glycol or glycerol (Eichner and Karel, 1972; Warmbier et al., 1976), decreases the rate of nonenzymatic browning at high a_w. The browning rate is significantly influenced by the concentration of reducing sugars and free amino groups from proteins which are the primary reactants (Lea and Hannan, 1949; 1950). The purpose of this study was to determine the influence of the sugar/amine molar ratio on the kinetics of the reaction in an intermediate moisture model system.

MATERIALS & METHODS

A MODEL FOOD SYSTEM with the composition shown in Table 1 was used. Moisture content was measured with a methanol extraction, gas chromatographic technique (Tjhio et al., 1969). Water activity (a_w) was measured by a vapor pressure manometric technique (Karel and Nickerson, 1964).

The ingredients were added together in descending order of appearance in Table 1, except water was the first component added for one-half of the samples (Sol Mix – SM) and the last component added for the other half of the samples (Direct Mix – DM).

Following formulation, the samples were held for 24 hr at room temperature in evacuated desiccators over a saturated magnesium nitrate solution (a_w = 0.52). Samples were then sealed in epoxy-lined 202 × 214 cans and stored at 45°C and periodically analyzed. Most samples were held at –29°C until analysis could be done. Duplicate samples were always taken and the average calculated.

The water activities of the samples varied from about 0.52–0.60. In that a_w region Warmbier et al. (1976) showed that the rate of browning varied by less than 10% so that the variation should not affect the rate significantly. Brown pigment accumulation was measured by the trypsin digest, aqueous extract procedure of Choi et al. (1949) as modified by Labuza (1971). The fluorodinitrobenzene (FDNB) method of Booth (1971), was used to measure available lysine content. Glucose content was measured with a glucose oxidase Blood Sugar test kit (Boehringer

Table 1—Model food system composition

Component	Grams
Potassium sorbate	0.3
Glucose	Variable
Glycerol	20.0
Casein	30.0
Apiezon B oil	20.0
Microcrystalline cellulose	20.0
Water	Variable
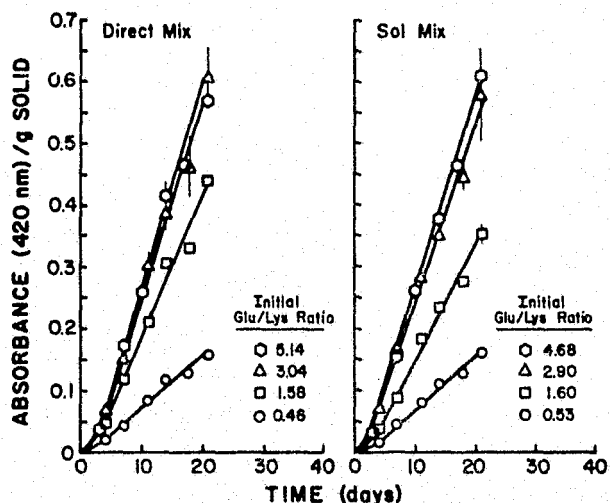
Fig. 1—Amount of brown pigment formed as a function of time for various G/L ratios, 45° C a_w 0.52.
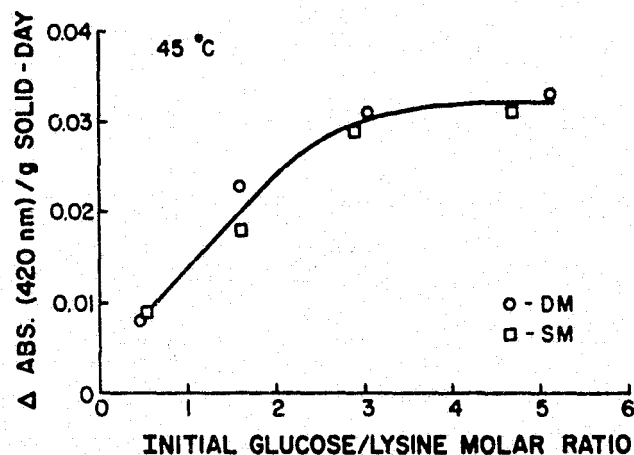
Fig. 2—Rate of brown pigment formation as a function of G/L molar ratio.

Mannheim Corp., New York, Cat. No. 15756. Method: adapted from Werner, W.H., Rey, G. and Willinger, H. 1960. Z. Anal. Chem. 252: 224).

RESULTS & DISCUSSION

THE INCREASE in nonenzymatic browning (NEB) pigment accumulation as a function of storage time is shown in Figure 1. All samples show an induction period of about 1 day and then a constant browning rate occurs. This is similar to that found by Lea and Hannan (1949) and Warmbier et al. (1976). This constant rate is indicative of a zero order reaction. Figure 1 shows that as the initial glucose to DNP-lysine molar ratio increases, the browning reaction rate increases.

The zero order reaction rate constants obtained from the slope of the linear portion of the curves in Figure 1 are tabulated in Table 2 and are presented graphically in Figure 2. The rate of pigment production approaches a plateau at an initial glucose to lysine (G/L) molar ratio of approximately three. This is similar to that found by Lea and Hannan (1950), for a

casein-glucose model food system humidified to a_w 0.7. Thus even though glycerol lowers the browning rate maximum in this system as found by Warmbier et al. (1976), it does not cause a change in the overall mechanism.

The fractional loss of glucose as a function of storage time is shown in Figure 3 on a first order semi-log plot. The results show that for up to at least 50% loss the initial condensation reaction of glucose with amines is first order, not zero order as the pigment production follows. If this were a zero order reaction the data points would begin to fall below the straight line after about 30% loss.

The slope of Figure 3, which is the loss rate constant, increases (Table 2) as the G/L ratio decreases. The reasons for this are unclear but may be due to the increased phase viscosity as the glucose level increases. At a ratio of from 1.5—5 the moisture content was about 10—12g H_2O/100g solids. At a 0.5 G/L ratio the moisture content was 14—16g H_2O/100g so the glucose concentration per unit volume is much less and the viscosity is much lower. Lee and Labuza (1975) have shown that phase viscosity has a direct effect on the first order break-

Table 2—Browning kinetic parameters

System	Moisture g H_2O/g solid	G/L ratio[a]	Browning rate Δ abs/g solid per day X 10^2	Glucose loss rate constant (day)$^{-1}$	Glucose initial loss rate mmoles/100g/day	Lysine loss rate constant (day)$^{-1}$	Lysine initial loss rate mmoles/100g/day	G/L loss rate ratio
Direct mix	10.4	5.14	3.3	0.062	3.74	0.181	2.12	1.76
Sol mix	10.5	4.68	3.1	0.062	3.74	0.232	2.97	1.26
Direct mix	10.8	3.04	3.1	0.087	2.91	0.156	1.71	1.71
Sol mix	11.8	2.90	2.9	0.084	2.88	0.163	1.92	1.50
Direct mix	11.3	1.58	2.3	0.117	2.26	0.128	1.57	1.44
Sol mix	11.8	1.60	1.8	0.083	1.54	0.074	0.85	1.81
Direct mix	14.2	0.46	0.8	0.120	0.73	0.064	0.84	0.87
Sol mix	16.2	0.53	0.9	0.110	0.67	0.042	0.46	1.46

[a] Initial glucose/lysine ratio
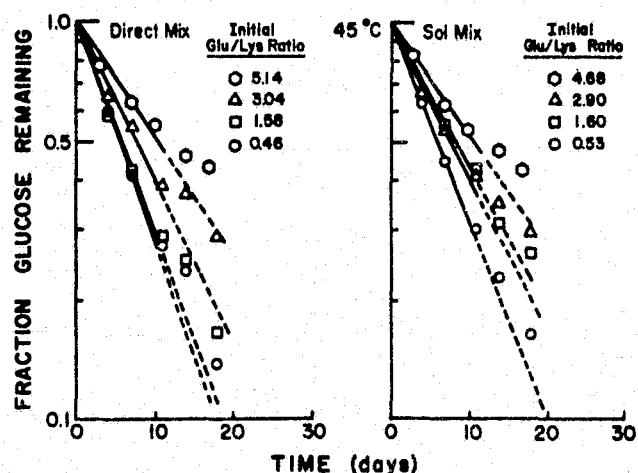
Fig. 3—Fractional loss of glucose as a function of time for various G/L ratios at a_w 0.52.
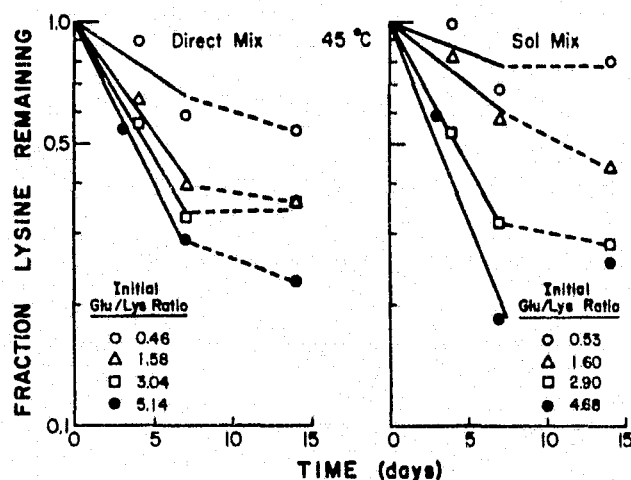
Fig. 4—Fractional loss of lysine as a function of time for various G/L ratios at a_w 0.52.

down of vitamin C with a higher value as the viscosity decreases. However, despite the reduced rate constant, the amount of glucose lost per unit time initially is greater as the G/L ratio increases. This increase as shown in Table 2 does not plateau out as did the browning rate. It is also obvious from the kinetics that the glucose/amine condensation reaction does not control the rate of pigment formation since the rate of browning does not decrease even though over 50% of the glucose disappears. This verifies that the polymerization steps are the rate controlling ones.

The reason that the browning rate increases as glucose level increases is most likely due to its closer proximity to the amino groups thus overcoming the greater diffusion barrier caused by the higher viscosity. This is indicated in Figure 4 by the lysine loss. The data show that an increase in the lysine loss rate constant occurs as the G/L ratio increases. Since the protein is set in the matrix of the system, diffusion should not affect it. The rate constants and initial loss rates are shown in Table 2.

Lea and Hannan (1950), found that the lysine loss rate was at a maximum at a G/L ratio of 3/1. However, our data show a continuous increase in accordance with the above model. They assumed zero order kinetics since their results are based on endpoint analysis (i.e., amount lost in some time equals rate). Our data also indicate a greater amount of glucose utilized per mole of lysine. Most likely, this is due to reactions with other available amino groups or regeneration of amine in the initial reaction steps. Finally, there seems to be no difference between the methods of addition of water to the systems.

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E. <u>Effect of Protein Substitution on Nonenzymatic Browning</u>

   <u>in an Intermediate Moisture System</u>

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Effect of Protein Substitution on Nonenzymatic Browning in an Intermediate Moisture Food System

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The effect of various proteins on the loss of lysine and rate of nonenzymatic browning was studied in an intermediate moisture food system containing glucose as 10% of the solids. The system was prepared to a water activity range of 0.6–0.8 and the samples were held at 35 °C to accelerate the reaction. It was found that proteins which showed long induction times before color development also had lower browning rates. The proteins also showed over 50% loss of available lysine soon after initial color development occurred. Free lysine added to the formulation caused very rapid browning. No pattern of browning rate and lysine loss existed with the total lysine availability.

Nonenzymatic browning reactions occur very widely during processing and storage of food materials. The colors produced range from pale yellow to very dark brown, depending on the type of food and/or the extent of the reaction. Reviews of the reaction have been made by Hodge (1953), Bender (1970), Carpenter and Booth (1973), Ellis (1959), and Reynolds (1963, 1965).

During food processing and storage, nonenzymatic browning can take place when reducing sugars and proteins react in the presence of H_2O to form brown pigments. This results in the production of off-flavors (Markova et al., 1972) and loss of solubility and protein nutritional value (Rao and Rao, 1972; Lea, 1958). This loss of protein nutritional value is due to the fact that lysine, an essential amino acid, is a primary reactant, although other amino acids are reactive. Nonenzymatic browning is especially important in intermediate moisture foods because the amount of water present results in a much greater reaction rate than in dry food systems (Lea and Hannan, 1949, 1950; Labuza, 1971). The rate usually has a maximum in the intermediate moisture food (water activity 0.6–0.85). Because of the high reaction rate in this a_w region, processors have a problem in the use of reducing sugars such

as dextrose or corn syrup solids for food formulations.

Many intermediate moisture foods have been developed recently, such as complete breakfast replacements and high nutrition dietary bars. The intermediate moisture range was chosen for these products because of the good palatability while still maintaining the stability of the food toward microorganisms (Labuza, 1971). Unfortunately, even though microbial stability can be achieved, chemical degradation cannot. For example, loss of protein nutritional value as discussed previously, rancidity, and vitamin losses all can occur unless the proper additives or formulations are used (Chou et al., 1973; Lee and Labuza, 1975). It is quite evident that more information is needed on the stability of these foods.

The purpose of this study was to examine the effect of protein substitution in an intermediate moisture food model system on the rate of nonenzymatic browning. The supply and/or the cost of the various proteins are such that food companies may find it necessary to substitute their usual protein source with a different protein source. The problem lies in that the new protein may alter the stability of the finished product, specifically with respect to the rate of nonenzymatic browning during storage.

There are three important factors related to nonenzymatic browning when considering using a protein for an intermediate moisture food formulation. The first consideration is the induction time prior to visual detection of an increase in brown color. Second is the overall change in color due to the pigment production during the expected shelf-life of the product. The last consideration is the

Ingredient	%
K-sorbate	0.3
Glucose	10
Glycerol	20
Protein	30
Apiezon B oil	20
Microcrystalline cellulose	20
Water	As per Table II

amount of available lysine lost.

MATERIALS AND METHODS

Model System. The proteins that were examined in this study were casein (Technical grade, Coleman Bell, Inc.), egg albumin (Technical grade, Coleman Bell, Inc.), whey protein (prepared at the University of Minnesota, spray dried, delactosed), fish protein concentrate (Bureau of Commercial Fisheries), wheat gluten, and spin textured soy protein (General Mills, Inc.). The wheat gluten also was studied with 10% lysine addition (weight/weight of protein). These proteins were substituted on a strict weight basis; thus the initial levels of available lysine (Table II) and inherent reducing sugars would be different. However, this was done as it might be used commercially. The amount of residual reducing sugars in the protein should make no difference in the rate of browning since the amount of glucose added exceeds the lysine by a 3:1 molar ratio as shown by Lea and Hannan (1950). Above this ratio the browning rate is at a maximum. The composition of the dry model system is shown in Table I.

The moisture content was determined on the "dry" mixed ingredients for each system by a GLC technique as described by Tjhio et al. (1969). Deionized water was then added to each system to achieve approximately 20% moisture in the final system. The final water activities were determined by the vapor pressure manometric technique (Labuza et al., 1976). The results are presented in Table II.

Storage of Samples. The samples were sealed in 202 × 214 cans, with the ends sealed with an epoxy resin to prevent water loss. The cans were stored at 35 °C for 60 days and sampled periodically for nonenzymatic browning, pigment production, and loss of available lysine. Duplicate samples were taken at each time used.

Browning Determination. The Maillard browning reaction was monitored by measuring NEB (melanoidin) pigment production by a trypsin digest, aqueous extraction procedure of Choi et al. (1949) as modified by Labuza (1971). The available lysine content was determined by the FDNB method of Booth (1971).

RESULTS AND DISCUSSION

The results of pigment production as a function of time are shown in Figure 1. The rate of browning is calculated
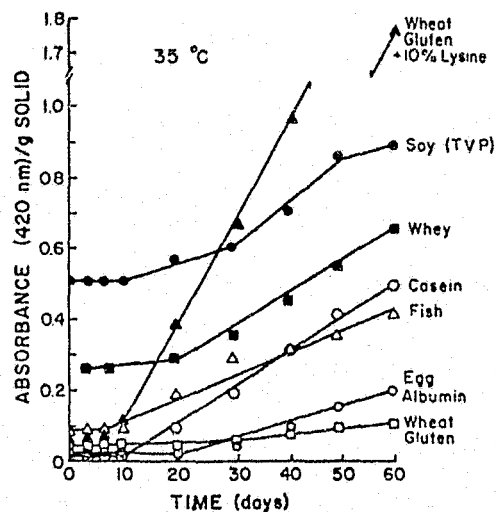
Figure 1. Extent of nonenzymatic browning in an intermediate moisture model food system with different proteins added at 35 °C.

by the slope of the line after measurable browning started occurring. The induction time was determined as the time up to where the amount of browning starts to increase by a zero-order reaction. These are presented in Table II. As seen, the wheat gluten has the largest induction time and the slowest rate, while the wheat gluten with lysine fortification has the shortest induction time and the greatest browning rate. Figure 1 also shows the different initial brown pigment which is due to preprocessing. This obviously has some effect on the build up of reacting intermediates. As noted in Table II, there is some influence between the induction time and browning rate, with a lower rate if the induction time is longer, except in the case of the fish protein concentrate system. Also, egg albumin reacts about as fast as the other proteins even though there is a low available lysine in the initial sample. This low value may have been due to initial overprocessing as generally one considers egg to be a high quality protein.

Table II also shows the time to reach a 50% loss of available lysine in the model systems. As seen, the destruction of lysine occurs very rapidly. In the case of the fortified gluten and the soy, this occurred prior to any increase in brown pigment over the initial value. For the other proteins over 50% loss occurred within about twice the induction time except for egg albumin. The fastest lysine loss occurs in the fortified product as would be expected.

For the other proteins a different pattern exists for lysine loss than found for nonenzymatic browning. Soy has the most rapid lysine loss as compared to casein which has the highest rate of browning. There is no pattern, however,

Table II. Results of Browning and Lysine Loss during Storage at 35 °C

Proteins	Total available Lys[a] content, mg/100 g of solids	Moisture content, g of H₂O/100 g of solids	a_w	Induction time, days	Browning rate, OD/day × 10³	Time for 50% lysine loss, days
Wheat gluten + 10% free lysine	3300	20.72	0.72	7	35.0	<1
Casein	1416	18.34	0.70	13	10.2	22
Whey	857	18.53	0.78	20	8.5	25
Soy (TVP)	762	20.00	0.73	20	7.5	7
Fish concentrate	1283	18.25	0.68	8	5.9	19
Egg albumin	360	19.76	0.63	20	4.9	>60
Wheat gluten	303	19.05	0.66	30	0.9	40

[a] By FDNB procedure.

for browning and lysine loss even based on initial lysine content. Obviously this variability is probably due to the reaction of other amino acids such as arginine, glutamine, asparagine, histidine, and tyrosine, as well as to structural availability of the amino acids.

This simple study shows that protein substitution in formulated IMF products is a complicated situation with respect to chemical and nutritional stability. The processor might substitute with a protein product of lower lysine content but this does not ensure either increased stability to browning or retention of the lysine content. In addition, this work verifies that supplementation with free lysine in an IMF product which contains reducing sugars cannot be done. Lastly, this work points out that some accelerated test for browning should be used in product development to determine applicability of a protein in an IMF product such as done by Mizrahi et al. (1970).

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Preparation of Optically Active Proline. Optical Resolution of N-Acyl-DL-proline by Preferential Crystallization Procedure

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To establish a practical method for the production of optically active proline, the optical resolution of DL-proline by a preferential crystallization procedure was investigated. DL-Proline was easily resolved by converting it to simple N-acyl derivatives, such as N-acetyl-, N-chloroacetyl-, N-n-butyryl-, and N-isobutyrylproline. The optically active acylproline obtained by this resolution method was hydrolyzed to the optically active proline without racemization. The undesired optically active acylproline was readily racemized into the racemic modification and could be reused for the resolution step.

Optically active proline is an important substance in the pharmaceutical and food industries. L-Proline, especially, as well as the essential amino acids, has been proven to be necessary in parenteral nutrition (Dolif and Juergens, 1971) and is widely used as a component of amino acid infusion.

L-Proline has been produced by hydrolysis of natural protein or by fermentation methods. To find a more economical method for the production of optically active proline, we have investigated the optical resolution of synthesized DL-proline. DL-Proline can be synthesized by several chemical methods, for instance, by the method of Albertson and Fillman (1949).

With respect to the optical resolution of DL-proline, chemical and enzymatic procedures have been reported (Greenstein and Winitz, 1961; Kovács et al., 1957; Vogler and Lanz, 1966). These conventional methods seem to be laborious and unsatisfactory for commercial production.

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On the other hand, the preferential crystallization procedure is considered to be one of the most useful methods for industrial application, since it enables the desired optically active isomer to crystallize preferentially from a supersaturated solution of the racemic modification. So far as proline is concerned, no report has appeared on optical resolution by this simple procedure. Therefore, the optical resolution of DL-proline by the preferential crystallization procedure has been investigated. The advantages of this simple resolution method and the screening methods for resolvable derivatives were described in our previous reports (Yamada et al., 1973a,b; 1975a,b).

DL-Proline itself had no properties suitable for this resolution method. It was reported by Hamer and Greenstein (1951) that the melting points of L isomers of N-acetylproline and N-chloroacetylproline are much higher than those of the corresponding racemic modifications. This satisfies one of the conditions under which they exist as a racemic mixture (conglomerate). Since the most desirable situation for the preferential crystallization procedure is that the racemic modification crystallizes as a racemic mixture, they might be expected to be resolved by this simple method. Therefore, N-acetyl-, N-chloro-

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F. Summary and Recommendations

It has been found that nonenzymatic browning is a significant
reaction leading to deterioration in IMF foods. In addition, signi-
ficant losses in the protein nutritional value occur due to the reaction
of lysine with reducing compounds. Alteration of the composition can
significantly affect the rate especially with respect to water activity.
The effect of water has been proposed to be a change in reaction pathways.
The specific findings and recommendations with respect to the study
of improvement of IMF stability are:

1. Tetrahymena pyriformis W (TPW) a protozoan can be used
as a biological technique to analyze for protein nutritional loss. In
IMF, TPW indicates less loss during early browning stages than would
be found by chemical means.

2. The use of the TPW assay for protein quality could save
significant time and costs in product development and shelf life testing
as compared to rat PER. Since rat PER studies require preparation of
the product in quantity to feed at least 20 animals for thirty days
for each point desired, it becomes impractical to use it for anything
other than an endpoint determination. Further, it would be impossible
for use in a shelf life study where reaction rate determination would
be needed especially if several moisture contents and temperatures
are included. The TPW assay makes all these possible.

3. Glycerol as well as other liquid humectants slow the
browning rate in the IMF a_w region. In addition, loss of lysine
follows a similar loss pattern degrading by a first order mechanism.

4. The glucose to lysine ratio significantly controls the browning rate at up to a 3:1 molar ratio in model systems. Above that further increases in reducing sugar have no effect on the rate. This information can be used in product development to insure minimizing the browning rate.

5. Substitution of proteins in IMF systems significantly affects both the browning rate and lysine loss. Generally, the higher the lysine content the greater the browning rate and loss of lysine but no direct correlation exists. Therefore, processors should shelf life test their products prior to marketing especially with respect to lysine nutritional loss.

III.   EFFECT OF HIGH TEMPERATURE AND A_W ON VITAMIN C LOSS IN THE
IMF REGION

A.   Introduction

The loss of nutritional quality during processing and
storage of foods has become increasingly important with the advent
of fortified foods and nutritional labeling regulations. Methods
to predict the extent of these losses can only be successful when they
are based on sound chemical principles. The study of chemical reaction
kinetics, a mathematical approach to chemical reaction rates, provides
a method which can be extremely valuable in the prediction of nutri-
ent losses in food. Labuza (1972) discussed the application of chemical
kinetics to deteriorative reactions occurring in dry and IMF foods.

Most of the available data concerning the kinetics of
nutrient degradation comes from the chemical literature. In the majority
of cases such data was obtained by studying the behavior of nutrients
in systems of simple design under specific conditions of pH, temperature
and ionic strength. While these studies yield valuable information
about the mechanisms involved in nutrient destruction, the resulting
kinetic parameters are difficult to apply to the more complex situation
encountered in a real food. Conversely, the food science literature
contains much information about the destruction of nutrients in specific
foods. However, most of this information concerns only the extent
of nutrient loss for a specific food subjected to a certain process.

Data resulting from this type of endpoint analysis is often impossible to analyze from a kinetic standpoint. This lack of kinetic data for nutrient loss in real foods makes accurate predictions of nutrient loss during the production or storage of food difficult. Endpoint analyses permit evaluation of existing processes, but give little information about what nutrient losses could be expected under different conditions of processing or storage. A knowledge of the kinetic parameters of nutrient degradation in specific foods makes possible accurate predictions of nutrient loss during processing or storage, under a wide range of conditions.

The relatively few studies which have been made involving the kinetics of nutrient degradation in food have been performed under temperature conditions more closely approximating those of storage, rather than of processing. Vojnovich and Pfeifer (1970) for example, studied the stability of ascorbic acid (vitamin C) in blends with wheat flour, corn-soya-milk and infant cereals. Samples stored at temperatures of 26, 37 and 45°C. Semi-log plots of percent retention of ascorbic acid vs. time yielded straight lines for all storage tests; hence the degradation of ascorbic acid in this case followed first order kinetics. Singh et al., (1976) investigated the kinetics of ascorbic acid oxidation in infant formula during storage at 7.2°C. In this case, the oxidation of ascorbic acid was found to follow second order kinetics when oxygen was limiting.

Workers in both of the above studies gathered kinetic data at or near normal storage temperatures. Other researchers have gathered

data at higher temperatures and by use of the Arrhenius equation (describing reaction rate as a function of absolute temperature) attempted to make predictions of storage stability at temperatures closer to those encountered in storage. Use of this technique allows kinetic data to be gathered rapidly at higher temperatures where rates are accelerated, rather than at lower temperatures, where low rates of reaction necessitate lengthy experiments. There is danger however, in extrapolating data over too wide a temperature range. The activation energy carries a slight temperature dependency and may not be constant throughout large temperature ranges. Furthermore, a change in temperature may have indirect effects on reaction rate via competing reactions, change in system composition, etc. Pelletier (1973) studied ascorbic acid degradation in multivitamin capsules and tablets held at 50, 60, and 70°C. Using the activation energies obtained from the resulting kinetic data, predictions were made for ascorbic acid stability in capsules and tablets held for up to 3 years, with good results. He reported degradation of ascorbic acid by a zero order mechanism (linear with time) at these temperatures. These results could just as easily have been interpreted as first order, however, since the degradation was only followed to a level of 70% retention. Zero order (linear) and first order (logarithmic) plots are difficult to differentiate during the initial stages of a reaction.

Just as it is dangerous to extrapolate kinetic data from high temperatures to low ones, it is difficult to apply kinetic data gathered at low temperatures to reactions occurring at high temperatures.

It is often tempting to gather data at low temperatures where reaction
rates are low, rather than at high temperatures where rates can be
extremely high and difficult to measure accurately. In addition,
high temperature studies are complicated by many other factors, most
notably those associated with heating or cooling lags and moisture loss.
If these problems can be controlled, it is definitely preferable to
gather data at or near the temperature conditions under which predictions
will be tested. Waletzko and Labuza (1976) have discussed the
methodology and theory of accelerated shelf life testing in foods.

In addition to using relatively low temperatures, most kinetic
studies of nutrient degradation have another aspect in common:
predictions based on kinetic data are tested under conditions of steady-
state. While conditions of storage may approximate steady-state temper-
atures, (but more often do not), processing conditions rarely do.
Nearly all processes which involve heating or cooling exhibit "come-up"
or "come-down" time, which is the time lag necessary for the product
to reach a constant temperature. In many cases temperatures may change
throughout the process.

Jen et al., (1971) and Teixeira et al., (1969) developed
equations to predict bacterial lethality and vitamin retention during
the canning process by integrating over the entire temperature curve.
These researchers approached the problem of vitamin destruction and
bacterial kill in the same manner however, thus limiting the appli-
cability of their equation to nutrients which degrade according to first
order kinetics.

Extrusion which is used in the manufacture of many IMF foods

is an excellent example of an unsteady-state process. Food passing

along the barrel of an extruder encounters a range of temperature condi-

tions. Several researchers have studied vitamin destruction during

extrusion. Beetner et al., (1974), (1976) and DeMuelenare (1969)

reported levels of vitamin retention after processing for several

vitamins in different extruded foods. In each case no kinetic analysis

was attempted, nor was the data presented sufficient for kinetic

analysis. Sizer and Maga (1976) studied the destruction of ascorbic

acid in extruder processed potato flakes. Their attempt to provide

a kinetic analysis of the resulting data was unsuccessful for several

reasons. First, all kinetic data was gathered using the extruder.

This was difficult because of the high temperatures and short residence

time of the product in the extruder. Second, the extrusion process

was treated as an essentially steady-state process, which it is not.

No allowances for temperature differences along the length of the

extruder barrel or come-up and come-down time were made. The product

temperature was not measured, but assumed to be equal to the temper-

ature of the barrel. Finally, all data was obtained by endpoint analyses.

As a result of these design inadequacies, the data obtained by these

researchers is impossible to analyze well from a kinetic standpoint.

A more preferable approach to the problem of nutrient

destruction during extrusion would be to determine the kinetics of

degradation for a particular nutrient under steady-state conditions.

The resulting data could then be applied to a prediction of nutrient

destruction during unsteady-state extrusion, which could then be tested

under actual conditions. The objective of this study was to determine

whether kinetic data gathered under steady-state conditions could be

used to predict ascorbic acid degradation during unsteady-state extrusion.

For this experiment we have modified an extruder to allow continuous

monitoring of extrudate temperature. This extruder was used to test

predictions of ascorbic acid loss based on kinetic data gathered under

steady-state conditions. Predictions of ascorbic acid loss under

conditions of linear temperature rise will also be tested. Because

Lee and Labuza (1975) have shown that water activity (a_w) has an

important effect on reaction rates of ascorbic acid oxidation in the

IMF region, water activity was used as an additional variable in

this study.

B. Materials and Methods

1. Model system preparation

The composition of the model system chosen for this

work is presented in Table 1. Water concentration is varied to

achieve the desired water activity. Three separate systems were

prepared, with approximate water activities of 0.6, 0.7, and 0.8.

Ascorbic acid was added at a level of approximately 400 mg/100 grams

dry solids. This level should insure accuracy of measurement while

providing a large enough percent destruction to differentiate between

zero, first, or second order kinetics.

Table 1

Composition of Model System

	%
Ground beef	35
Soy flour	25
Sucrose	23
Water	*
Lard	5
Ascorbic acid	0.2 (200 mg/100 gm)

* Proportions of water will be varied to achieve
   desired water activity.

2. Moisture and a_w measurement

To account for moisture changes during heating, ascorbic acid content was expressed on a mg/100 gm solids basis. All moisture analyses were performed using a methanol extraction followed by gas chromatographic separation. Samples were prepared for analysis by blending an accurately weighed sample of approximately 1.0 gram at high speed in 50 ml of anhydrous methanol. Moisture content was then determined by injecting 10 ml of this mixture into a Hewlett-Packard model gas chromatograph equipped with a thermal conductivity detector and a Povopak Q column operated isothermally at 110°C. The output was compared to a standard curve. Water activities were measured using the vapor pressure manometer (VPM) technique as reported in section V A.

3. Ascorbic acid measurement

a. Reagents, ascorbic acid standard, indophenol standard solution and standardization procedure are AOAC (1968) procedures.

b. Sample preparation

1. Transfer frozen sample weighing 5 - 10 grams into a tared blender jar and weigh accurately.

2. Add 99 ml of HPO_3 - HOAc extracting solution and blend at high speed for 30 seconds. Allow to settle and blend again for 30 seconds.

3. Centrifuge blended liquid for 2 min at a setting of 40 on an Adams model Dynac centrifuge.

4. Add approximately 1 gm of celite filtering
aid to the resulting supernatant and filter under suction.

5. Pipette two 10 ml aliquots of filtrate
into each of two 50 ml Erlenmeyer flasks.

6. Titrate to pink endpoint with indophenol
solution.

$$\frac{mg\ ascorbic\ acid}{100\ gm\ solids} = \frac{(ml\ dye - ml\ blank)\ (10^6)}{(ml\ dye/mg\ AA)\ (ml\ titrant)\ (gm\ sample)\ (100\%\ H_2O)}$$

4. Determination of steady-state kinetic data

The steady-state rate constants for ascorbic acid degra-
dation were determined using the system shown in Figure 1. A Haake
model FK2 constant temperature circulator circulates a heated glycerol-
water solution through a jacketed Brabender Farinograph mixing bowl
containing the model system. The mixing bowl has been fitted with a
specially designed jacketed pressure cover to minimize heat and
moisture loss during heating. The model system was mixed continuously
at a rate of 100 rpm to insure rapid heat transfer and even tempera-
ture distribution. The temperature of the model system was continuously
monitored using a 1/16 inch copper constantan thermocouple mounted
in the mixing bowl cover. Model system temperature was recorded
continuously using a Leeds and Northrup Speedomax model W temperature
recorder.

The entire system was allowed to warm up to operating
temperature before the model system was added. At that point 100 gm

TEMPERATURE RECORDER

MIXING BOWL WITH
THERMOCOUPLE

CONSTANT TEMPERATURE
CIRCULATOR
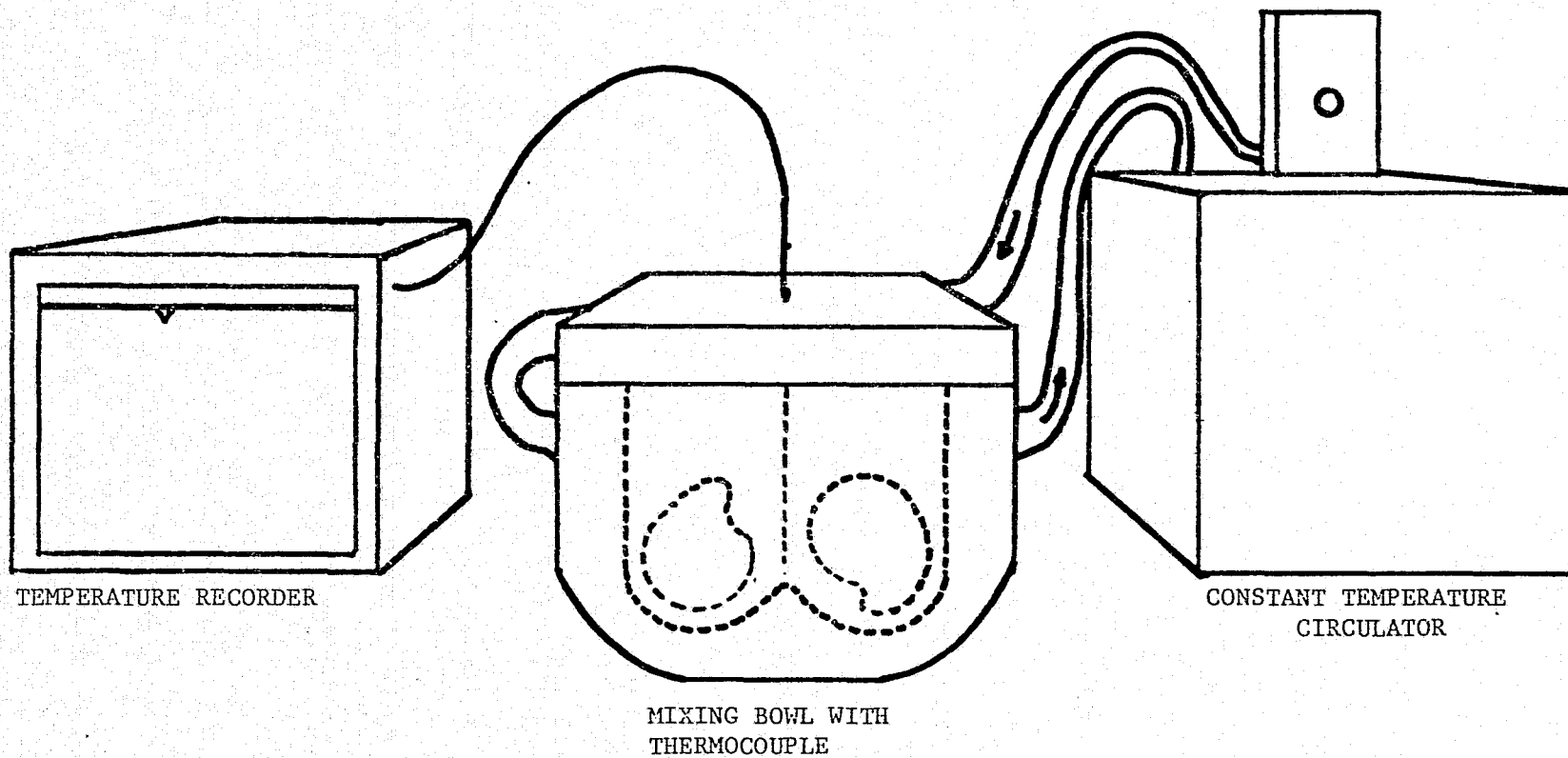
Figure 1.   Apparatus for determining steady-state rate constants of ascorbic acid degradation.

of fortified model system are preheated for 90 seconds in a covered

beaker in a Litton microwave oven and then are immediately placed

inside the mixing bowl. When a constant temperature was reached, mixing

is stopped momentarily and the cover is quickly removed to allow

removal of 5 - 10 grams of model system. The cover was then replaced

and mixing resumed immediately. The sample removed is frozen immediately

in liquid nitrogen, vacuum packed in foil pouches and stored in a frozen

state until later analysis for ascorbic acid and moisture. Subse-

quent samplings were taken in an identical manner at 5, 10, 15, 20

and 25 minutes after the first sampling, which was designated as time

zero. Portions of the system before and after heating were also used

for water activity analysis. This entire procedure will be performed

in duplicate for each of the three systems of varying water activity

at three separate temperatures. Experimental temperatures will be

in the range of 80, 93 and 105°C.

    5. Testing of steady-state predictions under unsteady-state

        conditions.

Conditions of unsteady-state approximating a linear

temperature rise will be obtained by linearly raising the temperature

of the circulating glycerol-water bath over the course of an entire

run. Model systems of each water activity will be analyzed for ascorbic

acid before and after heating to test predictions based on kinetic

data gathered under steady-state conditions.

6. Testing of steady-state predictions during extrusion.

A Brabender model 2003 3/4 inch laboratory extruder equipped with a model 2003 temperature control console and driven by a model D-3002 prep center has been modified as shown in Figures 2 and 3, for use in this experiment. Five 1/16 inch copper constantan thermocouples enter the extruder barrel perpendicular to its horizontal axis. Each thermocouple is held in place with a stainless steel pressure fitting. Teflon inserts insulate each thermocouple from the heating influence of the extruder barrel itself. A sixth thermocouple is located in the die assembly. Each thermocouple protrudes approximately 1/32 inch into the extruder barrel. The exposed portion of each thermocouple is protected by the walls of the groove through which it enters the barrel, as shown in Figure 3.

When in place, the thermocouples will measure the actual temperature of the extrudate at five points along the extruder barrel and in the die assembly. All temperatures will be recorded using the same equipment mentioned previously. The residence time of the product within the extruder will be determined by introducing carbon black into the feed. This result, combined with temperature data for each point along the extruder barrel, will yield the temperature history of the extrudate during extrusion. Endpoint analyses of ascorbic acid destruction during extrusion will be used to evaluate predictions made on the basis of kinetic data gathered at steady-state.
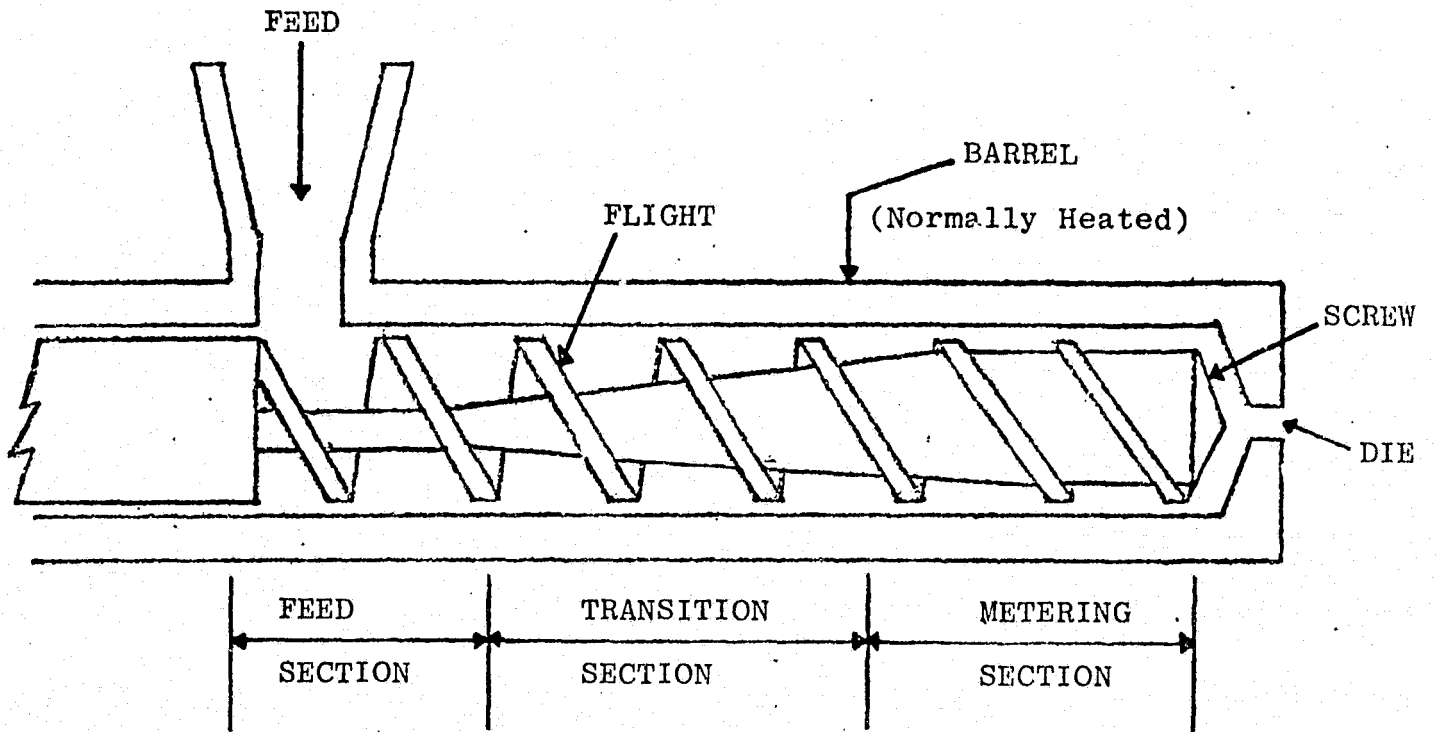
FIG. 2. - Functional sections of a typical cooking extruder.

Figure 3

Brabender Modified Food Extruder

a.

Feed Section	Transition Section	Metering Section	Die Assembly

Thermocouples

b.

Pressure Fitting

Teflon Insulator

Thermocouple

Groove

Barrel

Screw
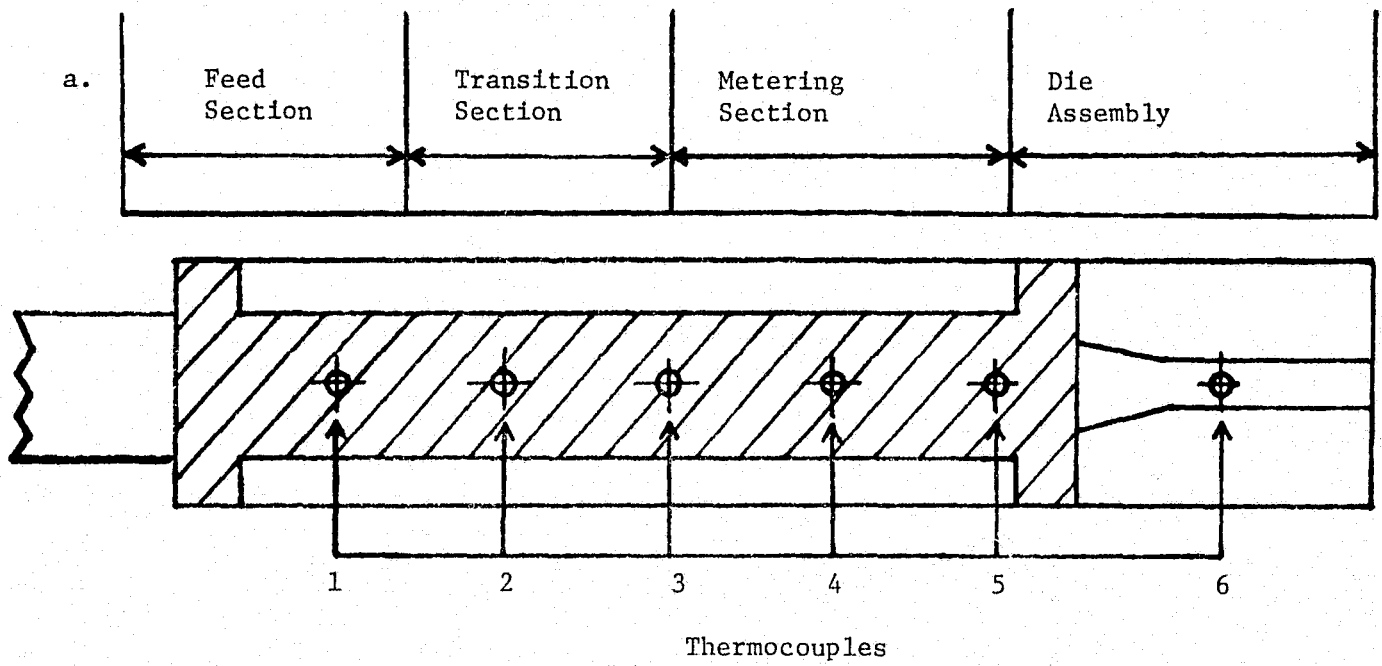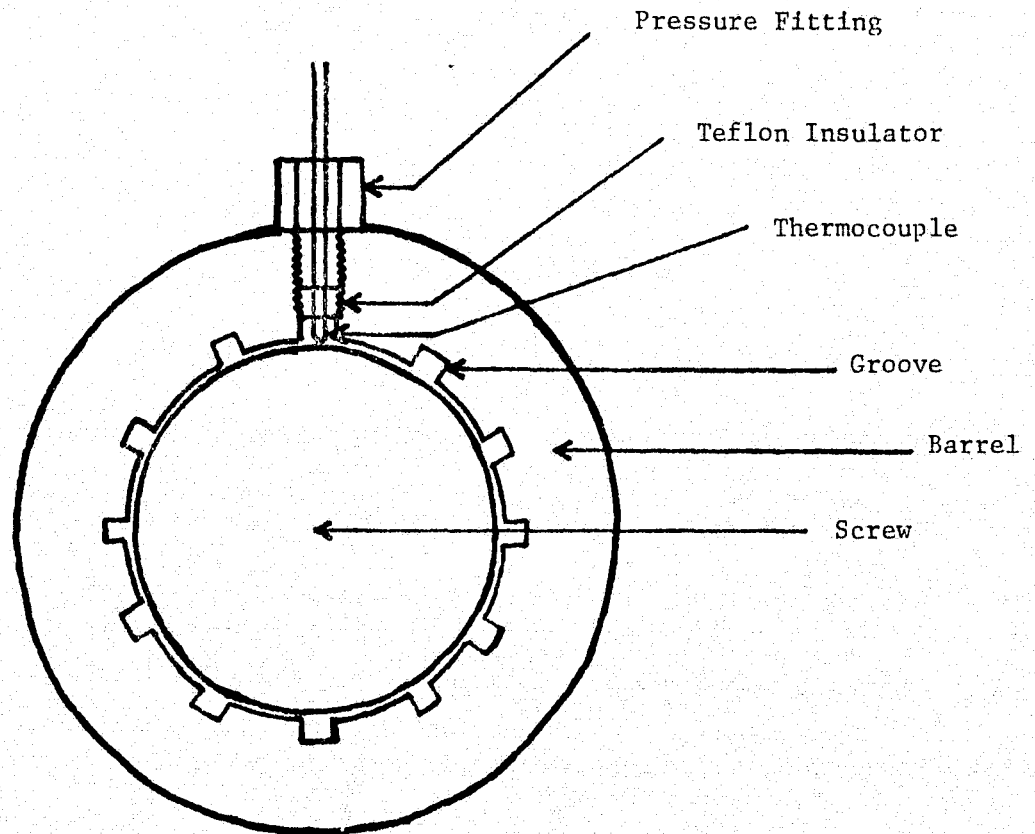
C. Results and Discussion

Preliminary data obtained in model systems similar to those to be used in this experiment is shown in Figures 4 and 5. Ascorbic acid content is expressed as $A_O - A$, where A_O is the concentration of ascorbic acid at time zero and A is the concentration at any time t (expressed in minutes). It can be seen from these plots that ascorbic acid loss with respect to time is linear in each case. In kinetic terms, the change in ascorbic acid concentration with respect to time can be expressed as:

$$\frac{-d(A)}{d\theta} = k \tag{1}$$

where k is a constant termed the rate constant. Rearranging equation (1) and integrating between limits of A_O and A yields:

$$A_O - A = k\theta \tag{2}$$

or
$$A = A_O - k\theta \tag{3}$$

Thus, a plot of $A_O - A$ vs. t yields a straight line, with the slope equal to k, as shown in Figures 4 and 5. As illustrated by equation (1) the rate of ascorbic acid disappearance in this case is independent of ascorbic acid concentration, and constant. A reaction which proceeds linearly with time according to equation (2) is termed a zero order reaction.

The preliminary data presented in Figures 4 and 5, indicate that in these systems under these conditions, ascorbic acid degradation
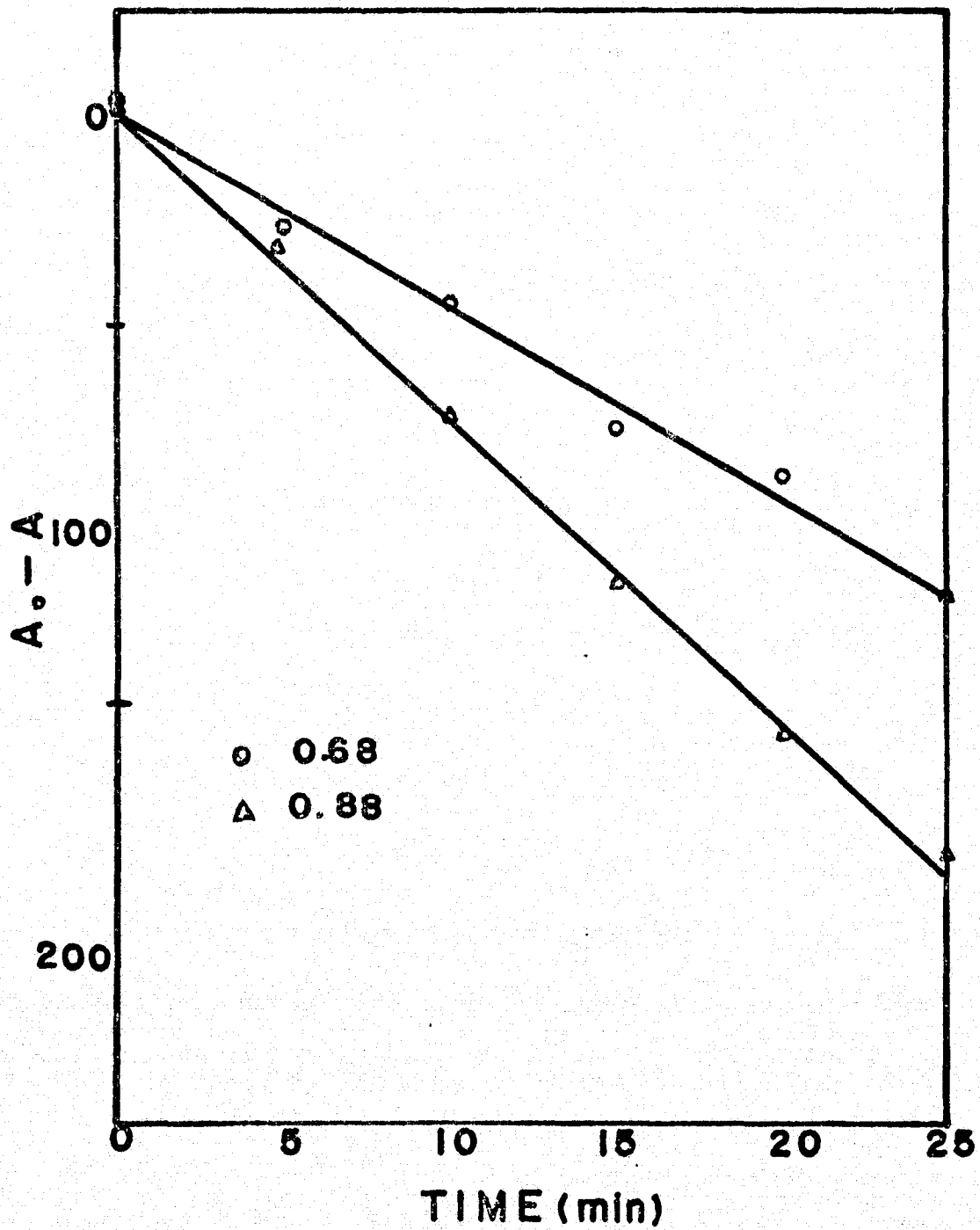
Figure 4. Destruction of ascorbic acid in systems of $a_w = 0.68$ and 0.88 at 83°C.
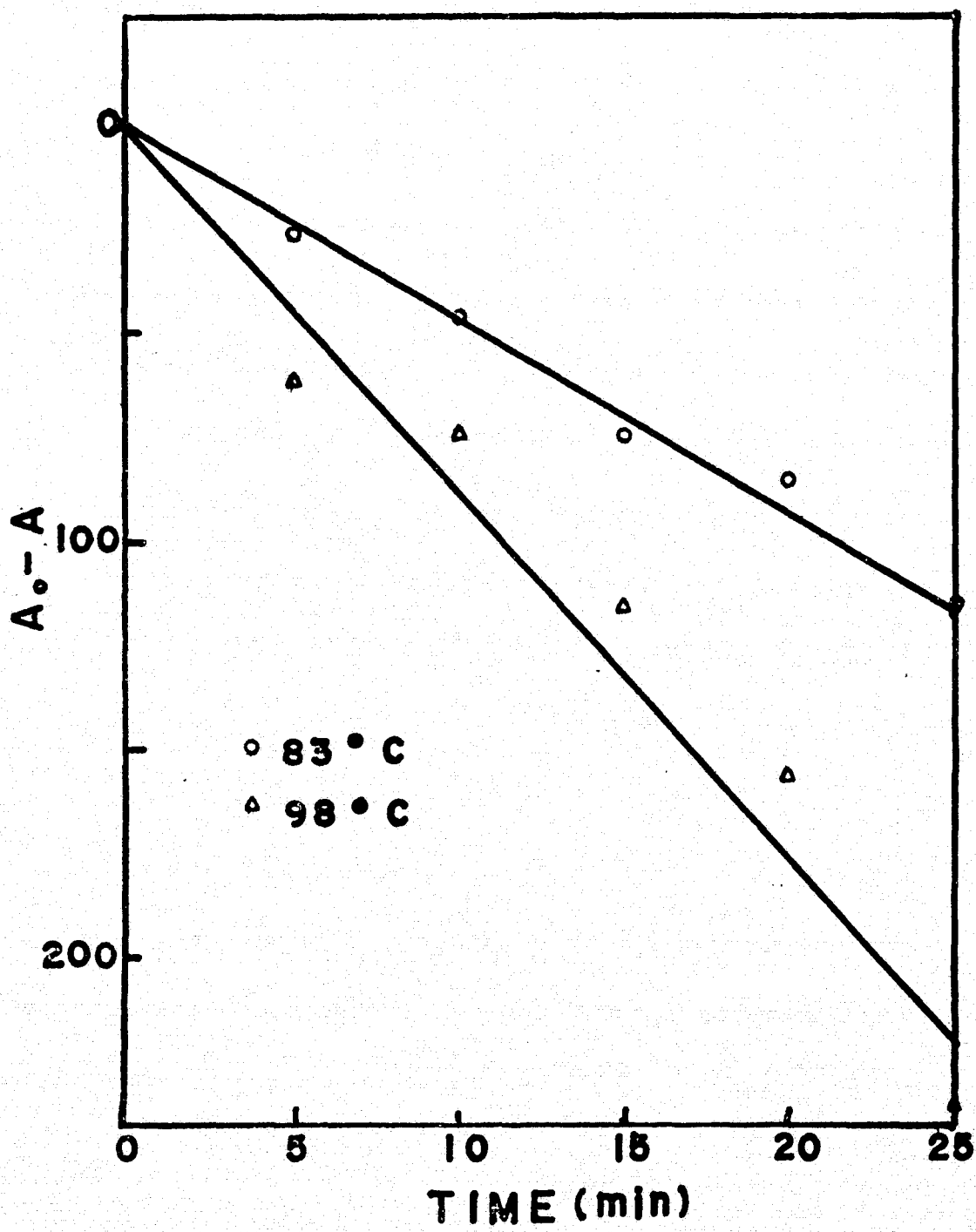
Figure 5. Destruction of ascorbic acid at 83°C and 98°C
(a_W = 0.68).

follows zero order kinetics. Figure 4 illustrates the effect of increasing

water acitvity on the rate of ascorbic acid destruction at 83°C. The

rate of degradation at an approximate water activity of 0.88 is signfi-

cantly higher than the rate observed at 0.68 (7.2 mg/min vs. 4.6 mg/min).

This is in accord with the findings of Lee and Labuza (1975) who found

that rates of ascorbic acid oxidation generally increase with increasing

water activity. Figure 5 illustrates the increase in rate of ascorbic

acid degradation with increasing temperature. Systems of the same

water activity showed considerable rate acceleration with a tempera-

ture change from 83 to 98°C. The reaction rate constant is related

to temperature according to the Arrhenius equation:

$$k = k_o e^{-E_a/RT} \qquad (4)$$

where
$$k_o = \text{pre-exponential factor}$$

$$e = \text{the base of the natural logarithm}$$

$$E_a = \text{activation energy (Kcal/mole)}$$

$$R = \text{the gas constant (Kcal/mole } ^oK)$$

$$T = \text{absolute temperature } (^oK)$$

Taking logs of both sides of equation (4) yields:

$$\ln k = \ln k_o - \frac{E_a}{R}\left(\frac{1}{T}\right) \qquad (5)$$

A semi-log plot, ln k vs. $\frac{1}{T}$ gives a straight line, with slope equal

to $\left(\frac{E_a}{R(2.303)}\right)$. Thus, the activation energy can be used as an indi-

cation of relative rate change with temperature. The activation

energy claculated from data presented in Figure 5 is approximately

10 Kcals/mole. This is within the range of activation energies for ascorbic acid degradation found by Vojnovich and Pfeiefer (1970). Provided that the slope (and hence, E_a) of the Arrhenius plot remain constant, this relation can be used to predict rate constants over a range of temperatures. Changes in reaction mechanism can cause a change in activation energy; for this reason at least three data points are required to construct an Arrhenius plot.

Knowledge of the Arrhenius parameters for a specific reaction gives information which can theoretically be used to apply steady-state kinetic data to unsteady-state conditions. For example, a system containing an initial concentration of ascorbic acid equal to A_o and following zero order kinetics will lose ascorbic acid according to this scheme under unsteady-state conditions of temperature:

$$A_o - A_1 = k_1\theta_1 \qquad (6)$$

$A_o - A_1$ is equal to the amount of ascorbic acid lost during a time interval θ, at a temperature corresponding to k_1. When the Arrhenius parameters for this reaction are known, k_1 can be determined directly from temperature by use of the Arrhenius plot. When this system is now placed at a new temperature for a new time:

$$A_1 - A_2 = k_2\theta_2 \qquad (7)$$

where $A_1 - A_2$ is equal to the amount of ascorbic acid lost during a time interval θ_2 at a new temperature corresponding to k_2. Adding

equations (5) and (6) yields:

$$A_o - A_2 = k_1\theta_1 + k_2\theta_2 \qquad (8)$$

which describes the total amount of ascorbic acid lost during the time interval $\theta_1 + \theta_2$ and under conditions of temperature corresponding to k_1 and k_2. If intervals of time are taken to be constant, equation (7) can be written:

$$A_o - A_2 = \theta(k_1 + k_2) \qquad (9)$$

For a series of n temperature changes, ascorbic acid loss can be described by:

$$A_o - A_n = \theta(k_1 + k_2 + k_3 + \ldots k_n) \qquad (10)$$

where $A_o - A_n$ is equal to the total loss of ascorbic acid and $\frac{A_n}{A_o}$ is equal to percent retention of ascorbic acid during time $(\theta \cdot n)$. For a variable time temperature sequence a similar equation exists:

$$A_n = A_o - \sum_{i = o}^{i = n} (k_i\theta_i) \qquad (11)$$

Arrhenius parameters determined under steady-state conditions, the temperature history of the model system and equation (9) can be combined on a computer program to make predictions for ascorbic acid loss under unsteady-state conditions. A similar computer analysis has been used previously by Mizrahi et al., (1970) to make predictions of extent of browning in dehydrated cabbage using moisture/time as the variable instead of temperature/time. Simple endpoints analyses will prove whether these predictions are valid.

D. Conclusions and Recommendations

The conclusions resulting from this work should give some idea as to whether kinetics determined under steady-state conditions can be used to make predictions under conditions of unsteady-state. These results will probably also indicate the need for further study in other areas of processing and with other nutrients since the preliminary work shows that vitamin C degrades by a zero order rather than a first order reaction at high temperature.

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IV. EVALUATION OF ANTIOXIDANTS IN ACCELERATED SHELF LIFE STUDIES
OF IMF SYSTEMS

A. Introduction

Rancidity due to lipid oxidation is one of the most significant reactions leading to deterioration of IMF systems. The next pages consist of the text of a chapter written by the principal investigator which appeared as a chapter in Water Relations of Foods, R. Duckworth, editor, Academic Press, New York.

Oxidative Changes in Foods at Low and Intermediate Moisture Levels

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I. OXIDATIVE DETERIORATION OF FOODS: AN OVERVIEW

Dehydrated and intermediate moisture foods are an important part of the food process industry. Despite the lowered moisture content, they are subject to many deteriorative chemical reactions. Labuza (1971) and Karel (1973, 1974) have reviewed the many problems of storage stability especially with respect to oxidation problems. It was concluded in these reviews that the initial quality of the food, the moisture content, the oxygen partial pressure, the amount of oxygen available in the package and the package permeability to O_2 and H_2O all are important in controlling shelf-life. Deteriorative reactions depending on oxygen include:

1. lipid oxidation;
2. carotenoid degradation;
3. oxidation of myoglobin causing a discoloration in meat;
4. oxidation of other food components such as proteins;
5. enzymatic oxidation;
6. non-enzymatic browning;
7. oxidation of ascorbic acid.

For every one of these reactions, the water content of the food is of primary importance. Water reacts in many ways; hydrating polar or ionic groups which can affect their reactivity, dissolving and mobilizing species allowing them to react, hydrating and swelling surfaces exposing new reactants and finally, decreasing reactant concentration and adsorbed phase viscosity as moisture content increases at high a_w. It is found that for most aqueous phase reactions the effect of water on reaction rate ceases below the monolayer (Labuza, 1975) since that water is adsorbed tightly and cannot participate.

This, however, is not the case for oxidation of unsaturated lipids where some effects occur below the monolayer as shown by Karel (1975).

In all these cases there exists a minimum extent of reaction at which the product becomes organoleptically unacceptable. The challenge to the processor is to devise means of control of oxidation through control of O_2 levels, the water content–water activity relationship of the food and the packaging permeability. The problem exists that little information is available about the kinetics of such reactions in these areas that can be used to help predict shelf-life. In addition, Herlitze et al. (1973) have shown that in certain products the history sequence (time–temperature–%r.h.) of the product may change the extent of oxidation at which the product becomes unacceptable. This chapter will discuss some of the above reactions with respect to dehydrated and intermediate moisture foods as a function of water and oxygen levels.

II. OXIDATION OF ASCORBIC ACID IN DRY AND INTERMEDIATE MOISTURE FOODS

L-Ascorbic acid is one of the most essential vitamins for man and is of technological significance since it is considered to be the most unstable (Harris and von Loesecke, 1960). The mechanism of its degradation is very complicated and involves metal catalysis, pH and the oxygen concentration in the environment. Pathways for both anaerobic and aerobic degradation are present and usually follow a free radical mechanism as reviewed by Bauernfeind and Pinkert (1970). It has been found, however, that the degradation in foods can be treated as a simple first order reaction (Vojnovich and Pfeifer, 1970).

Vojnovich and Pfeifer (1970) studied the degradation of vitamin C which was added to various dehydrated food mixtures. The results are shown in Table 1 for the half life as a function of moisture content for the various mixtures. The a_w values were not in fact reported, but an estimated value is presented. As can be seen, the rate of destruction increases significantly as a_w increases and the rate is very different in different products.

Karel and Nickerson (1964) studied the effects of oxygen and a_w on the destruction of ascorbic acid in orange juice crystals. They found a direct linear relationship of the loss rate with a decrease in moisture content down to the BET monolayer moisture value. There was also no difference between the rate in O_2 or in N_2 atmospheres. This could be due to the poor solubility of oxygen in the crystals so that no differences would show up between O_2 and N_2 gas; the latter always contains some residual oxygen.

In order to explain the effects of increasing water content on ascorbic acid destruction, Lee and Labuza (1975) made a study using a cellulose–oil–glycerol model system (pH6) stored in air. Representative results are shown in Fig. 1. As seen, the rate of destruction increases rapidly as the moisture increases at

TABLE 1. Destruction rates of ascorbic acid in various dry cereal blends

	Water content	a_w	T (°C)	Half life $\theta_{\frac{1}{2}}$ (days)
Corn soya milk	11.8%	0.7	45	3
			37	22
			26	231
	10.4%	0.6	45	23
			37	83
			26	537
	8.0%	0.55	45	372
			37	809
			26	2423
Wheat flour	14.6%	0.7	45	35
			37	372
			26	1616
	13.7%	0.6	45	99
			37	537
			26	2423
	12.9%	0.5	45	323
			37	1213
			26	4047
Mixed cereal	10.7%	0.6	45	12
			37	15
			26	19
	7.0%	0.5	45	14
			37	25
			26	48
	5.0%	0.4	45	24
			37	45
			26	103

From Vojnovich and Pfeifer (1970).

low moisture contents. The a_w increases from 0.32–0.68 over the range of 5 g H_2O/100 g solids to 15 g H_2O/100 solids. Above about 25 g solids there is little further increase in the oxidation rate. Using n.m.r. to measure the viscosity of the adsorbed aqueous phase they found a direct linear decrease of viscosity with increasing a_w. The destruction rate results were explained on the basis of a first order reaction in which the dilution of the aqueous phase decreases viscosity thereby increasing reactant mobility. This in turn increases the rate constant k. The overall rate increase could be balanced out by the
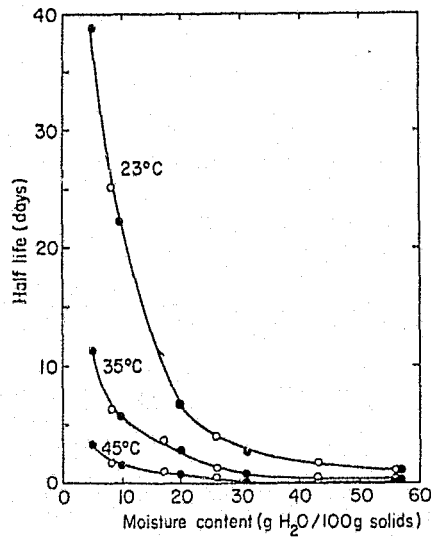
FIG. 1. Half life for ascorbic acid destruction as a function of moisture content. DM(●) = desorption system, DH(○) = adsorption system. (Lee and Labuza, 1975.)
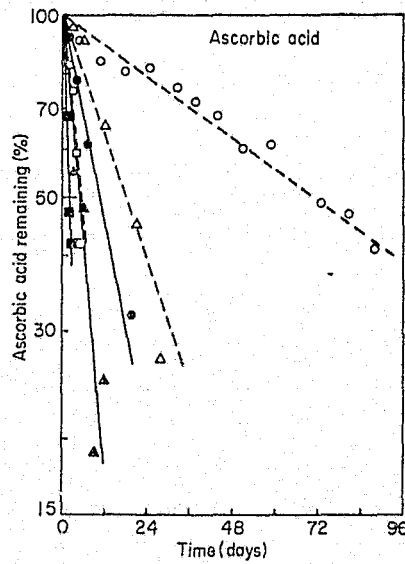
FIG. 2. Loss of ascorbic acid in an intermediate moisture food stored in air (——, closed symbols) and a zero O_2(– – –, N_2/H_2, open symbols) atmosphere at three temperatures: (●) 25°C; (▲) 35°C; (■) 45°C. (Labuza, 1974).

dilution of the reactants, although no decrease in rate was found. At high moisture contents, any further increase in water content does not cause any further significant viscosity decrease, therefore the rate should remain constant as was found.

Labuza (1974) studied this further in an intermediate moisture food (i.m.f.) stored at three temperatures and in two atmospheres (air and in a special paladium catalyst pouch with a N_2/H_2 atmosphere—American Can Co., Maraflex 75 Scavenger Web). The results are shown in Fig. 2 for the system at a_w 0·86. As expected, the rate increases with temperature in air and in an oxygen-free atmosphere. However, contrary to the orange juice crystal experiments, at 25°C there is a significant reduction in rate when oxygen is removed completely. The rate difference becomes minimal at 45°C suggesting that the solubility of oxygen is an important factor, since it decreases at high temperature. Fig. 3 shows further results in an i.m. food at two a_w values. As
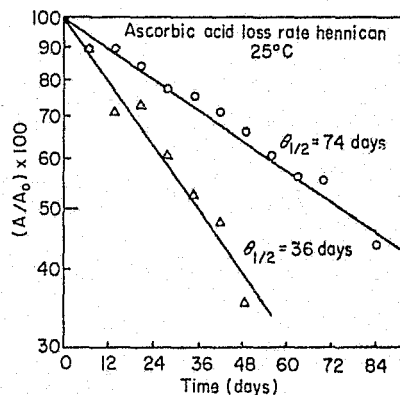
FIG. 3. Fraction of ascorbic acid remaining (A/A_0) at 25°C and two water activities. (Labuza, 1974.)

expected the half life was less at the higher a_w. The overall results show the real difficulty in supplementing a non-acid food with vitamin C. Either very high overruns are needed, or the ascorbic acid must be coated with an edible, low-water-permeable coating to prevent losses and meet nutritional claims.

III. OXIDATION (OTHER NON-LIPID FOOD COMPONENTS)

Unsaturated fats are highly oxidizable and their degradation contributes to rapid loss of shelf-life and food quality. Other components oxidize as well leading to degradation. Some of these reactions are controlled by water content of the food through the properties of solubilization, mobilization and reaction.

Haas *et al.* (1974) studied the oxygen absorption of various dehydrated vegetables. Their results, calculated with respect to a_w, moisture content and rate of O_2 uptake, are shown in Table 2. Since the vegetables were not blanched,

TABLE 2. Oxygen absorption rate of dehydrated vegetables*

Vegetable	Moisture content (g H₂O/100 g solids)	a_w‡	Oxygen uptake (ml O₂/h/g solids)
Celery:			
Fresh	19	1·0	0·48
Dried to 24% of ww†	3·8	0·95	0·04
Carrots:			
Fresh	7·33	1·0	0·32
Dried to % of ww:			
39	2·3	1·0	0·06
27	0·8	0·95	0·02
15	0·2	0·85	0·002
Dried to 29% ww and rehydrated to:			
64%	4·33	1·0	0·20
79%	6·6	1·0	0·10

* Hass *et al.* (1974).
† ww = wet fresh weight, dried in air at 65°C.
‡ estimated.

the data presumably reflect enzymatic reactions, especially since the rates are very high as compared to non-enzymatic lipid oxidation rates (usually about 0·003 ml/g h). Two effects must be occurring; first, some enzymes would be expected to be denatured during the drying and this is borne out by the results for the rehydrated, dried carrots in which over 50% of the activity appears to have been lost; second, the rate of O_2 uptake decreases as the a_w is decreased, an effect similar to that found by Acker (1969) for enzymes studied in model systems. Most likely, as a_w decreases the increased aqueous phase viscosity and decreased reactant mobility reduce the reaction rate. Haas *et al.* (1974) also found that rehydration after drying was less if the vegetables were not blanched before drying. Thus it could be that during drying the oxidation that occurs may cause chemical and structural changes that lead to reduced hydratability.

Labuza *et al.* (1969) found that a pure protein, egg albumin, reacts with oxygen during storage. The rate increased with a_w from 0·11–0·6. The average oxygen uptake just above the monolayer was 0·0005 μl O_2/g h, a very low value. However, this could also lead to chemical and structural rearrangements which cause toughening, a major problem in the storage of dry foods.

Kapsalis *et al.* (1961) have studied oxygen uptake in freeze-dried beef. The rate of oxidation trippled on going from the dry state (a_w 0·01) to an a_w of 0·32 which is above the monolayer. A concomitant loss in the red pigment myoglobin due to eventual oxidation to metmyoglobin also occurred in about two months giving the meat a brown color. It is well-known that in the wet state (a_w 1·0), this can occur in meat very rapidly even at refrigerated temperatures unless the product is held in a vacuum. Thus, at low a_w the low water content decreases the rate of this discoloration.

The oxidation of various fat-soluble pigments such as carotenoids is coupled to lipid oxidation and will be discussed in the next section. It should be obvious, however, that with dehydrated foods, the oxidation reactions increase in rate with an increase in a_w and in the presence of oxygen. Thus, one must keep the water content as low as possible and evacuate the storage container to maximize shelf-life. As an example of the effect of oxygen, the U.S. Army Natick Laboratories, in a series of studies (Tuomy and Hinnegart, 1968; Tuomy *et al.*, 1968, 1969, 1970; Bishov *et al.*, 1971) found that dehydrated raw beef patties (about 3% moisture) became unacceptable after one month in air at 37°C, but had a shelf-life of 6 months at a vacuum of less than 20 mmHg. Using a palladium catalyst system similar to the film mentioned previously afforded a one year shelf-life at "zero" oxygen content. The oxygen uptake rates were about 0·00004 cc O_2/g food per h indicating that the low water content prevented enzymatic reactions but allowed other slower reactions to occur. Speiss (1963) has found a similar pattern for freeze-dried cooked beef.

IV. OXIDATION OF FOOD LIPIDS

A. Hydrolytic Rancidity

Lipid oxidation or rancidity has been associated with two general mechanisms. In general, the term rancidity in the dairy industry refers to hydrolytic rancidity. This is the action of lipase on dairy fat triglycerides splitting off free fatty acids. Since milk fat contains about 4% butyric acid this can lead to acceptability problems since butyrate, as the free fatty acid, is very obnoxious. Since lipid hydrolysis by enzymes increases with an increase in a_w from the dry state (Acker and Wiese, 1972) one should either prepasteurize at temperatures to denature lipase or keep the moisture content as low as possible. In intermediate moisture foods, one must also prevent growth of any micro-organisms, as the organisms could produce exogenous enzymes which would lead to hydrolysis.

B. Lipid Oxidation—General Scheme

The major lipid oxidation mechanism is the oxidation of unsaturated fatty acids as reviewed in the previous chapter by Karel. The overall scheme is a free radical mechanism in which polyunsaturated methylene-interrupted double bonds react with oxygen , controlled by moisture content, oxygen level, light and trace-metal catalysis. The overall scheme illusis trated in Fig. 4. Besides the production of objectionable odors and flavors, the free
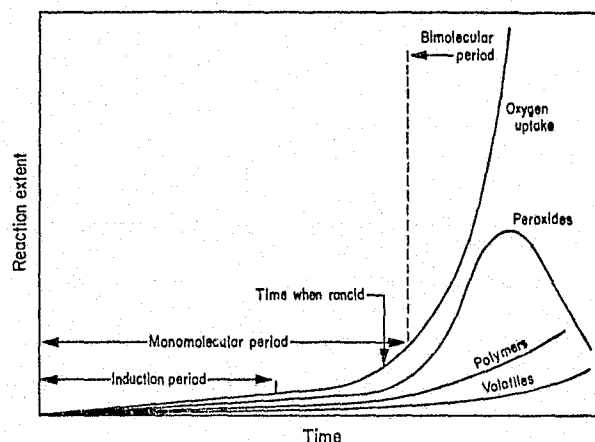
FIG. 4. Extent of lipid oxidation reactions as a function of time.

radicals and peroxides produced can react with pigments bleaching them, can react with proteins causing toughening and reducing digestibility, can destroy vitamins and can lead to the production of possibly toxic material (Labuza, 1971). In addition, products of oxidation such as aldehydes and ketones can react with proteins through Maillard browning and also causing toughening leading to darkening and reduced digestibility and therefore the reduced nutritional value of proteins.

C. Effect of Water on Lipid Oxidation

In early work on cereal and cereals products, it was found that stability of the product was a definite function of the moisture content. As moisture content was lowered, the products became rancid much sooner (Matz *et al.*, 1955; Halton and Fischer, 1937; Steven and Thompson, 1948; Martin, 1958). Salwin (1959) proposed that at the BET monolayer, the water formed a protective barrier, preventing the oxygen from reaching the underlying unsaturated fats. Halton and Fischer (1937) in a similar manner proposed that the water retarded the diffusion of oxygen to the sites of the unsaturated double

bonds. This was based on the fact that oxygen diffusion is 10^4 times faster in air than it is in water.

Fig. 5 shows an example of the effect of water on oxidation of a dehydrated food (freeze-dried salmon, Martinez and Labuza, 1968). As can be seen, as the
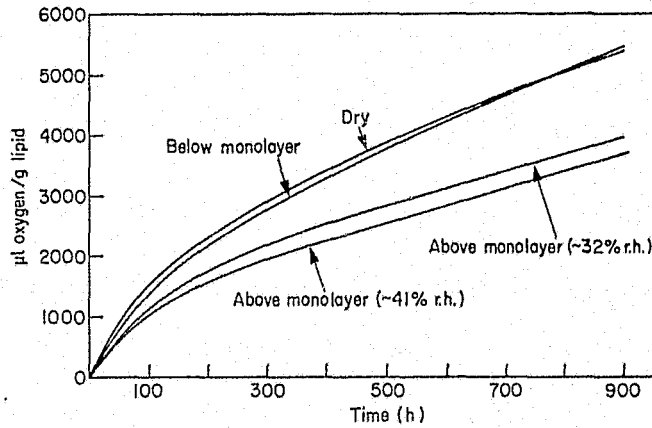
FIG. 5. Oxidation of freeze-dried salmon at different equilibrium % r.h. values and at 37°C. (Martinez and Labuza, 1968.)

a_w increases, the rate of oxidation decreases. This is exactly coupled to the bleaching out of the lipid-soluble red pigments present in the salmon as seen in Fig. 6.
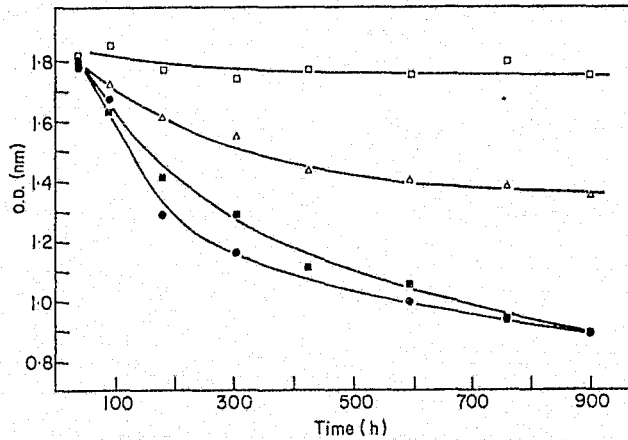
FIG. 6. Loss of pigment (o.d. ratio) of freeze-dried salmon at 37°C stored at different relative humidities: (□) above monolayer (~41% r.h.); (△) above monolayer (~32% yr.h.); (■) below monolaer; (●) dry. (Martinez and Labuza, 1968.)

Similar results were found in studies of the oxidation of pure lipids. For example, Labuza et al. (1969) reported comparisons of the monomolecular reaction rate constant for methyl linoleate oxidation in different systems. As seen in Table 3, as the a_w increases, the rate decreases. However, in an emulsion

TABLE 3. Oxidation rates for methyl linoleate at 37°C

System	a_w	Km (mol/mol)$^{\frac{1}{2}}$ h^{-1}
Bulk lipid	0·00	13×10^{-3}
Lipid–cellulose	<0·01	4×10^{-3}
Lipid–cellulose	0·32	2×10^{-3}
Lipid–cellulose	0·40	$1·75 \times 10^{-3}$
Lipid–cellulose	0·50	2×10^{-3}
H$_2$O–lipid emulsion	1·00	32×10^{-3}

the rate of oxidation of linoleate is even higher than in the bulk oil. This would seem to preclude any protective layer or diffusion effect of water at or above the monolayer.

Karel and co-workers in a systematic study to determine the effects of water at low a_w on lipid oxidation found the following mechanisms to be important (Maloney et al., 1966; Labuza et al., 1966; Karel et al., 1967, 1974).

1. Water hydrogen bonds with the hydroperoxides produced during the free radical mechanisms. This essentially lowers the peroxide concentration and decreases overall oxidation rate.

2. Many trace metals act as significant oxidation catalysts. As they become hydrated with water their effectiveness decreases, thereby decreasing the rate of oxidation.

3. Water promotes free radical combinations and reactions with other food components thereby reducing overall reaction rate.

4. Some metal catalysts may react with water to produce an insoluble non-reactive hydroxide.

These mechanisms explain the antioxidant effect of water at low a_w but do not account for the increased rate that was observed in the aqueous system. Heidelbaugh and Karel (1970) and Chou et al. (1973) studied oxidation in the region of intermediate moisture content using linoleate model systems. Their results show that once the monolayer region of water is exceeded the solution properties of water now affect the oxidation reaction in several ways causing the rate to eventually increase. As a_w increases, the metal catalysts, although less effective, are more easily mobilized to reaction sites from their aqueous

environment. In addition, new catalytic sites may become exposed due to dissolution of precipitated crystals or because of swelling of bound surfaces. Water has a direct effect based on the total amount present as was shown by using systems which exhibited moisture sorption hysteresis. When the induction time for oxidation was plotted $vs.$ moisture content for both adsorption and desorption systems at constant trace metals content, the rates fell on the same line (Fig. 7) whereas when plotted $vs.$ a_w they did not (Labuza and Chou,
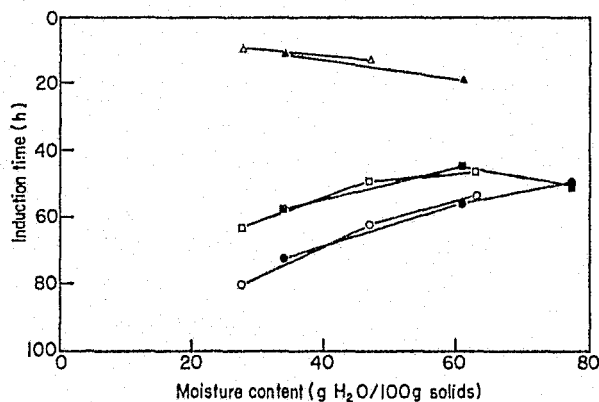
FIG. 7. Induction time for linoleate oxidation in a cellulose model system as a function of moisture content. DM = desorption (solid symbols), DH = adsorption (open symbols). Circles, control; squares, 20 p.p.m. metal; triangles, 1000 p.p.m. metal. (Labuza and Chou, 1974.)

1974). Therefore, at constant a_w the higher moisture system oxidized faster than the lower moisture system, the difference increasing with an increase in sorption hysteresis. Chou et $al.$ (1973) also showed that the increased moisture content decreases aqueous phase viscosity allowing more rapid mobility of the catalysts. This, however, could not account for the total increase in rate of oxidation.

Further studies (Labuza and Chou, 1974) illustrated these overall oxidation effects as a function of the total metal concentration added to the system. At low metal content (10–50 p.p.m.), as moisture increases the rate of oxidation increases due to the decreased viscosity, increased mobility and the swelling which exposes new catalytic sites. However, in systems containing high concentrations of trace metals, the rate at high a_w decreases as a_w increases and is slower for the higher moisture systems. This is shown in Fig. 8. The reasons given are that at high metal concentration, metal catalysis is a predominant force in inducing formation of free radicals. Thus, any substantial decrease in the concentration depresses the rate steeply, as would be the case when the water content is increased.
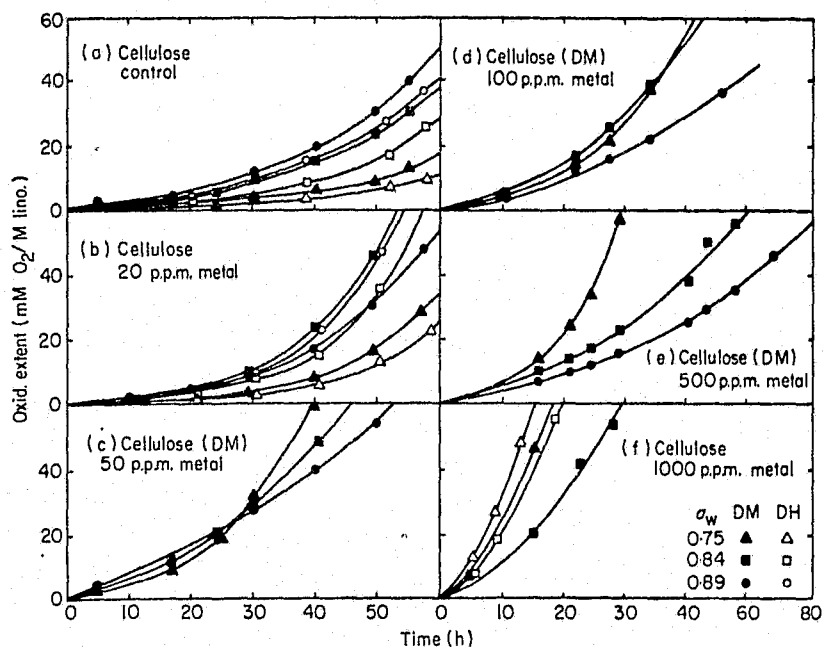
FIG. 8. Effect of water activity and method of preparation on linoleate oxidation as a function of trace metal concentration. (Labuza and Chou, 1974.)

Overall then, water acts in several ways on the mechanism of lipid oxidation:

1. antioxidants effects:

    a. hydration of trace metals;

    b. hydrogen bonding of hydroperoxides;

    c. promotion of radical recombination or reaction with other components;

    d. dilution of metal concentration.

2. pro-oxidant effects:

    a. reduced viscosity promoting mobility;

    d. dissolution of precipitated catalysts;

    c. swelling of solid matrices exposing new catalytic surfaces.

As would be expected, a minimum–maximum type curve should result from these effects as shown schematically in Fig. 9. It can be seen that as a_w increases from the dry state, the rate reaches a minimum in the monolayer–multilayer region and then increases again. This increase may be large as found by Quast and Karel (1971) for potato chips (Table 4). In a chicken system Labuza et $al.$ (1969) found the rate of oxygen uptake to be twice as fast at a_w 0·75 as at 0·01.

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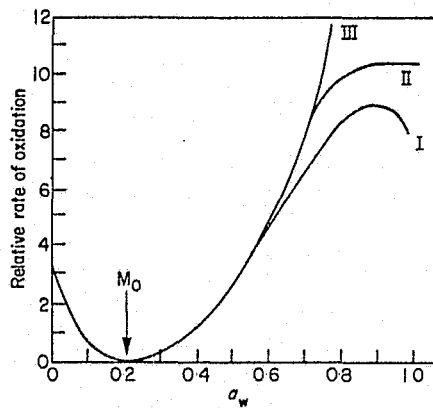
FIG. 9. Overall effect of water activity on rates of lipid oxidation: Case I—dilution exceeds viscosity effect; Case II—dilution balances viscosity effect; Case III—viscosity effect predominates.

TABLE 4. Oxidation of potato chips*

a_w	Relative oxidation rate–room light	Time to reach 1200 μlO_2 (STP)/g	
		Diffuse sunlight	Dark
0·01	1	1400	1400
0·11	0·87	—	—
0·20	—	2100	2250
0·32	0·47	—	—
0·40	0·40	3000	4250
0·62	1·27	—	—
0·75	10·7	—	—

* Quast and Karel (1972).

However, dilution of the trace metals may occur at higher a_w causing the rate to flatten out again or even decrease. In studies with an intermediate moisture food, this decrease in rate at high a_w has been observed, as Fig. 10 shows for oxygen uptake (Labuza, 1973). The food contained about 30% meat and 10% raisins and was thus high in trace metals. Similarly, in studying a pork and a chicken i.m.f. system, Labuza et al. (1972) found a decrease in shelf-life of one-half in going from a_w 0·61 to 0·75, but little further decrease at 0·85 (a Case II reaction type).

Oxygen absorption is not the only factor correlated with lipid oxidation which can be shown to be affected by moisture content. As shown by Labuza et al. (1969) for a model system and confirmed in potato chip studies by Warner et al. (1974), the production of pentane, an off-odor product of oxidation, decreases as a_w is increased from the dry state into the monolayer region.

Quast and Karel (1972) also found that water had an effect on the rate of oxidation as induced by light. Table 4 illustrates some results from their data. As can be seen, light has a greater pro-oxidant effect at a_w 0·40 than in the dry
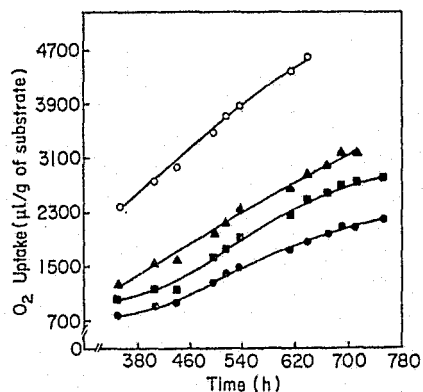
FIG. 10. Rate of oxidation of an intermediate moisture food as a function of water activity with and without antioxidants. a_w values: (▲) 0·68; (■) 0·75; (●) 0·85; (○) EDTA + BHA ($a_w = 0·75$). (Labuza, 1973.)

state. The reason is not known. These authors also showed that at the higher humidities, the rate of oxidation was less affected by a decrease in oxygen level in the atmosphere. Again, this is an area that needs further exploration.

D. Oxidation of Intermediate Moisture Foods

It should be obvious from the above studies that intermediate moisture foods should be very subject to deterioration by lipid oxidation despite references to the contrary (Brockman, 1970, 1973; Loncin et al., 1968). Labuza et al. (1972) and Labuza (1973, 1975) have studied rancidity in actual intermediate moisture foods. A chicken–peanut–sugar system as shown in Fig. 11 became rancid very quickly at 35°C even though packaged in a vacuum. The formulation was based on the American Indian trail food *pemmican*. Foods of similar nature were prepared by Armour and Co. (E. Binkerd, personal communication) for Admirals Perry and Scott on their Arctic voyages and for the U.S. Navy to use as emergency rations on submarines. These foods were failures because of the extreme rancidity that occurred during storage. As illustrated by Fig. 11, one could easily be misled into concluding that in this case oxidation had not occurred if peroxides were measured only at inadequate intervals and no organoleptic analyses were made. The Fig. shows the usual increase in peroxides at the point of rancidity followed by a decrease to almost zero. It was obvious from the above studies on intermediate moisture foods that prevention of oxidation of lipids was a necessity to ensure an adequate shelf-life.
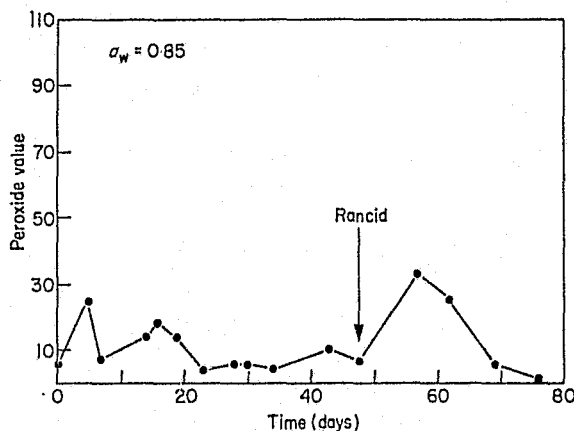
FIG. 11. Increase in peroxide value of an intermediate moisture food at 35°C. The point of rancidity determined organoleptically. (Labuza, 1973.)

E. Prevention of Oxidation

Since oxidation of lipids is a problem, different techniques can be used to control it. One important approach is to decrease the oxygen content by gas flushing or by pulling a vacuum on the food and package. In addition, the food can be packed in a low-oxygen barrier to prevent any regain of oxygen. As an example, Zscheile (1973) found that there was essentially no loss of carotenoids from alfalfa leaves stored in the dark at room temperature and 10^{-4} torr for 27 years. Of course, foods cannot be packed in vacuum glass ampoules and most flexible containers have some rate of oxygen permeation.

Karel (1974) has recently discussed the vacuum-packaging technique for increasing food shelf-life. Simon et al. (1970), Quast and Karel (1972) and Quast et al. (1972) have developed mathematical models for predicting shelf-life of dehydrated food products undergoing oxidation as a function of the rate of penetration of water and oxygen through the pouch.

The other main method of preventing oxidation is to add antioxidants. Labuza (1971) has reviewed the mechanism of the antioxidant effect in decreasing oxidation rate and has pointed out some of the problems. Basically, antioxidants have been shown to be very effective in pure oils and in model systems but little evidence exists for their value in real foods.

Labuza et al. (1971) studied the effect of antioxidants in linoleate model systems as a function of a_w. Lipid-soluble antioxidants which combine with free radicals are essentially unaffected by water content. This includes BHA, BHT and tocopherol. Metal chelating agents, however, are strongly affected by increasing a_w. The effectiveness of EDTA and citric acid increase with a_w

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above the monolayer reducing the rate significantly. Chou and Labuza (1974) found that EDTA reduced the rate of oxidation by a factor of 5–10 at a_w 0·75 as compared to a control at the same a_w and the rate was 20 times slower than in dry samples. The EDTA was twice as effective as BHA. The reason for the better effectiveness of chelating agents is their increased solubility in the aqueous phase as moisture increases. Ascorbic acid, however, could not be used at high a_w as a chelating agent as it is degraded itself and contributes free radicals, thereby increasing lipid oxidation.

Despite the good results found in model systems, results in foods are not as good. Fig. 10 shows that the combination of antioxidants in an i.m.f. system actually promoted oxidation (Labuza, 1973). Walter and Purcell (1974) found nitrogen packing of dehydrated sweet potatoes to be very effective in reducing oxidation, whereas antioxidants had no effect. The poor effectiveness of anti-oxidants in most foods was pointed out as early as 1955 by Reimenschneider. The reasons need further investigation. However, Labuza et al. (1969) found that antioxidants were less effective in the presence of proteins. Perhaps the food proteins bind the antioxidant thereby causing the loss in effectiveness.

V. EFFECTS OF LIPID OXIDATION

As pointed out earlier, lipid oxidation products can participate in other reactions. One mechanism coupled to oxidation is non-enzymatic Maillard browning since the aldehydes and ketones formed can react with protein. The amount of browning resulting from oxidation increases as a_w increases above the monolayer, as found by Martinez and Labuza (1968) in the storage of
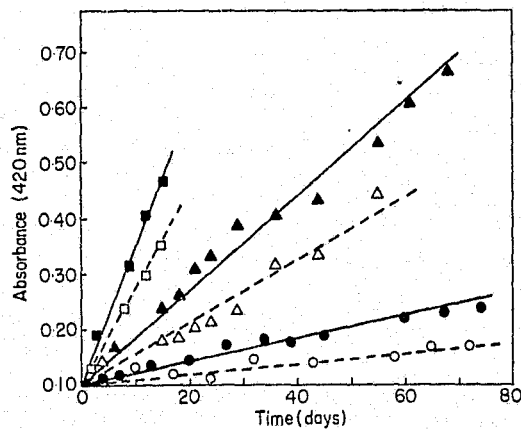
FIG. 12. Non-enzymatic browning of an intermediate moisture food (a_w 0·86) stored in air and an "O_2 free" atmosphere. (●) 25°C; (▲) 35°C; (■) 45°C; ——, air (closed symbols); ———, N_2/H_2 (open symbols). (Labuza, 1974.)

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salmon. Since browning rates are usually at a maximum at intermediate moisture contents, one would expect a rapid browning rate to occur along with the rapid oxidation that was discussed earlier. This is shown in Fig. 12 for an i.m. food (a_w 0·85) stored at three temperatures and in two environments (air *vs.* a zero O_2 pouch system). The difference in browning rate between the two atmospheres is the difference between browning as a result of lipid oxidation and browning as a result of ascorbic acid oxidation. It should be noted that the antioxidants BHA, BHT and EDTA were added in this case (Labuza, 1974).

During browning, the protein present reacts to form insoluble polymers and causes a toughening of the protein structure. This toughening also occurs as a result of lipid oxidation as has been shown by several authors (Narayan and Kummerow, 1963; Andrews *et al.*, 1965; Desai and Tappel, 1963). Karel in the previous chapter has reviewed his recent research in this area, showing that the interactions between protein and lipid-derived free radicals increase as a_w increases and parallels peroxide increases.

Table 5 and Figure 13 show results for the increase in peroxides and in the degree of toughening for the same i.m.f. product as in Fig. 12. As can be seen, very little peroxide is produced in air; no peroxides were formed in the O_2 free atmosphere and there is no difference in the amount of toughening between the

TABLE 5. Hennican shelf-life study peroxide values

| | mequiv./kg fat | | | | | |
| | 25°C | | 35°C | | 45°C | |
Day	Air	N_2/H_2	Air	N_2/H_2	Air	N_2/H_2
0	N*	N	N	N	N	N
2	0·39	N				
5	0·88		2·08	N	3·12	
10	4·06	N			3·56	
15			3·80	N	3·72	
20	2·62	N	2·27	N	2·48	
25	4·34	N			5·05	
30	4·52	N	2·93		4·38	N
35	3·54	N				
40	3·09	N				
45	3·00	N	2·40	N		
50	4·03	N	3·28	N		
60	4·03	N				
65	4·24	N	4·09	N		
70			1·67	N		
75	3·03					
80	3·79		3·31			
85			3·93			
90	3·23					

* N = no detectable peroxides.

FIG. 13. Increase in toughening of an intermediate moisture food (a_w 0·86) stored in air (solid symbols) and an O_2 free (open symbols) atmosphere.

two systems. The activation energies calculated for browning and toughening were both about 21 kcal/mol. Since this suggests that lipid oxidation is not occurring, the increased browning measured must be the result of some other degradation reaction which is a function of oxygen and moisture content. Fig. 2 illustrates the results for the degradation of ascorbic acid added to this food. Most likely the degradation products of this have caused the increased browning. This study shows the difficulty in explaining the causes of changes in food quality unless one studies all the degradation reactions which are taking place.

As a last example of the effects of moisture content and oxygen, Table 6

TABLE 6. Loss of infectivity of influenza virus*

		Days to loss of 1 log cycle of infectivity				
Temperature	% H_2O:	0·4	1·0	1·7	2·1	3·2
+10°C		4	24	145	68	7
−20°C		16	480	>·1000	>·1000	40

* Greiff (1971).

shows results for the loss of infectivity of influenza viruses stored in a low-oxygen atmosphere at different moisture contents (Greiff, 1971). The loss is rapid at low moisture, decreases to a minimum and then increases again as the water content is increased. The results exactly parallel the effect of moisture on lipid oxidation. Since this virus has a lipid coat which is about 50% unsaturated lipid, it appears that oxidation destroys the coat in such a way as to make the virus ineffective.

VI. CONCLUSION

It has been shown that many deteriorative reactions in foods are strongly influenced by oxygen and moisture content. In most cases, minimizing oxygen in the package head space and dehydrating the food to the monolayer value will prevent rancidity, loss of ascorbic acid, destruction of pigments and toughening resulting from protein oxidation and non-enzymatic browning. Intermediate moisture foods are especially liable to these reactions because at the higher a_w enough water is present to act as a suitable reaction medium. Thus oxygen control must be exercised to prolong their shelf-life.

Acknowledgements

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B. Evaluation of Current Accelerated Shelf Life Tests

On the following pages is the text of a review paper written
as part of this contract, which will appear in the International
Journal of Food Chemistry.

ACCELERATED SHELF--LIFE TESTING FOR

OXIDATIVE RANCIDITY IN FOODS - A REVIEW

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Abstract

Accelerated shelf life test (ASLT) methods for processed foods are receiving greater attention. In this paper, current ASLT methodology for fatty foods, is reviewed with particular emphasis on the testing of antioxidant effectiveness.

In all the classical ASLT methods temperature is the dominant acceleration factor used. Its effect on the rate of lipid oxidation is best analyzed in terms of the overall activation energy, E_A for lipid oxidation. It is an inherent assumption in these tests that the E_A is the same in the absence and presence of antioxidants. An analysis of the rate equations for the uninhibited versus the inhibited oxidation indicates however, that the E_A may be considerably higher in the latter case. ASLT data collected at 60-65°C bear this out and show that such tests lead to sizeable, but predictable underestimation of the shelf life extension by antioxidants for room temperature. In comparison, data collected at 98-100°C are much less predictable. At this higher temperature E_A-variations are generally smaller and both under- and overestimation of shelf life is found. In addition, the use of such high temperatures for complex foods is ruled out because of secondary reactions of other food components.

Other acceleration parameters for shelf life used are the oxygen pressure, reactant contact and the addition of catalysts. The effect of these factors, although much less important than that of temperature, is discussed.

ACCELERATED SHELF-LIFE TESTING FOR OXIDATIVE RANCIDITY OF FOODS

A. Introduction

Rancidity of edible oils and fatty foods due to lipid oxidation is a serious problem in some sectors of the food industry. Factors which have contributed to this problem in recent years are the increased emphasis on polyunsaturated dietary lipids and the fortification of certain foods with iron. Because of the unfortunate consequences of lipid oxidation in foods it is critical that information about the oxidative stability of susceptible food items be obtained before they are marketed.

The food manufacturer would like to employ methods which can give a reasonably accurate indication of the product shelf-life in a relatively short period of time. Many accelerated shelf-life tests (ASLT) are available. Since the rate of a reaction increases exponentially with the absolute temperature, this parameter is usually singled out to speed up the oxidation in such tests (for explanation of symbols and abbreviations used see Table 1).

The first step in a typical ASLT study is to select a suitable method for testing the food product under consideration. Next, a sample is placed under the conditions of the test and the induction period θ_s is measured, i.e., the time, usually in hours, required to reach a specified end-point. The last and the most difficult step is to translate the value for the induction period obtained into actual product shelf-life in months of storage. Usually this is done with some arbitrary factor based on prior experience. As most highly oxidizable foods contain added antioxidants these methods are often used to evaluate the effectiveness of antioxidants.

There are several reasons why a review of the ASLT methodology is presented here. Originally these methods were designed to be used for homogeneous lipids such as animal fats and vegetable oils. Unfortunately only a few studies have been carried out to evaluate these methods critically. At least two such studies (Pohle et al., 1964; Paul and Roylance, 1962A) raise serious doubts about the usefulness of these tests even when applied to fats and oils. Pohle et al., (1964) concluded from their data that in order to obtain any useful information from the current ASLT methods, each test must be calibrated for each individual fat formulation.

Another serious drawback of the currently used ASLT methods is the fact that with the exception of the Active Oxygen Method, they are not standard methods. Different workers use different versions and modifications, which greatly complicates matters.

Finally, there is a growing interest in applying ASLT methods to formulated foods. The feasibility of using existing methods for this purpose should be evaluated.

Basically four parameters are manipulated in ASLT procedures to speed up the oxidation and development of rancidity in foods or oils. These are listed in Table 2. Increased temperature is the most common and effective means of accelerating the oxidation. The rate of the reaction is exponentially related to the temperature thus shelf life should decrease logrithmically with increasing T. In single-component lipid systems oxidation can be represented by the following equation:

$$RH + O_2 \xrightarrow{K} ROOH \longrightarrow Secondary\ Products = rancidity\ or\ end\ of$$
$$shelf\ life$$
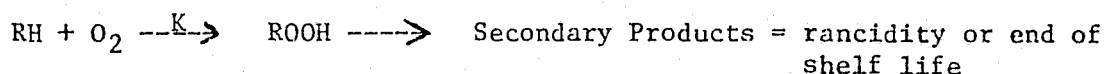
where:

RH = polyunsaturated lipid substrate

O_2 = oxygen

$ROOH$ = lipid hydroperoxide (primary product)

K = overall rate constant for ROOH production

On a kinetic basis $K = Ae^{-E_A/RT}$ where A, the pre-exponential factor, and E_A, the activation energy, stay approximately constant as long as the mechanism does not change. Some workers in analyzing storage studies use Q_{10}-values to determine the accelerating effect instead of activation energies. These quantities are related through the following equation:

$$\log Q_{10} = (2.189\ E_A)/(T + 10)T = \frac{shelf\ life\ at\ T + 10}{shelf\ life\ at\ T}$$

where:

Q_{10} = increase in rate or decrease in shelf life for a $10^\circ C$ increase in T

T = temperature in $^\circ K$

E_A = energy of activation in cal/mole

Unlike the activation energy, the Q_{10}-value is a strong function of temperature and thus is a poor criterion of the temperature sensitivity of the rate constants. As can be seen, the higher the E_A the larger will be the increase in rate resulting from an increase in temperature. If the Q_{10} for the control is known it is usually applied to the test system containing the antioxidant in question. Obviously if E_A changes this Q_{10} factor is meaningless as will be discussed.

Lipid oxidation can be broken down into a number of elementary step reactions where each step has its own activation energy. The controlling overall activation energy E_A can thus change as a result of a temperature elevation alone. Also, the addition of antioxidants, change in the oxygen pressure and other factors can alter the mechanism and hence the activation energy. These E_A changes, therefore, could cause erroneous prediction of the shelf-life at room temperature on the basis of data collected at a higher temperature, when the shelf-life of a control system is compared to that of one with added antioxidants.

In multi-component formulated food systems the effect of temperature can be very complex. A number of changes, both physical and chemical may occur as the temperature is increased as listed in Table 3. Some of these changes could drastically affect the chemical reactivity through their effect on the distribution of reactants, metal binding properties, viscosity, metal prooxidant effectiveness and other variables. The water activity will increase as the temperature is increased and water can evaporate from the food. The fat - if solid at room temperature - can undergo crystal structure changes and eventually melt, contact new catalytic surfaces or drip out of the food. Once melted, oxidation will no longer be limited to the surface of the fat. Some end-products of the browning reaction between carbohydrates and proteins have antioxidative properties (Kirigaya et al., 1968). Various proteins and enzymes will be denatured including lipoxigenases, peroxidases and other heme-proteins, with subsequent alteration in their pro-oxidative potential (Ericksson et al., 1971). Heating can expose SH-groups which can act as antioxidants. Heme-bound metals may adopt a low-spin configuration resulting in a loss

of catalytic activity (Love and Pearson, 1974). Thus, the probability of a change in the mechanism occurring as a result of an increase in temperature is much greater in complex foods than in simple lipid systems. Some of the reactions listed in Table 3 can become significant at temperatures as low as 40 to 50°C. This temperature range should therefore not be exceeded in ASLT studies for foods. The question remains as to whether such a moderate temperature elevation alone provides a sufficient acceleration in the oxidation rates for evaluation of shelf life.

Other parameters besides the temperature which may accelerate the oxidation are listed in Table 2. At oxygen pressures close to atmospheric and higher, little effect of the oxygen pressure on the rate is obtained. However, at higher temperatures the effect of oxygen can become considerable (Bateman, 1954), especially for fats which are high in polyunsaturated fatty acids. Similarly, stirring, air bubbling and the use of "inert" carriers or other means of promoting the reactant contact should have little effect provided the lipid is completely melted, the temperature is relatively low and no trace contaminants are introduced in the process. At the high temperatures of most ASLT methods, however, increasing the contact between oxygen and substrate can have an effect on the rate. Finally, by adding prooxidants such as transition metals one can accelerate the oxidation. This parameter is seldom used, as it may cause changes in the mechanism of lipid oxidation. In conclusion, the temperature is generally by far the most important acceleration parameter used in ASLT methods.

B. The Effect of Temperature on Lipid Oxidation Rates

Lipid oxidation is caused by a free-radical chain reaction occurring through a series of steps. Each one has associated with it a rate constant. The most important steps in the mechanism of the oxidation of polyunsaturated fatty acid esters are shown in Table 4. As indicated the reaction can be divided into four phases: Initiation, propagation, termination and inhibition.

The initiation can be either metal-, light- or thermally-catalyzed. Singlet oxygen initiation has been implicated in the early initiation process (Rawls and Van Santen, 1970). When the lipid contains suitable sensitizers such as chlorophyll or polycyclic hydrocarbons, singlet oxygen initiation may dominate throughout in the presence of ultraviolet or visible light (Labuza, 1971; Grosch, 1975). Normally, however, as soon as hydroperoxides have formed, reactions 2 and 3 predominate. Waters (1971) does not believe that reaction 2 is important in metalcatalyzed lipid oxidation. Kochi (1973) in contrast has suggested that reaction 2 does occur and is rate-limiting in nonpolar and poorly coordinating solvents such as lipids, especially in the presence of transition metals which are powerful oxidizing agents such as Co and Mn.

The propagation occurs through steps 4 and 5. At ambient conditions the concentration of oxygen and methyl linoleate in purified methyl linoleate are on the order of 10^{-3} molar and 1 molar respectively; thus reaction 5 is rate-limiting and the overall rate is approximately independent of the oxygen pressure. As the energy of activation for reaction 5,

E_p is much higher than E_o (Korcek et al., 1972) – the same is not true

at $100^{\circ}C$. If weak, non-hindered antioxidants are present the A radical

can also act as a chain carrier through reactions 6 and -10, especially

at high temperatures and at high antioxidant concentrations.

The termination is dominated by reaction 7 at atmospheric pressure.

In the presence of primary antioxidants termination steps 8 and 9 also

occur. The former is normally "fast" whereas reaction 9 is very slow for

2,4,6-tri-substituted phenols (Ingold, 1973).

The inhibition takes place via reaction 10. Howard and Furimsky

(1973) measured the E_A for a number of amines and phenolic antioxidants.

For highly hindered phenols and amines the E_A was approximately 0-1 kcal/

mole whereas the corresponding value for phenol and aniline was about

5 kcal/mole (Chenier et al., 1974).

Mahoney (1969) has pointed out that for hydroquinones and sterically

hindered phenols (hereafter referred to as ideal inhibitors) reactions 8

and 9 are much faster than reactions 6 and -10, whereas for non-hindered

phenolic antioxidants (i.e. non-ideal antioxidants) the four reactions

can proceed at comparable rates. Commercial primary food antioxidants

generally belong to the former category.

The low value of E_A obtained for hindered phenols and aromatic

amines has been explained in terms of the formation of a hydrogen-bonded

free-radical complex prior to the transfer of hydrogen (Howard and Furimsky,

1973). The activation energy for the back-reaction, E_{-A} may be roughly

estimated by subtracting the bond dissociation energy of AH, D_{AH} from that

for ROOH, D_{ROOH}. Generally the latter is about 88 (Howard, 1973) while

the former is about 80 for hindered phenols, e.g., 81 kcal/mole for 2,6-di-tert-butylphenol (Chenier et al., 1974). Therefore E_{-a} in this case is close to 7 kcal/mole. Hence at high temperatures and once significant hydroperoxide has built up, the back-reaction can become important giving the antioxidant some prooxidant properties. Reaction 6 also has a relatively high activation energy and would be expected to dominate the chain transfer process at low hydroperoxide concentration.

In the absence of primary antioxidants the limiting equation at "high" oxygen pressures has been shown to be (Labuza, 1971):

$$[ROOH]^{1/2} = (k_i [M]/k_t)^{1/2} k_p [RH] \Theta/2 = K_M \Theta/2 \tag{11}$$

thus $E_A = E_p + 0.5(E_i - E_t) \approx 6 + 0.5(E_i - 0)$ $\qquad$ (12)

where:

Θ = time

K_M = overall uninhibited reaction rate constant

It should be noted that the rate does not depend on the oxygen pressure. At "low" oxygen pressures however, an oxygen-dependent rate-expression with a lower E_A is found.

Because of the change in termination with the presence of ideal primary inhibitors the following first order rate-expression is obtained (Labuza, 1971):

$$\ln [ROOH] = \ln [ROOH]_o + k_i [M] k_p [RH] \Theta \big/ k_A [AH] = \ln [ROOH]_o + K_A \Theta \big/ [AH] \tag{13}$$

thus

$$E_a = E_i + E_p - E_a \approx E_i - E_a + 6 \tag{14}$$

where:

$$[ROOH]_0 = \text{initial hydroperoxide concentration}$$

$$K_A = \text{overall inhibited reaction rate constant}$$

In this case the rate is also oxygen-independent, even at quite low oxygen pressures. As shown the rate of lipid oxidation is inversely proportional to the antioxidant concentration.

A comparison of equations 12 and 14 shows that the E_A will be considerably higher in the presence of a primary antioxidant than for the corresponding control if E_i is the same. This is of course quite logical and indicates that these inhibitors lower the rate of the oxidation at least partly by increasing the overall energy of activation. It must be realized, however, that equation 13 and hence equation 14 applies only for ideal primary inhibitors. Especially for relatively non-hindered phenolic antioxidants at high antioxidant concentrations, much more complex rate expressions with lower E_A-values can be expected. The same is probably true for the ideal inhibitors. At high temperatures chain transfer reactions become more important and equations 13 and 14 no longer apply.

To illustrate the significance of equations 12 and 14 one can consider a reaction, in this case lipid oxidation ($RH + O_2 \xrightarrow{K} ROOH$), which can occur via two different routes, path 1 and path 2, where $K_1 = A_1 e^{-E_1/RT}$ and $K_2 = A_2 e^{-E_2/RT}$. If path 1 represents lipid oxidation in the absence of primary antioxidants and path 2 in the presence of primary antioxidants then $E_2 > E_1$. The Arrhenius plots corresponding to $A_1 = A_2$ are shown in Fig. 1a. As shown, the degree of protection by the primary antioxidant increases as the temperature is decreased.

In order to apply these principles to ASLT methodology one must first assume that the overall rate constant (K_1 or K_2) is proportional to the reciprocal of the "induction period," Θ_S, where the induction period (or the shelf-life at room temperature) is defined as the time to reach a constant percent oxidation of the substrate or end of shelf-life. Hence one can plot "either" $\ln K$ or $\ln \Theta_S$ versus $1/T^{O}K$ (or $T^{O}C$, if the temperature interval is small) and obtain linear plots as shown in figures 1a and b. These plots both show that the effectiveness increases as T decreases. Thus the overall protection predicted at high temperatures for an antioxidant will usually be less than that found at lower temperatures. On the other hand, if the E_A decreases when the antioxidant is added, the degree of protection projected from high temperatures would overestimate the true shelf-life.

Bolland (1949) assumed that E_i = 30 kcal per mole for all purified olefins. In foods the E_i is much lower. Labuza (1972) found that in the range of 37-52°C for lipid oxidation in a chicken/cellulose/glycerol food system, E_A was about 10 in the absence of added antioxidants, indicating that E_i may have been as low as 8 kcal ole. In the presence of added EDTA the E_A rose to about 17 presumably because the metal catalysts were inactivated, allowing an increase in E_i (to about 22 kcal/mole).

These considerations have important implications for high temperature testing of antioxidants at a single temperature. When fats such as animal fats, which contain very low levels of primary antioxidants (cf.

equation 12), are tested against the same fat containing ideal antioxidants (cf. equation 14) the temperature coefficients are quite different. Thus, any quantitative prediction for lower temperatures is impossible unless several temperatures are used. Besides, the order of effectiveness (ranking) at the higher temperature may not be the same at lower temperature, since E_A may vary depending on the structure of the antioxidant. The same is true for the evaluation of chelating agents since the E_i will change as compared to the control. However, if the only objective is to qualitatively rank antioxidants of similar structure (e.g., hindered phenols) at a single temperature, the test is probably useful.

Some other important considerations include: (1) Especially for weak, non-hindered antioxidants, very complex rate equations have been obtained indicating that different mechanisms can occur (Scott, 1965, Ingold, 1970). (2) Volatility of antioxidants can become important at high temperatures, e.g., for BHT (Klaui, 1971). (3) If two phases are present such as water and fat, the solubility in each phase and pH may become important especially for the low-molecular weight gallates (Cornell et al., 1970).

Lea (1960) prepared purified tocopherol-free cottonseed, linseed and cod liver oil esters which respectively were high in linoleate, linolenate and more unsaturated fatty acid esters. From the induction period (time to reach PV = 100 meq/kg) the E_A-values for the control oils respectively were about, 20, 11, and 13 kcal/mole. For samples containing various hindered phenols the corresponding E_A ranges were

35-40, 27-35 and 13-20. Agreement with equations 12 and 14 thus seems

to have been excellent for cottonseed oil if E_i is taken as 30 kcal/mole.

In this case the gallates and hydroquinones behaved very similarly to the

phenols. Agreement could be considered fair for the other substrates if

one assumes lower values for the E_i. However, in the linseed oil the

gallates and hydroquinones did not show the same behavior as did the

phenolic antioxidants and thus the E_A-value was somewhat different.

C.   ASLT Methods for Predicting Oxidative Stability

(a)   The Schaal Oven Test (SOT)

This method was developed in the baking industry in the twenties.

No published reference by its originator exists (Dugan, 1955). Joyner and

McIntrye (1938) recommended that 50 g samples be held in 250 ml beakers with

watch-glass on the top and maintained at about 63°C. The samples were

smelled daily until the rancid point was reached. Lea (1962) advocated

the use of peroxide values to monitor the oxidation and the use of much

smaller samples (0.2 ml) which were kept in small glass cups, the oil

forming about a 2 mm layer on the bottom.

The temperature called for in this method is much lower than in

most other ASLT procedures. This method can therefore be recommended

as the one having the fewest possible problems. Hartman et al., (1975)

feel that this method gives a better correlation with an actual shelf-life

test than the Active Oxygen Method. However, especially for complex foods,

a temperature of 60°C is too high. The end-points used – either a rancid

odor or a peroxide value of 70-120 – are appropriate for correlating

with the shelf-life at lower temperature.

(b)   Oxygen Absorption Methods (OAM)

Many versions of the Oxygen Absorption Method are available. The
most commonly used procedures are those of Sylvester-Martin and Eckey.
In the former method (Sylvester et al., 1942) as modified by Martin (1961),
100 to 1,000 mg samples of lipid are kept in 30 ml flasks connected
to mercury manometers. These are connected to a pressure recorder. The
sample is kept at atmospheric pressure in oxygen at $100^{\circ}C$. The end-point
was taken as the time when a marked drop in pressure occurred. If the
sample absorbed oxygen only gradually throughout, the end-point was taken
at the organoleptic rancid point. In order to get a sharp end-point with
vegetable oil, Sylvester found it necessary to replace the air with oxygen.

Eckey (1946) proposed a somewhat similar design in which 1 g of lipid
was suspended in 12.5 g of "pure silica sand" in a 50 ml flask. The
temperature was maintained at $80^{\circ}C$. The end-point was taken as the time
for the sample to absorb 3 ml of oxygen as calculated at $0^{\circ}C$, 760 mm. The
author used air as the surrounding atmosphere, but suggested a modified
design that could be used with pure oxygen.

The temperature used in these methods is considerably higher than
that used in the Schaal Oven Test. This is a serious disadvantage even for
simple lipids. Of the two methods considered the Eckey method involves a
somewhat lower temperature, but this method has two additional disadvantages.
First, the sand is bound to introduce trace prooxidants. Generally the use
of the sand increased the rate 1.4 to 6 times. The least acceleration
occurred with a sample containing phosphoric acid. This acid can act as
a metal chelating agent indicating that the acceleration was probably due

to metal contaminants. The other problem is the end-point chosen. The significance of the induction period obtained depends on the degree of unsaturation of the oil. If the lipid to be tested contains 10% polyunsaturated fatty acids (PUFA), 1 g of lipid contains about 0.3 mmoles PUFA. Three ml O_2 at 1 atm and 0^oC corresponds to about 0.13 mmoles O_2. Thus at the end-point the sample would be about 40% oxidized. In contrast, rancidity in foods usually occurs when the lipids are 0.1 to 3% oxidized (Labuza, 1971).

A considerable disadvantage of both methods is that at the relatively low oxygen pressures used (3 and 15 psia in the Eckey and Sylvester-Martin methods respectively) the rate can easily become dependent on the oxygen pressure and the rate of oxygen dissolution. There are three reasons why this can occur. At the high temperatures used (a) a shift in the mechanism occurs so that now higher oxygen pressures are needed to remove the oxygen dependence of the reaction rate (Bateman, 1954), (b) the solubility of oxygen in the lipid decreases (Bateman et al., 1951) and (c) the rate of oxidation increases dramatically decreasing the oxygen concentration in the lipid more and more as the reaction proceeds (Bateman and Gee, 1951). The dependence on oxygen will be expected to increase with the degree of unsaturation of the lipid.

Sylvester et al., (1942) found that for palm kernel oil the induction period was almost cut in half when the air was replaced with pure oxygen. Pohle et al., (1962) using a considerably higher oxygen pressure found less than a 10% increase in rate as the oxygen pressure was increased from 65 to 115 psia. At higher temperatures the dependence was found to be even greater as expected (Bennett, 1964).

(c) The Active Oxygen Method (AOM)

The Active Oxygen Method or the Swift Stability Test, as it is
sometimes called, was originally proposed by King et al., (1933) and later
modified slightly by Riemenschneider et al., (1943). Twenty ml samples of
lipid are kept in 1" x 8" glass tubes and clean dry air at 2.33 cm^3
per sec is bubbled through. The temperature is maintained at 97.8°C.
Periodically about 0.2 ml samples are withdrawn and the peroxide value (PV)
is determined until it reaches 120 meq/kg. Unlike most other ASLT methods
this one has been rigorously standardised.

The main problem with this method is the high temperature used.
Generally an arbitrary multiplying factor is used based on previous experience
to give an estimate of the shelf-life at room temperature. The method
obviously cannot be used with formulated foods.

Another problem is the arbitrary air bubbling rate. Since one is
trying to compare the data with room temperature conditions where the rate
is indeed oxygen-independent, ideally the rate in the AOM test should be
oxygen-independent also. The use of bubbling does of course speed up the
rate of dissolution. Air at atmospheric pressure, however, will not suffice
to achieve oxygen independence even if one assumes that the oxygen concen-
tration in the lipid equals the oxygen solubility under the conditions used.
As oxygen solubility depends almost linearly on the oxygen concentration
in the head space, the only way to maintain oxygen-independence is to use
higher oxygen pressures. The final problem is that antioxidants such as
BHT which are relatively volatile can evaporate from the sample (Klaui,
1971).

Luckman et al., (1953) used specially prepared and cleaned iron tubes

instead of glass tubes to shorten the induction period. A two to six-fold

increase in the rate was obtained with hydrogenated vegetable oil. In

contrast only a 20% increase was obtained when the fat contained 0.08% added

isopropyl citrate. This modification has not been generally accepted. The

easiest and most accurate way of increasing the level of prooxidants is to

add the metal directly in the form of a salt.

(d)   The ASTM Oxygen Bomb Method (OBM)

This method has long been used (A.S.T.M. Committee D-2, "A.S.T.M.

Standards on Petroleum Products and Lubricants," pp. 254-257, 1955) to determine

the resistance of petrols to gum formation (Scott, 1965). Gearhart et al., (1957)

were the first to use this method for food lipids. The authors added 15-30 g

of lipid to a glass container which was fitted into the bomb. The oxygen pressure

used was either 65 or 115 psia and the temperature 99°C. The induction period

was taken as the time to reach the mid-point of the first hour during which

a pressure drop of at least 2 psia/hr was obtained. The reproducibility

has been shown to be excellent (Pohle et al., 1962). Stuckey et al., (1958)

modified the method by using a smaller sample which was spread on tissue

paper in order to increase the contact between the lipid and the oxygen.

For lard a 5-8 fold increase in the rate was obtained in this way.

Three problems occur with this method. First, the temperature is

too high. Secondly, as in the Eckey method the 'egree of oxidation of the

sample at the end-point depends on the % PUFA in the lipid. When a 6 g

sample of 10% PUFA-lipid is to be tested in a 200 ml bomb the sample will be

about 40% oxidized at the end of the induction period, if one assumes a total pressure drop of 2 psia. Finally, unwanted contaminants can be introduced from the tissue paper.

Pohle et al., (1963) proposed a modification whereby a fat soluble copper-salt was added to the oil at a level of 25 or 100 ppm. Approximately a 10-fold increase in the rate was obtained in this manner. This method is not generally accepted.

(e) Other Methods

A number of other methods have been suggested for specific applications. The Weight-Gain Technique (Sherwin, 1968) is based on the increase in weight of the lipid as it continues to absorb oxygen. This method is not recommended. Unless the weight-gain is calibrated against oxygen uptake data the method is meaningless.

A variety of methods have been proposed specifically to evaluate antioxidant effectiveness. Cort et al., (1975) used a modification of the Schall Oven Test (Lea's version) which is carried out at 45°C and which they term the Thin Layer Test. This method is strongly recommended, but it will be slow when potent antioxidants are tested.

Because of the problems experienced with the high-temperature ASLT methods, alternative means of acceleration have received more interest recently. Most of these tests use some form of metal-containing prooxidants. According to Betts and Uri (1968) metal ions, when they act as prooxidants, usually increase the rate in proportion to their concentration to the one-half or first power. Hence the addition of such prooxidants can be used to give a powerful catalytic effect.

Uri (1961B) has pointed out that since the temperature coefficients
of antioxidant efficiency vary with the nature of the antioxidant, high-
temperature ASLT studies are open to criticism. Furthermore, since lipid
oxidation is generally trace-metal catalyzed, adding metallic prooxidants
may be a more meaningful method of acceleration. The author used ferrous
phthalocyanine as a catalyst for linoleic acid oxidation in an ethyl
benzoate solvent at 25°C. He compared the effectiveness of five anti-
oxidants (propyl gallate and four flavonoids) at a concentration close to
50 ppm. The order of effectiveness was found to be similar to that
obtained using a control containing no added prooxidant. It should be
pointed out that the advantage of using complexed iron is that it is
equally effective in the presence of metal chelating agents.

Berner et al., (1974) used hemin catalyst which they added to a lipid
emulsion at 45°C, pH 7.2 and measured the oxygen uptake using an oxygen
analyzer. The method was used for lard containing 0.02% of one of several
antioxidants. The AOM method was used for comparison. As expected, the
two methods ranked the antioxidants quite differently. In particular the
more water-soluble antioxidants (PG and TBHQ) were much less effective
in the former method, probably in part because of their higher partition
into the water phase.

The use of metallic prooxidants in ASLT studies, perhaps in
conjunction with a moderate temperature elevation, should be given careful
consideration. One of the problems involved is that some foods contain
higher levels of endogenous metals than others. Therefore, different

amounts must be added to different foods to give the same percent acceleration. If metal salts are used, problems will be encountered when metal-chelating compounds are present. By using metal complexes such as phthalocyanins or hematin-catalysts, this may be avoided. However, the possibility of a change in the mechanism can become much greater, e.g., when hematins are used (Kendrick and Watts, 1969). In selecting a suitable catalyst, the emphasis should be on using one which is already present in the product and which is likely to be the dominating catalyst for lipid oxidation in that particular food. One would question, therefore, the use of heme-catalysts for accelerating the oxidation of vegetable oils.

D. ASLT Study Result Analysis

Unfortunately ASLT studies where the overall rate constant K_A and K_M are calculated are almost non-existent. In calculating E_A-values for such studies one must rely on data for the induction period obtained. The use of different end-points and the concurrent presence of other acceleration parameters makes the calculated values inaccurate at best. An added obstacle, when it comes to interpretating the data, is the loose characterization of the substrates given. Neither trace metal content nor the fatty acid distribution is generally known. Obscure terms such as "stabilized lard," "shortening A" and "hydrogenated vegetable oil" are often the only information given.

Because of the different temperatures used for different methods it is convenient to divide this survey into two parts depending on the T-zone under consideration.

(a) Ambient to 65°C

In this range the Shelf-Life Study carried out at or close to room temperature and the Schaal Oven Test are the most commonly used methods. The most interesting data available are those of Pohle et al., (1964) who compared various different ASLT methods. When comparing Shelf-Life Test data collected at 29.5°C with data obtained using the Schaal Oven Test at 60°C an E_A-value of about 14 kcal/mole can be calculated for both lard and tallow. When 0.01% BHA was added, the value increased to 18-20 kcal/mole. In contrast the E_A for hydrogenated vegetable oil (HVO) was about 18-20 and did not change when BHA was added. These results are in line with what would be predicted on the basis of equations 12 and 14 and the fact that most vegetable oils already contain close to an optimum concentration of primary antioxidants (tocopherols).

(b) Ambient to 100°C

The most important ASLT methods which are carried out in the vicinity of 100°C are the AOM, OBM and the two oxygen absorption methods. Of these methods only the OBM, especially if used with a dispersant, will be close to giving oxygen-independent kinetics. The dispersant, however, is bound to be a source of prooxidative contaminants. Of the other methods the AOM is intermediate and the absorption methods are the most severely oxygen-dependent.

Pohle et al., (1962) found the Eckey OAM at 100°C to be twice as fast as the AOM. Stuckey et al., (1958) found the OBM without a dispersant

to be 1.4 times faster than the AOM when lard was the substrate With

dispersant the OBM was 3-6 times faster still. Based on these considerations,

the OBM seems to be the best of the high-temperature ASLT methods.

Pohle et al., (1964) made extensive comparisons between different

high-temperature ASLT methods and concluded that the OBM was the most precise

and gave the best correlation with product stability. When comparing their

shelf-life test data at 29.5°C with AOM data, the calculated E_A for lard is

20 and for tallow is 14 kcal/mole. In the presence of 0.01% BHA these values

dropped to 16 and 11 kcal/mole respectively instead of increasing as expected.

In this case, predictions about antioxidant effectiveness, based on the

control, would cause the degree of protection afforded by the antioxidant

under normal storage conditions to be overestimated. Why the E_A dropped

is unexplainable.

When the OBM-data for these same fats are compared to the Shelf-Life

Test data the calculated E_A becomes about 19 kcal/mole for lard and 10 kcal

for tallow. The corresponding values in the presence of 0.01% BHA are 19 and

13 kcal/mole. Similarly the value for HVO is 16 kcal/mole both with and

without added BHA.

Thompson and Sherwin (1966) collected data at 43.3°C (storage studies)

and 98.9°C (AOM) for safflower oil. Their data show that E_A can vary from

13-24 kcal/mole depending on the antioxidant used. Generally, however, the

value increased from 17 for the control to 19-20 when antioxidants were

added. In this case predictions would lead to underestimation of protection

at room temperature.

Paul and Roylance (1962) collected data at 10-20°C (Shelf-Life Test) and 98.4°C (AOM) for two batches of peanut oil, (1) factory oil, refined in iron vessels, (2) further purified oil, using batch 1 as starting material. The E_A for the batch 1 control was about 9 and dropped to 7.5 kcal/mole in the presence of some primary antioxidants (0.01% level). This difference may seem slight, but in this case indicated a 60-70% increase in the AOM-induction period whereas no corresponding increase in shelf-life occurred. For batch 2 the E_A for the control was about 17 kcal/mole indicating that the purification procedure (Crossley et al., 1962) removed active prooxidants, possibly trace metals. In the presence of primary antioxidants (0.01 or 0,02% level) the E_A appears to have been considerably lower.

The data of Paul and Roylance show that the AOM is unreliable, not only for testing antioxidants but also for testing batches of oil processed in different ways. The shelf-life test data collected indicated that the purification procedure increased the shelf-life of the control in some cases by over 100% while the AOM data showed a dramatic drop in the induction period and thus a shorter shelf-life.

These results illustrate that temperature-accelerated tests procedures for rancidity are generally more accurate for vegetable oils and stabilized animal fats. However, even for such lipids, high temperature test procedures can give very misleading results. In some cases the protection at 100°C is greater than at room temperature, whereas in other cases the reverse is true. The Schaal Oven Test seems to yield much more predictable results although more data are needed to prove this. It seems quite possible that in this test one may be

able to correct for E_A-differences between samples with and without anti-oxidants using equations 12 and 14.

The erratic behavior at high temperatures is undoubtedly caused at least in part by the increased importance of prooxidative side-reactions of the antioxidants, when the antioxidant radical, A· becomes an effective chain carrier as discussed above, causing a change in mechanism of antioxidation and possibly a drop in the activation energy. It seems that in the studies reviewed here, these reactions are not sufficiently important at 60°C to greatly affect the results. Shelf-life studies for both model systems and foods are currently being carried out in our laboratories using oxygen absorption in the range of 25-45°C. Results will be presented in the future.

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TABLE 1

ABBREVIATIONS AND SYMBOLS USED

Symbol	Meaning
a	Inhibition, reaction 10
A	Preexponential factor
$A\cdot$	Antioxidant radical
AH	Primary antioxidant
AOM	Active Oxygen Method
ASLT	Accelerated shelf-life test
BHA	Butylated hydroxyanisole
BHT	Butylated hydroytoluene
c	Chain transfer, reactions 6 and -10
d	Dimerization, reaction 9
D	Bond dissociation energy, kcal/mole
E_a, E_i, E_p, E_t	Activation energies for elementary steps
E_A	Overall activation energy
EDTA	Ethylene diaminetetraacetic acid
f	Reaction 8
HVO -	Hydrogenated vegetable oil
i	Initiation, reaction 2
k	Rate constant for elementary step
K	Overall reaction rate constant
K_A	Overall inhibited rate constant
K_M	Overall uninhibited rate constant
M	Metal ion
M^{n+}	Metal ion, lower oxidation state
$M^{(n+1)+}$	Metal ion, upper oxidation state
o	Propagation, reaction 4
OAM	Oxygen Absorption Method
OBM	Oxygen Bomb Method
p	Propagation, reaction 5
PG	Propyl gallate
psia	lbs/sq. in. absolute pressure
PUFA	Polyunsaturated fatty acid
PV	Peroxide value, meq ROOH/kg lipid
Q_{10}	Temperature coefficient, k_{T+10}/k_T
R	Universal gas constant, 1.987 cal/$^\circ$K-mole
$R\cdot$	Lipid radical
RH	Lipid substrate
$RO\cdot$	Oxyl radical
$ROO\cdot$	Peroxyl radical
ROOH	Hydroperoxide
SOT	Schaal Oven Test
t	Termination, reaction 7
T	Temperature in $^\circ$C or $^\circ$K
TBHQ	Tertiary butylhydroquinone
Θ	Time
Θ_s	Induction period (elevated T) or shelf-life (room temperature)

TABLE 2

COMMON ACCELERATION PARAMETERS

Parameter	Normal Range
Temperature (^{o}C)	60-140
Oxygen pressure (psia)	3-165
Added Metals (ppm in lipid)	25-100
Reactant Contact	variable

TABLE 3

EFFECTS OF "HIGH" TEMPERATURES ON FOODS

Component	Process	Critical T-Zone
Water	Transfer	Above ambient
	Evaporation	Above 60°C
Fat	Crystal changes	Close to melting point
	Melting	Above melting point
	Transfer	Above melting point
Starch	Gelatinization	Above 60°C
Carbohydrates	Non-enzymatic browning	Above 50°C
	Carmelization	Above 100°C
	Charring	Above 100°C
Proteins	Denaturation	Above 40°C

TABLE 4

MECHANISM OF LIPID OXIDATION

		$k_{30°C}$	E_a
Initiation			
1.	$RH + M^{(n+1)+} \longrightarrow R\cdot + H^+ + M^{n+}$	f(metal)	f(metal)
2.	$ROOH + M^{(n+1)+} \xrightarrow{k_i} ROO\cdot + H^+ + M^{n+}$	f(metal)	f(metal)
3.	$ROOH + M^{n+} \longrightarrow RO\cdot + OH^- + M^{(n+1)+}$	f(metal)	f(metal)
Propagation			
4.	$R\cdot + O_2 \xrightarrow{k_o} ROO\cdot$	$10^7 - 10^9$	0
5.	$ROO\cdot + RH \xrightarrow{k_p} ROOH + R\cdot$	10^2	5-7
6.	$A\cdot + RH \xrightarrow{k_c} AH + R\cdot$	slow	5-10
Termination			
7.	$ROO\cdot + ROO\cdot \xrightarrow{k_t} ROOOOR$	10^7	0-3
8.	$ROO\cdot + A\cdot \xrightarrow{k_f} ROOA$	fast	0-3
9.	$A\cdot + A\cdot \xrightarrow{k_d} A_2$	slow	0-3
Inhibition			
10.	$ROO\cdot + AH \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} ROOH + A\cdot$	10^4	0.8

O_2	=	oxygen
RH	=	lipid substrate = methyl linoleate or linolenate
$R\cdot$	=	substrate radical (di- or tri-allyl)
$RO\cdot$	=	lipid oxyl radical
$ROO\cdot$	=	lipid peroxyl radical
ROOH	=	lipid hydroperoxide
AH	=	primary antioxidant = butylated hydroxy toluene, BHT
$A\cdot$	=	antioxidant radical (phenoxy)
$k_{30°C}$	=	rate constant at 30°C, 1/mole-sec
M^{n+}	=	metal ion, lower oxidation state
$M^{(n+1)+}$	=	metal ion, higher oxidation state
E_a	=	energy of activation, kcal/mole

Uri (1961A); Ingold (1973); Howard (1973); Howard and Furimsky (1973): Bateman (1954); Korcek et al., (1972); Ingold (1968).
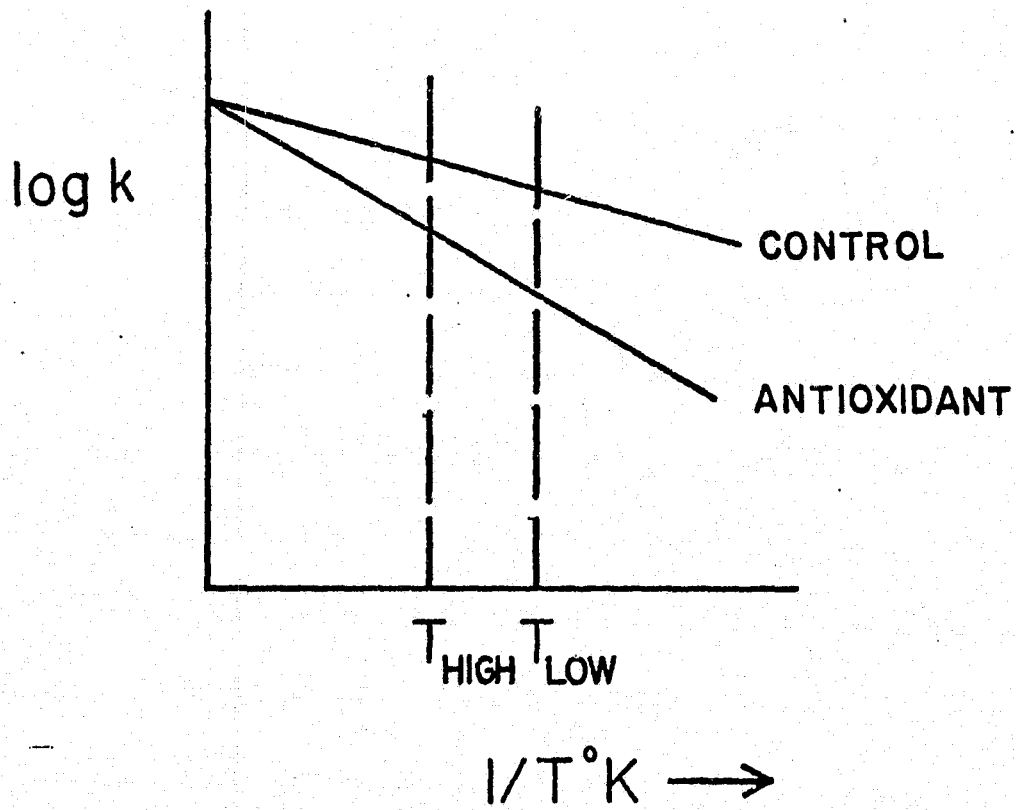
FIGURE 1A

ARRHENIUS PLOTS FOR TWO PARALLEL REACTIONS
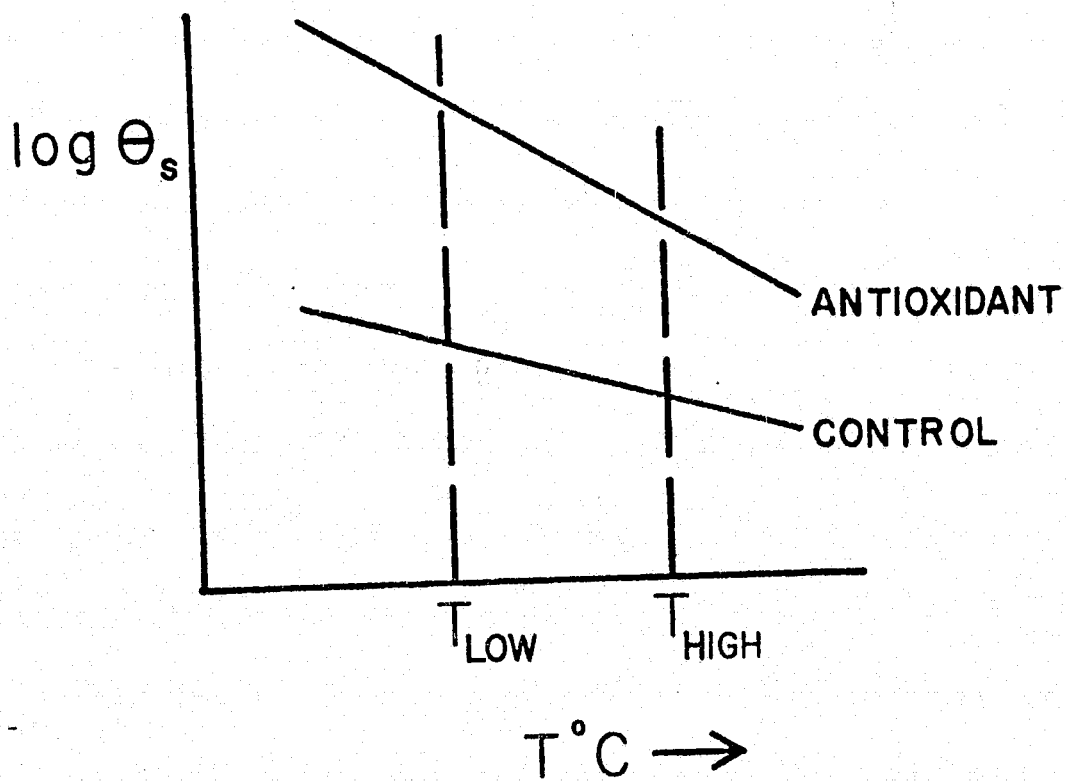
FIGURE 1B

ASLT Plots For Two Parallel Reactions

C. __Shelf Life Test of Antioxidants in Dry and IMF Model Food__

   __systems__

   1. Introduction

        There is a great need for critically evaluating the entire
accelerated shelf life methodology for stability of fats and oils.
One of the major reasons is the wide variation in methods, conditions
and endpoints used in these tests. Basically an accelerated shelf
life test (ASLT) method uses harsh conditions including temperature
elevation to speed up the oxidation. But a great deal of work remains
to be done in relating these conditions to the underlying mechanisms
of lipid oxidation. Since the most commonly used acceleration parameter
is temperature, the effect of temperature on lipid oxidation should
be clarified. This is especially true because the usual temperature
employed in these tests is 60 to 140°C. Even more important, however,
is the fact that all of these ASLT methods were originally designed
for use with pure oils. Application to the real food systems has not
been done.

        When using an ASLT method for a pure oil the danger is
that the mechanism of lipid oxidation may change when the sample is
exposed to the harsh conditions of the test. With real foods the
situation becomes much more complex. This is because at high tempera-
tures other food components may react and undergo changes which can
influence lipid oxidation. As an example, starches gelatinize in the

temperature range 60 - 100°C and can affect water binding. Proteins

denature in the same temperature range and may affect metal availability.

Carbohydrates enter caramelization, browning and charring reactions with

possible antioxidant effect. The fat also may melt and drip from the

food and also penetrate regions of the food previously unaccessable

where it could contact catalytic surfaces.

Table 1 shows the most common ASLT methods. The fastest

one is the Modified Oxygen Bomb Method of Pohle et al., (1963). The

slowest one is the Schaal Oven Method. The table clearly shows the

wide range in test conditions used in these tests.

If these ASLT methods are to be critically evaluated

they must be related to the underlying mechanism of lipid oxidation.

Uninhibited lipid oxidation basically occurs in three stages:

initiation, propagation and termination stage. When primary anti-

oxidants are added - antioxidants which interfere directly with the

reaction chain by inactivating free radicals - a further inhibition

stage is added. These steps can be written as follows:

Initiation	Initiator	$\xrightarrow{k_i}$	Free radicals	(1)
Propagation	$R\cdot + O_2$	$\xrightarrow{k_o}$	$ROO\cdot$	(2)
	$ROO\cdot + RH$	$\xrightarrow{k_p}$	$ROOH + R\cdot$	(3)
Termination	$ROO\cdot + ROO\cdot$	$\xrightarrow{k_t}$	Non-radicals	(4)
	$R\cdot + ROO\cdot$	$\xrightarrow{k_t'}$	Non-radicals	(5)
	$R\cdot + R\cdot$	$\xrightarrow{k_t''}$	Non-radicals	(6)
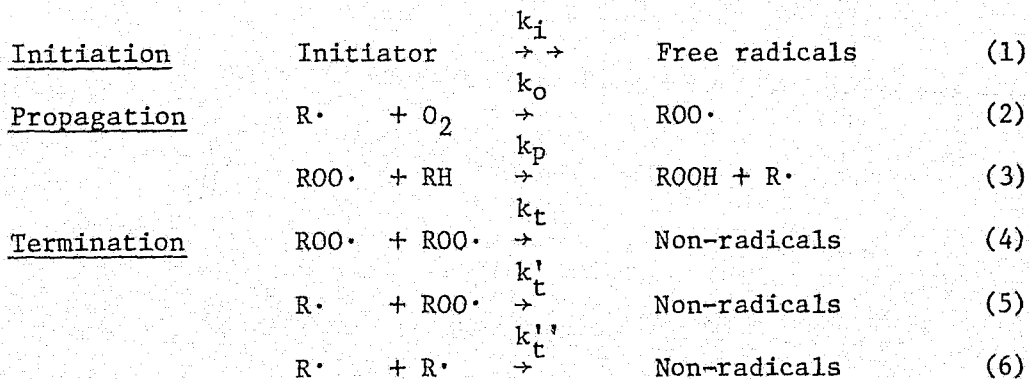
Table 1

Analysis of Common ASLT Methods

Accelerating Variables	AOM*	OBM	MOBM	OAM	SOM
$T^{\circ}C$	97-100	100-135	100	80-140	37-70
PPM Cu	0	0	25-100	0	0
Reactant	2.33 ml	Inert	Inert	Inert	Oil Film
Contact	Air/Sec	Dispersant	Dispersant	Dispersant	
P_{O_2}	ATM	50-150	50	3-15	ATM
Endpoint	PV=100	2PSI/HR	2PSI/HR	40 MM Hg	Rancid

* Titles of Tests

AOM	Active Oxygen Method
OBM	Oxygen Bomb Method
MOBM	Modified Oxygen Bomb Method
OAM	Oxygen Absorption Method
SOM	Schaal Oven Method

Inhibition	$ROO\cdot$	$+ AH_2$	$\xrightarrow{k_a}$	$ROOH + AH\cdot$	(7)
	$R\cdot$	$+ AH_2$	$\xrightarrow{k_a'}$	$RH + AH\cdot$	(8)
	$AH\cdot$	$+ AH\cdot$	$\xrightarrow{k_a''}$	$AH_2 + A$	(9)
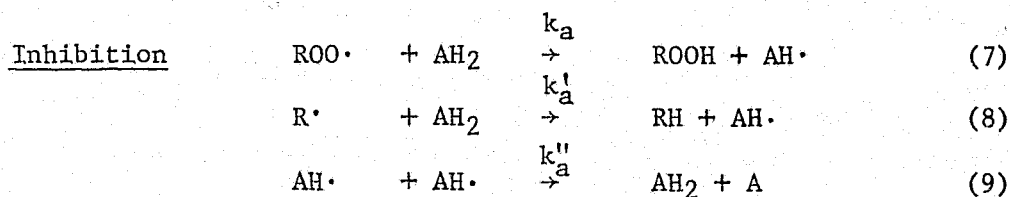
RH $\quad\quad\quad$ = fat or oil substrate molecule

ROOH $\quad\quad$ = Hydroperoxide

AH_2 $\quad\quad\quad$ = primary antioxidant

$R\cdot$, $ROO\cdot$ et. = free radicals

The initiation step may be brought about by metal catalysts, heme-proteins, light (especially UV-light) and heat. The most frequent type of initiation is probably metal-catalyzed or a combination of metal and thermally initiated lipid oxidation, since metal ions are present in all fats and oils as trace contaminants. Five reactions are thought to be involved in the initiation process brought about by metal ions: (Ingold, 1962)

$RH + M^{(n+1)+}$	$\rightarrow$	$R\cdot$	$+ H^+ + M^{n+}$		(10)
$O_2 + M^{n+}$	$\rightarrow$	$O_2^-\ldots\ldots M^{(n+1)+}$			(11)
$O_2^-\ldots\ldots M^{(n+1)+} + RH$	$\rightarrow$	$R\cdot$	$+ HO_2^\cdot + M^{n+}$		(12)
$ROOH + M^{n+}$	$\rightarrow$	$RO\cdot$	$+ OH^- + M^{(n+1)+}$		(13)
$ROOH + M^{(n+1)+}$	$\rightarrow$	$RO_2^\cdot$	$+ H^+ + M^{n+}$		(14)
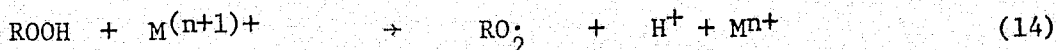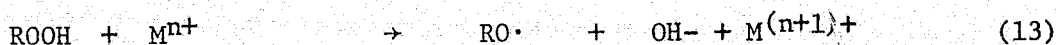
Generally in nonpolar media reactions 10, 11 and 12 (as well as others) are expected to be important only in the very beginning. As soon as the hydroperoxide concentration starts to build up hydroperoxide breakdown via reactions 13 (reduction activation, very fast) and 14 (slower and rate-determining) becomes the main route for initiation. This means

that after the oxidation has started it makes no difference whether the metal ions are added in the higher or the lower valency state.

The propagation step shows that theoretically only one R· radical is needed to start the chain reaction. Also it shows that each oxygen molecule absorbed is converted to one hydroperoxide molecule. Hence, provided the hydroperoxide decomposition is slow, it does not matter whether one measures oxygen uptake or peroxide values to monitor the reaction.

By applying the Bodenstein steady-state approximation the basic derivation for the kinetics of lipid oxidation can be obtained. It is a complex equation involving the various rate constants given in equations (1) to (7) above. Through the pioneering work of chemists at the Brit. Rubber Prod. Res. Assoc. and others, the various rate constants and their associated energies of activation have been evaluated for ethyl linoleate and some other substrates. By a pure coincidence it turns out that for pure ethyl linoleate at 25°C the rate constants are such as to allow one to make a drastic simplification so long as the oxygen pressure is close to atmospheric. The rate equation in this case becomes:

$$d(ROOH)/dt = k_p r_i^{1/2}(RH)/(k_t)^{1/2} \qquad (15)$$

where r_i = rate of initiation = $k_i(ROOH)^n$ in the case where the initiation is brought about by breakdown of hydroperoxides and $n = 1$ in the beginning and often changes to $n = 2$ at higher hydroperoxide concentrations.

One must be very careful in applying this equation to oxidation of food systems. For example, it turns out that even for pure ethyl linoleate at 100°C and at atmospheric pressure this simplified equation (equation 15) can no longer be used. Real food lipids contain linoleic acid esterified not to ethyl or methyl alcohol groups, but to glycerol or glycerol-phosphate. Besides, there may be other PUFA than linoleic acid and the food may contain water, proteins and other food components which may modify the kinetics.

In the model systems used in the present study, methyl linoleate was used as the substrate. The highest temperature used was 45°C. Under these conditions one may expect to be able to use equation 15. This equation has been integrated (Labuza, 1971). At low levels of oxidation when hydroperoxides are decomposing via a monomolecular mechanism one gets:

$$(ROOH)^{1/2} = K_M t/2 \qquad (16)$$

where K_M = overall rate constant for lipid oxidation in the monomolecular period = $k_p k_i^{1/2} (M)^{1/2} (RH)/(2k_t)^{1/2}$.

Labuza (1971) derived the basic equation for lipid oxidation in the presence of primary antioxidants assuming equation (7) to be the main mode of action:

$$\ln(\; (ROOH)_i/(ROOH)_o \;) = K_A t_i/(AH_2) \qquad (17)$$

where:

t_i = induction period, time to reach rancidity

$(ROOH)_o$ = initial peroxide value

$(ROOH)_i$ = peroxide value at the rancid point

K_A = overall rate constant for the inhibited

reaction = $k_p k_i (M)(RH)/k_a$

Equations (16) and (17) were the mathematical models which were put to the test in the model system study. These two models are summarized in table 2.

2. Materials, methods and calculations

The materials, methods and calculations used in this study were fully described in NAS 9-12560 Phase III Final Report (1974-1975). The model system composition is shown in table 3. The antioxidants used are shown in tables 4 and 5. Basically oxygen uptake was measured by peroxide determination and Warburg's and the above kinetic equations were applied.

3. Results

The square-root model for linoleate oxidation in absence of primary antioxidants is shown in table 2, as well as the model suggested by Labuza (1971) for use with linoleate oxidation inhibited by primary antioxidants.

Table 6 summarizes the methods used to calculate the Q_{10} and the energy of activation, E_a for lipid oxidation.

Table 2
Rate Equations for LO

(A) <u>Monomolecular Period</u> $ROOH \rightarrow$ Free Radicals

Rate Equation: $(ROOH)^{1/2} = K_M t/2$

K_M = Monomolecular Rate Constant

(B) <u>Inhibition W/1° AH</u> $ROO\cdot + AH \underset{\rightarrow}{\leftarrow} ROOH + A\cdot$

Rate Equation: $\ln(ROOH) = \ln(ROOH)_o + K_A t/(AH)$

K_A = Antioxidation Rate Constant

Table 3

Model System Composition

MAJOR COMPONENTS	%(DRY SOLIDS BASIS)
Microcrystalline Cellulose	50
Glycerol	40
Methyl Linoleate	10
	100

MINOR COMPONENTS	PPM(LIPID BASIS)
Ferrous Ions (Fe(II))	50
Antioxidant	200

IN ADDITION: Water Added to Give Either:

DEHYDRATED SYSTEM $a_w = 0.11$

INTERMEDIATE MOISTURE SYSTEM $a_w = 0.75$

TABLE   4
PRIMARY ANTIOXIDANTS USED

BUTYLATED HYDROXYANISOLE, BHA
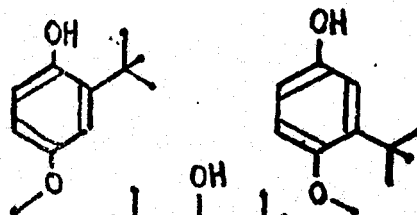
BUTYLATED HYDROXYTOLUENE, BHT
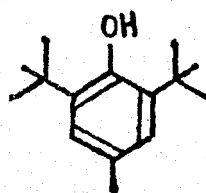
PROPYL GALLATE, PG
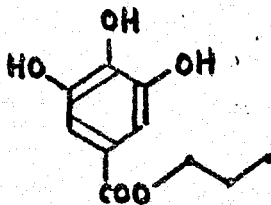
ALPHA-TOCOPHEROL, E
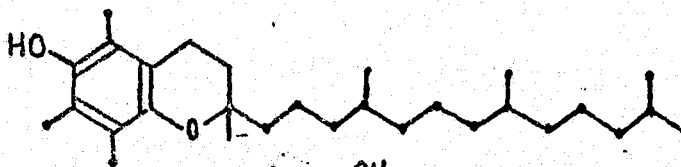
TERTIARY BUTYLHYDROQUINONE, TBHQ
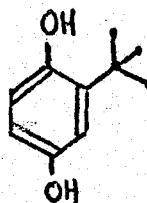
TABLE 5
SECONDARY ANTIOXIDANTS USED

ASCORBIC ACID, <u>AA</u>
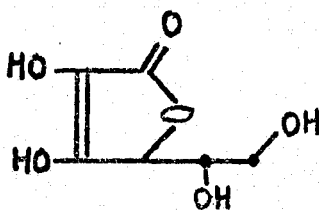
ETHYLENEDIAMINETETRA-
ACETIC ACID, <u>EDTA</u>
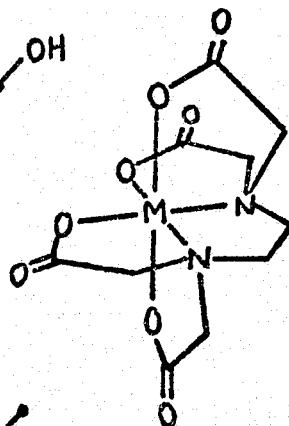
ISOPROPYL CITRATE, <u>IPC</u>
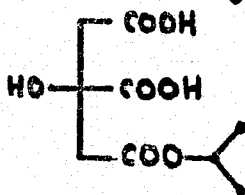
Table 6

Calculation of Q_{10} and E_a

$$Q_{10} \text{ (Induction Period)} = \frac{\text{Induction Period At } T^oC}{\text{Induction Period At } T+10^oC}$$

$$Q_{10} \text{ (Reaction Rate)} = \frac{\text{Reaction Rate At } T+10^oC}{\text{Reaction Rate At } T^oC}$$

$$Log_{10} (Q_{10}) = \frac{2.186 \ E_a}{T(T+10)} \qquad (T \text{ In } {}^oK)$$

E_a = Energy of Activation (Kcal/mole)

Typical oxidation versus time curves are shown in Figure 1 for control, EDTA and BHA at $45^{\circ}C$ in the IM system. Similar curves were obtained for other systems. As shown the oxygen uptake as measured by the Warburg technique coincides almost exactly with the oxygen uptake as calculated from the peroxide values.

The above mathematical models were applied to all the raw data. All controls and systems containing secondary antioxidants were found to obey a half-order rate equation (square-root plot) thus confirming the applicability of equation (16). Similarly systems containing effective primary antioxidants all obeyed a first-order rate equation (logarithmic plot) thus confirming the applicability of equation (17).

The application of these rate equations to the raw data given in Figure 1 are shown in Figures 2 and 3. As shown for the control and EDTA a straight line is obtained in Figure 2. Only for the EDTA is the curve broken, but not for the control. From the slope of these lines in the early ranges of the reaction one can get the overall rate constants, K_M. For EDTA the break in the curve can be taken to mark the end of the induction period.

Similarly for BHA the straight line obtained in Figure 3 the value of K_A can be obtained from the slope. The break in the curve was used as indication of the end of the induction period.

CONTROL EDTA BHA

10,000

uL O$_2$/G

5,000

3 % OXIDATION

TEMPERATURE
OF RUN = 45°C
IM SYSTEM
OPEN SYMBOL: O.U.
CLOSED SYMBOL: PV

0

0   20   40   60   80

TIME (HRS)
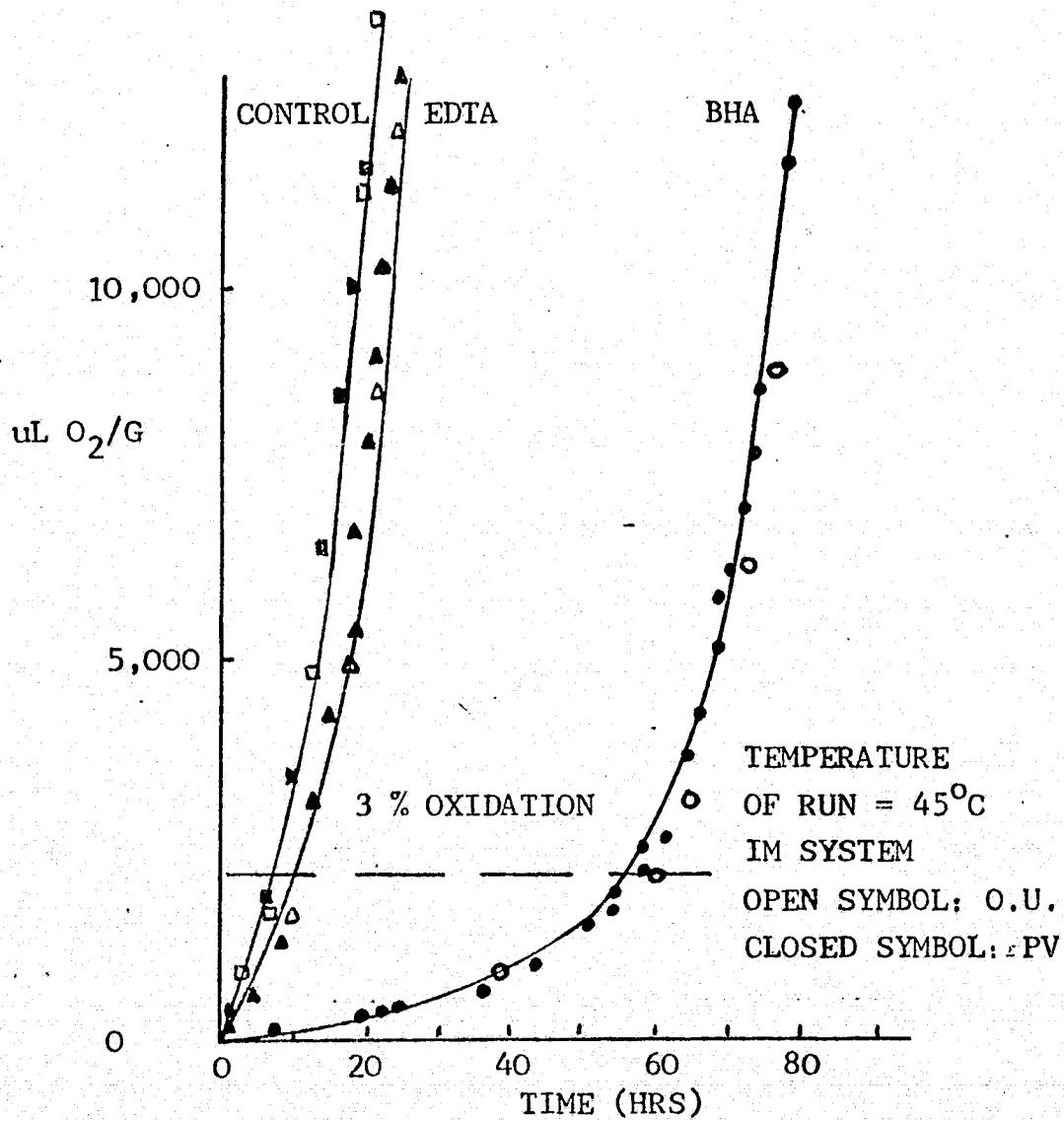
TYPICAL OXIDATION-TIME CURVES

FIGURE 1

FIGURE 2
MONOMOLECULAR MODEL APPLIED TO C & EDTA
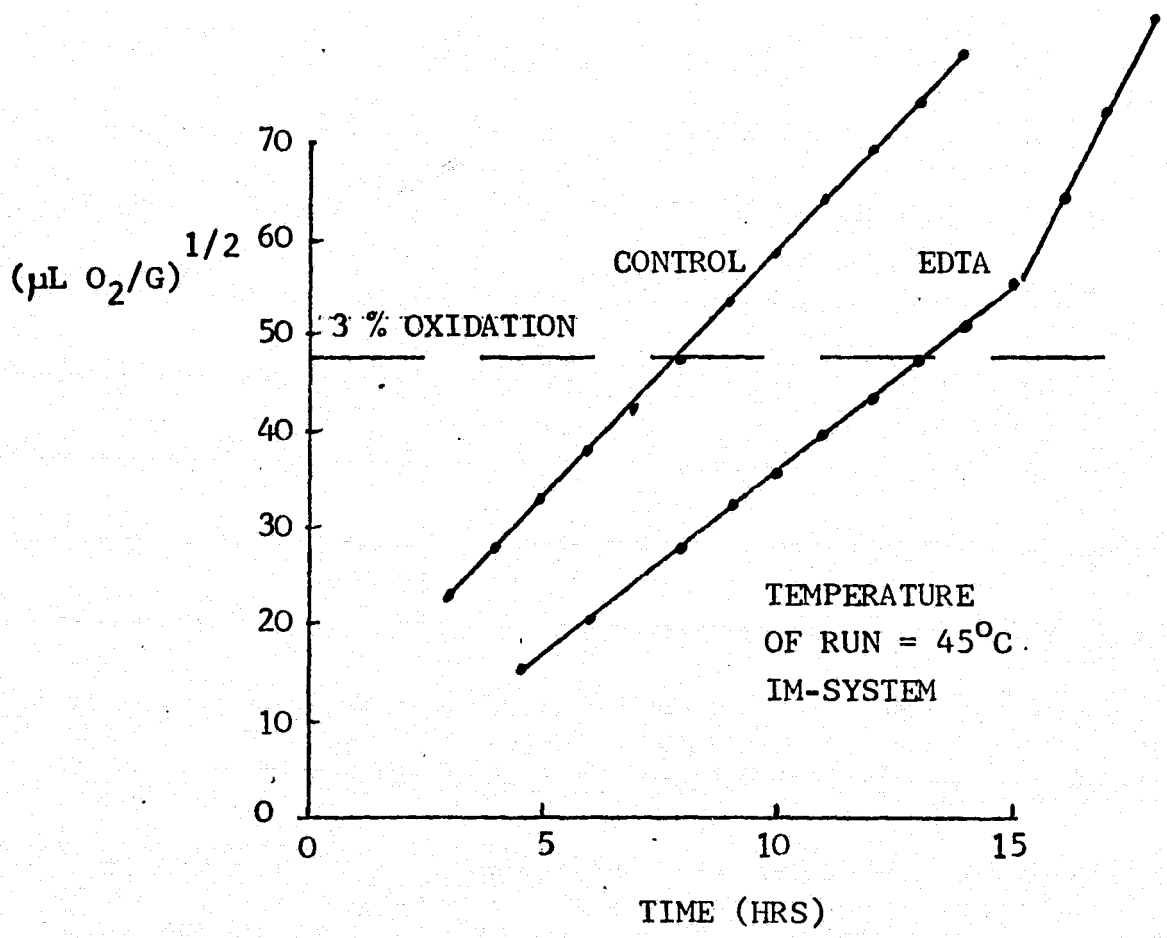
FIGURE 3
FIRST-ORDER MODEL APPLIED TO BHA
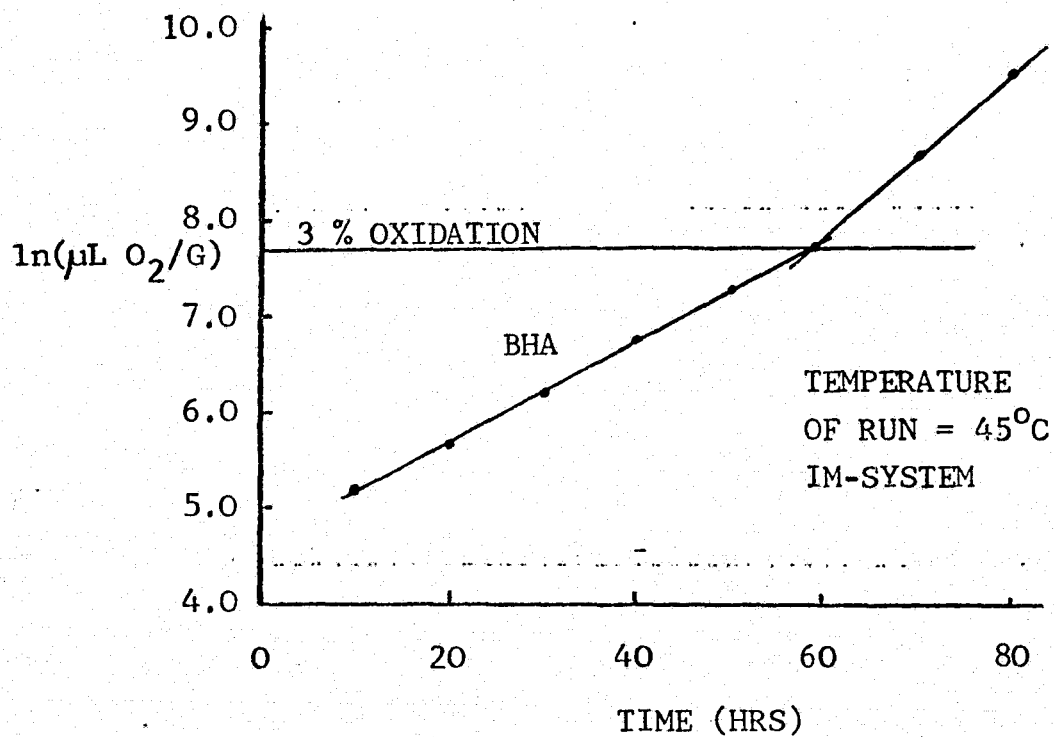
As seen from these figures there are two parameters which emerge as being important in order to be able to describe the kinetics: the overall rate constant and the induction period. From a kinetic standpoint the latter is of limited validity since a break did not occur in all cases. When it did occur it always corresponded to about 2-4% oxidation. From a practical standpoint however, the induction period is the only data of interest. Hence a 3% level of oxidation was somewhat arbitrarily picked as the end of the induction period and was applied to all samples including the controls.

Table 7 shows the induction periods obtained for the dry systems. C represents control without any antioxidants added. As shown all the primary antioxidants were active with the exception of TBHQ which gave very limited protection. . In contrast the secondary antioxidants were all relatively useless with ascorbic acid actually behaving as a prooxidant. The low activity of these antioxidants may reflect the low water activity of the dry systems (a_w = 0.11).

Table 8 shows the induction periods obtained for the IM systems. Again the primary antioxidants are very effective, but not as effective as in the dry systems. Again the secondary antioxidants are relatively ineffective with ascorbic acid again behaving as an antioxidant. Interestingly propyl gallate behaves as a prooxidant in this system. This may partly reflect the relatively high water solubility of propyl gallate.

TABLE 7
EFFECT OF AH ON INDUCTION PERIOD - DRY SYSTEM

		PRIMARY						SECONDARY		
T^{o}C	C	TBHQ	PG	E	BHT	BHA	BHT + BHA	AA	IPC	EDTA
25	57	-	180	260	1300	1500	1800	21	-	62
35	14	16	28	58	290	330	-	-	14	-
45	8.6	-	20	32	130	175	-	5.0	-	12

INDUCTION PERIOD = TIME TO REACH 3 % OXIDATION, HRS

TABLE 8
EFFECT OF AH ON INDUCTION PERIOD - IM SYSTEM

		PRIMARY						SECONDARY		
T^{O}C	C	PG	TBHQ	E	BHT	BHA	BHT + BHA	AA	IPC	EDTA
25	23	15	-	85	380	500	550	10	-	55
35	13	6.5	17	24	75	125	-	-	16	-
45	8.0	3.8	-	16	37	58	-	3.9	-	11

INDUCTION PERIOD = TIME TO REACH 3 % OXIDATION, HRS

Table 9 summarizes the above data for the most effective antioxidants. This table shows the normalized induction periods obtained simply by setting the induction period for the control = 1 hour. The tremendous protection offered by BHA and BHT (about 5 to 30-fold) can be seen. It should be pointed out, that in practice a doubling of the shelf life may be quite adequate to make the food sufficiently shelf stable for the required distribution.

Figure 4 shows how the induction periods (t_i) measured at different temperatures may be calculated simply as shown previously in Tables 8 and 9. Since only three temperatures were used, 25, 35 and 45°C, only two values for Q_{10} can be calculated. These are shown in Table 10. As shown the most striking feature of the data is that the Q_{10} between 25-35°C is always higher than that obtained between 35-45°C. This is especially true at the higher water activity (i.e. in the IM system, $a_w = 0.75$).

From a theoretical standpoint the Q_{10}'s based on the rate constants are more correct. Figure 5 shows how one can evaluate the Q_{10} from a graph of either K_M or K_A versus temperature in accordance with the formula in Table 6. As shown the curve is steeper at the lower temperature confirming a similar trend as was seen previously with the induction periods.

The actual Q_{10} values are shown in Table 11. As shown, the Q_{10} values are always higher in the range between 25-35°C than

TABLE 9
NORMALIZED INDUCTION PERIODS FOR SOME 1° AHs

$T °C$	DRY SYSTEM					IM SYSTEM			
	C	PG	E	BHT	BHA	C	E	BHT	BHA
25	1	3.2	4.6	23	32	1	3.7	17	22
35	1	2.0	4.2	21	24	1	1.8	5.8	9.6
45	1	2.3	3.7	15	20	1	2.0	4.6	7.3

t_i(HRS)

BHA

CONTROL
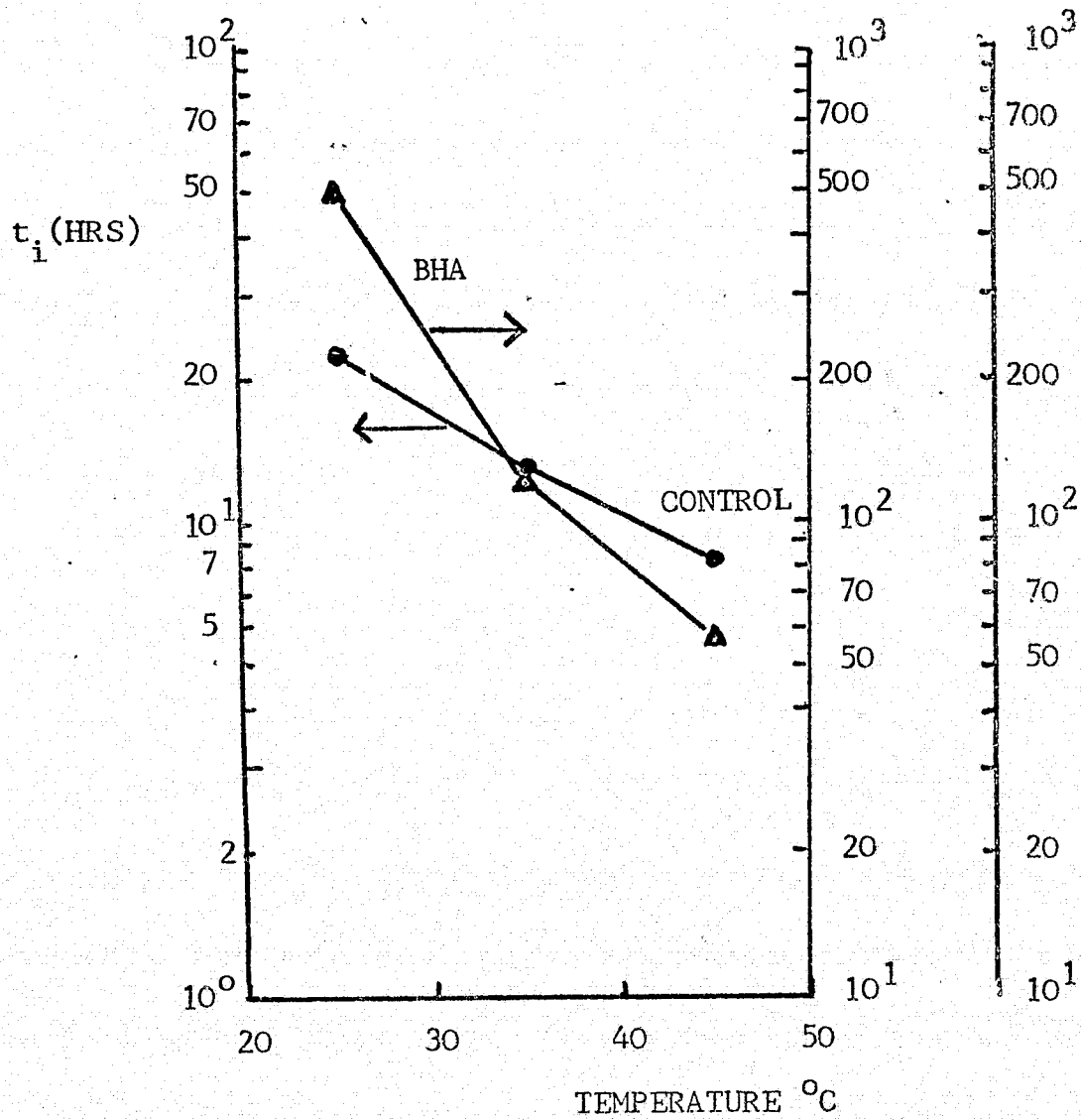
PLOT OF LOG(t_i) AGAINST TEMPERATURE

FIGURE 4

TEMPERATURE ^{o}C

TABLE 10
Q_{10}-VALUES BASED ON INDUCTION PERIODS

SYSTEM	T-RANGE $^{\circ}$C	C	PG	E	BHT	BHA
DRY	25-35	4.1	6.4	4.5	4.5	4.5
	35-45	1.6	1.4	1.8	2.2	1.9
IM	25-35	1.8	2.3	3.5	5.1	4.0
	35-45	1.6	1.7	1.5	2.0	2.2
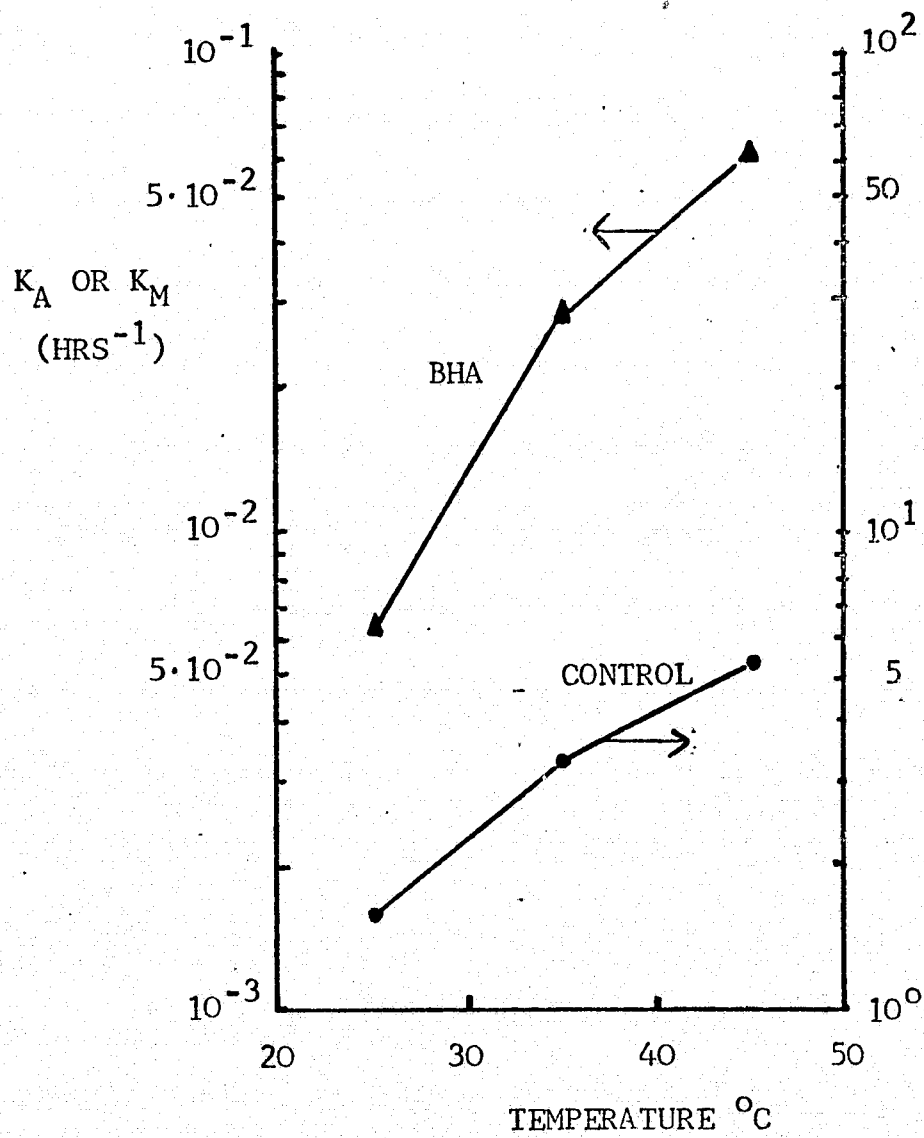
PLOT OF LOG(K_A OR K_M) AGAINST T

FIGURE 5

TABLE 11

Q_{10}-VALUES BASED ON RATE CONSTANTS

SYSTEM	T-RANGE $^{\circ}C$	C	PG	E	BHT	BHA
DRY	25-35	3.8	5.3	6.9	8.3	10
	35-45	2.3	2.6	1.4	1.7	1.2
IM	25-35	2.1	2.2	3.6	4.3	4.1
	35-45	1.6	2.6	1.3	2.3	2.3

they are in the range between 35-45°C. This trend is especially pronounced

in the dry system. Compared to the Q_{10} values which were obtained

with the induction periods these Q_{10} values show a more drastic trend.

The high values obtained in the dry system for BHA, BHT and vitamin E

are particularly striking between 25 and 35°C.

4. Discussion and conclusions

The data given in this report illustrate well the need

for using two different kinetic parameters, the rate constant (K_M or

K_A) on one hand and the induction period (t_i) on the other. Theore-

tically, the former is the basic kinetic parameter that should be used

if meaningful comparison between treatments is to be made. The latter

parameter has practical importance. In this study the 3% level of

oxidation which was chosen as the end of the induction period corres-

ponds reasonably well with the rancid point.

The data show that (a) different Q_{10} values are obtained

depending on whether one uses rate constants or induction periods as

the kinetic parameter. This is to be expected as the Q_{10} - concept

should only be used with the former. Secondly (b) the Q_{10} value is

considerably different between 25-35°C than between 35-45°C. This

difference is more pronounced in the dry system than in the IM

system and was more pronounced when rate constants rather than induction

periods were used as the kinetic parameter.

These results show the dangers involved in attempting to predict shelf life at room temperature from data collected at high temperatures using ASLT methods. Since the Q_{10}-values differ for different antioxidants one cannot be sure that the order of effectiveness of the various antioxidants is the same at two different temperatures. These studies were made with model systems. These results will be compared with data collected using real food systems in which inorganic-iron catalyzed oxidation of linoleate is the major mode of deterioration.

Another important aspect of the results is antioxidant solubility. The most water-soluble primary antioxidant, propyl gallate, is inactive (actually a prooxidant) in the IM system. This probably occurs because of a partition between the lipid and the water phase (this system contains 3:1 water:lipid) with most of the antioxidant going into the aqueous phase where it would be useless. With the secondary antioxidants the effectiveness is greater in the IM system than in the dry system. This is probably because these antioxidants are highly water-soluble. Unlike propyl gallate, however, these antioxidants retain their antioxidant effect since they chelate metal ions, pull them out of the lipid phase and hence retard lipid oxidation.

In the introduction the various ASLT methods were surveyed. It was shown that four out of the five methods considered use a high temperature as the dominant acceleration parameter. Out of these four, three use (a) a very advanced endpoint which is totally meaningless from a practical standpoint and (b) are often used in conjunction

with so-called "inert" carriers or dispersants onto or into which the sample is dispersed. In such cases the simultaneous contamination with prooxidative impurities is likely to occur making reproducibility at a later time impossible.

The data given in this report indicate that the use of high temperatures in ASLT methods may lead to completely meaningless results when one is testing for antioxidant effectiveness. Thus the only method which can be recommended at present is the Schaal Oven Method. A major problem with this test however, is that it may take weeks to get the data needed. Since time is usually critical, another acceleration parameter must be superimposed on the temperature elevation already present.

The discussion in the introduction shows that apart from temperature, the only useful accelerator available is the addition of prooxidants. This must be done in a reproducible manner, however, and not as an accidental consequence of the addition of a carrier. Also, one must be very careful when choosing such a prooxidant since a shift in catalyst type or solubility will change the mechanism of initiation and hence will yield meaningless results. One must therefore know beforehand what is the dominant prooxidant in the food and then incorporate a specified amount of this particular catalyst into the food fat before testing it.

If this approach is used with foods which contain inorganic iron as the dominant prooxidant, a specified amount of an inorganic

iron-salt can be added to the food fat in question. When done in conjunction with a relatively mild temperature elevation, one may be able to get meaningful results in a short period of time. It should also be pointed out that (a) the use of carriers should be discontinued altogether and (b) in some foods, especially those high in iron, because of fortification, a temperature elevation may be all that is needed to get data quickly.

D. Summary and Recommendations

Lipid oxidation leading to rancidity is a major problem in intermediate moisture foods since at high a_w the water acts to catalyze the reactions. In product development of IMF as well as other foods, accelerated shelf life testing procedures are usually used to predict the deterioration problems that would occur at normal shelf temperatures. This study shows that:

1. The normal accelerated shelf life test methods for oxidation are inapplicable for intermediate moisture foods since the temperature range is too high (65-120°C).

2. In determining shelf life predictions, studies should be made at three temperatures so that true kinetic data may be found. In this way, over or underprotection can be eliminated.

3. A plot of log (induction time) vs. temperature can be used to determine whether the kinetics changes and thus the effect on the Q_{10} may be evaluated. Thus processors can use this method for shelf life testing.

4. For primary antioxidants the rate of oxidation follows a first order reaction.

5. Antioxidant solubility in the aqueous phase decreases their activity. Thus ascorbate, propyl gallate and isopropyl citrate are ineffective antioxidants.

6. The two primary antioxidants BHA and BHT have the best effectiveness in model systems whereas TBHQ is ineffective. Thus processors should use the former antioxidants to protect IMF foods.

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V. EVALUATION OF WATER ACTIVITY DETERMINATION AND PREDICTION
   TECHNIQUES

   A. Detailed Procedure of the Vapor Pressure Manometric (VPM)
      Technique

      1. Introduction

         No literature exists for giving the detailed procedure
      and drawings for the VPM a_w technique. The following is a descrip-
      tion of the technique and a drawing. It should be noted that over
      eight of these have been built by various companies for research
      purposes.

         2. Procedure for measuring a_w with the vapor pressure manometer

            I. Prepare Sample
               A. Temperature – all samples should be at room
                  temperature before being placed in the sample flask.
               B. Size of sample
                  1) Fill the sample flask about ½ full (50 to 60 ml).
                  2) Solid samples should be cut into small pieces
                     (i.e., no larger than ½ cm.)
            II. Start System – Refer to Figure 1 for positions of
                stopcocks.
               A. Stopcocks B, D, E, F, G, and H are closed –
                  stopcocks A and C are open.

B. Fill cold trap with liquid nitrogen.

C. Turn on vacuum pump and vacuum meter, close A

(3-way stopcock, open to pump and system; closed

to outside air), open B, D, and G.

D. Evacuate system to 120-150 microns, close G

(dessicant).

III. Put on Sample

A. Break vacuum in previous sample flask (if one

is in place).

1) Close E (sealing sample section from rest of

system)

2) Open F (letting air in)

3) Close F (resealing system)

B. Take off last sample flask and put on next using

Dow Corning High Vacuum Grease. Spread a liberal

amount of the grease on the fitting and rotate

the flask in position several times until all

air bubbles have moved out of the grease.

IV. Take ΔH_1

A. Open E for 0.5 - 1.0 minutes, to evacuate the air

space in the sample flask. This time should be

kept to a minimum to prevent any drying of the

sample itself. Samples with high gas and volatile

content should be evacuated for the longer time

to give a smaller ΔH_2 and better accuracy.

B. Close C and wait for equilibrium of the vapor

pressure of the sample. Time to reach equilibrium

varies but is usually 40 to 60 minutes. Air tempera-

ture and sample temperature (water bath temperature)

must be the same throughout equilibration and the

water bath temperature should never exceed the air

temperature. (Note: The present method uses

ASTM 60 to 80°F thermometers (0.2°F intervals)

in the water bath and hanging in the air next to

the manometer, to monitor sample and air temper-

atures. However, the VPM is designed for a thermo-

couple to be inserted into the glass tubing above

the oil in the leg to the right of C. A thermocouple

can also be inserted through the side of the sample

flask (via a handblown extension). Both thermocouples

can be connected to the same digital thermometer.)

C. When there is no change in the height of the oil

column for 10 minutes at a constant temperature

on both thermometers, read the manometer, record

ΔH_1, and the temperature on both thermometers

(same). Read to 0.5 mm oil.

V. To Take ΔH_2

A. Close E and open G while C is still closed. Wait

10-15 minutes so the dessicant absorbs the water

vapor collected in the system from the sample.

B. Read the manometer difference and record ΔH_2

at the same two temperatures.

VI. Calculate the a_w

A. From Figure 2 determine the vapor pressure of water

in terms of cm of the oil at the sample temperature

(P_o @ T in cm oil).

B. $a_w = \dfrac{(\Delta H_1 - \Delta H_2)}{P_o \text{ @ T in cm oil}}$

VII. Put on Next Sample

A. Open C and evacuate dessicant to 200 microns.

B. Close G

C. Return to Section III and proceed from there for

next sample.

VIII. Shut Down System

A. Close E (should already be closed)

B. Open F

C. Close F

D. Close D and B (leave C open)

E. Open A (3-way stopcock, open to air, system and

pump)

F. Switch off pump and vacuum meter

IX. Regreasing vacuum stopcocks using the special high

temperature, high vacuum stopcock grease.

A. Release the vacuum on both sides of the stopcock

to be regreased.

B. Heat the stopcock and fitting for one minute with
a heating blow gun held as close as possible.
This will melt the grease so the stopcock can be
gently pulled out.

C. Clean the stopcock and the inside of the fitting
thoroughly using acetone, kimwipes and pipe cleaners.

D. Reheat the fitting for one minute with the blow
gun and then reheat the stopcock for another minute.

E. Apply a very thin, even coating of new high tempera-
ture and high vacuum grease to the stopcock with
a glass rod. Insert the hot stopcock into the hot
fitting and turn several times to seal it properly
and get rid of air bubbles. Excess grease buildup
in the stopcocks or tubing should be avoided.

X. "Blowing" the VPM

A. Causes

1) The usual reason that oil is blown out of its
column and into the rest of the system is:
leaving C closed while opening some part of
the system to the right of C that is not
under vacuum.

2) Oil can also be blown even with C open by
opening E too quickly on a volatile or liquid
sample. Putting a piece of glass wool into the
fitting below E (G on large VPM), can help

prevent this. All of the stopcocks should
be opened slowly and gently while running the
VPM – to avoid breakage as well as blowing.
They should also be held by the body to prevent
this.

D. Cleaning the system.

1) Shut down and release the vacuum on all parts
of the VPM to the left of D.

2) Take out the stopper at the thermocouple fitting,
the vacuum gauge, and the oil trap (as in
Section IX).

3) Blow out the rest of the oil with an air stream
through rubber tubing – gently to avoid blowing
out C. Flush the manometer legs with acetone
several times – until the acetone comes off
clear. Heat gently to help blow all the
acetone out.

4) Take out C.

5) Clean the oil off all glass surfaces using
acetone, kimwipes, and pipe cleaner.

C. Replacing the oil

1) De-gas new oil

a) Pour enough new oil to refill the manometer
into a glass beaker.

b) Heat the oil.

c) Place the hot oil in a dessicator and pull

a vacuum on it until it stops bubbling

(about $\frac{1}{2}$ hr).

2) Pour the oil slowly through a narrow rubber tube

inserted into one of the manometer legs. Fill it

to between 40 to 45 cm (close to 45 cm).

3) Reassemble the system. Pull a vacuum on the system

and let it run overnight or until no more air or

acetone is left in the oil. Heating the outside

of the oil columns gently with the blow gun will

release the air from the oil more quickly.

Table 1

List of Stopcocks and Fittings

Stopcock A: pyrex T 4 mm 3-way

Stopcocks B, C, H, D, E, F, and G: hollow 2-way vacuum stopcocks with
    bulbs, Eck and Drebbs 5100

Cold trap: pyrex 200 mm with T 40/50

Oil trap: pyrex 100 mm with T 34/45

Thermocouple fitting: pyrex T 10/30 custom sealed to Veeco
    Type DV-1m vacuum gauge.

Sample and dessicant flasks: handblown 100 cc flasks with pyrex T
    24/40

The glass tubing used throughout is 9 mm OD medium wall

Table 2

Suppliers

Apiezon B oil: James G. Biddle Co.
                                                  Township Line & Jolly Rd.
                                                  Plymouth Meeting, PA 19462

High Temperature, High Vacuum   Robert Austin, Ph.D.
    Stopcock Grease: Box 5374
    (Spectrovac Type II)   Pasedena, CA

High Vacuum Grease Silicone   Dow Corning
    Lubricant:

Vacuum pump: Precision Scientific model 75

Vacuum gauge (type DV-1m) and   Veeco Instruments, Inc.
    Vacuum meter (thermocouple   Terminal Drive
    gauge control TG-6):   Plainview, NY 11803

vacuum gauge

vacuum meter

8 cm vacuum gauge or thermocouple
fitting (10/30)

C

10 cm   5 cm   7 cm   1-4 cm-1 ← 15 cm → 4 cm → 1

B   H

← 8 cm →   11 cm   13.5 cm   ← 11 cm →

rubber
tubbing

34/40   7.5   E   5 cm   G   5 cm

A   oil trap

ASTM   2   3 cm →   1 cm

40/50   thermometer
60-90°F   F   sample   Dessicant
flasks
with

vacuum   3-way
pump   stop-cock   water bath   24/40
with sample

97.5
cm

LN₂

cold trap

Apiezon B
oil level
∿ 45 cm

X - vacuum stopcock
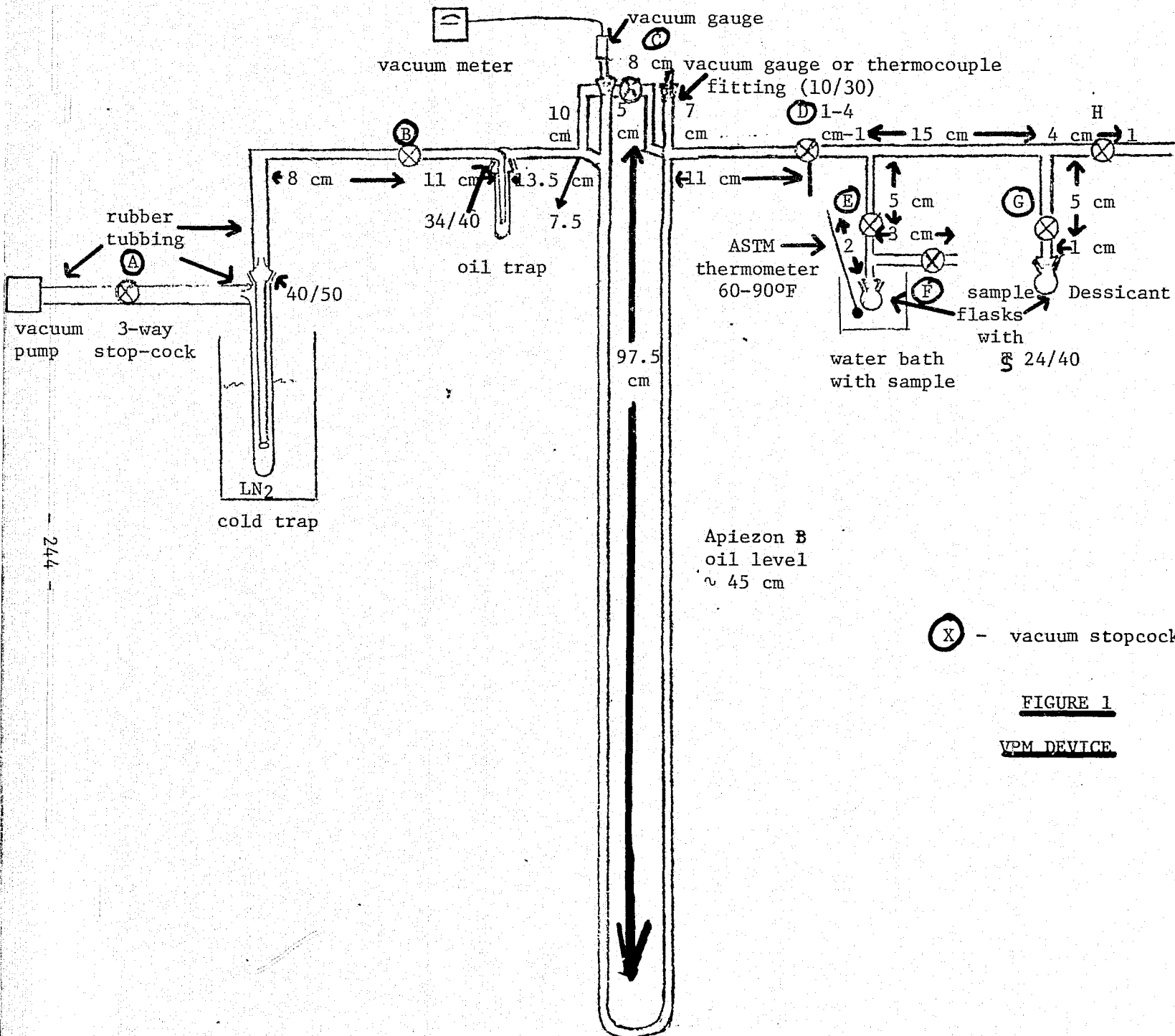
FIGURE 1

VPM DEVICE

FIGURE 2

Vapor Pressure of H_2O in cm of Apiezon B oil at 10°C to 30°C

Temperature °C

cm Apiezon B Oil

B. **Statistical Comparison of a_w Methods**

1. Introduction

Water activity (a_w) directly controls most chemical reactions as well as microbiological activity in food systems and formulations (Labuza, 1974) and thus is an important consideration in the manufacture of food items. For this reason there is a demand in the food industry for relatively accurate, convenient and inexpensive methods of measuring a_w that can be used in food processing plants by regular personnel. In this study three methods of a_w measurement were compared in greater detail than in the paper presented by Labuza (1975) and as reported in Phase III. In addition, a quality control filament hygrometric device used to a great extent in the German meat food industry was included.

2. Method used

a. The Abbeon Analyzer

The Abbeon a_w Value Analyzer Model #5803 made in West Germany and marketed by Abbeon Cal Inc., 123-1A Gray Avenue, Santa Barbara, CA 93101, offers one such possible method. It consists of a sensing system set in a round metal casing. On the top of the casing is a dial calibrated in .01 a_w units from 0.40 to 1.0 with an indicator needle that reads the a_w value when the sample, air and sensor have come to equilibrium. The dial gives further units between 0.70 to 1.0, the range for which it is intended. Equilibration

according to the instructions, takes about three hours at room temperature. Since the analyzer is sensitive to temperature a thermometer is enclosed with the dial. The analyzer comes with a table indicating the amount of correction to be made to the reading for every $1^{\circ} \pm 5^{\circ}$ from $20^{\circ}C$. The analyzer is calibrated at $20^{\circ}C$ using a saturated solution of barium chloride ($a_w = 0.90$) that is supplied by Abbeon. To calibrate it, a set screw is turned which moves the indicator. According to the instructions, this should be done periodically.

The bottom of the casing is a metal plate with holes that admit the air to the sensor which is a monofilament attached to finely calibrated springs. The increased elasticity of the filament when exposed to water vapor gives the a_w value indicated on the dial.

The sample is placed in a metal container that is clamped to the casing containing the sensing system and dial. The system is set in a $20^{\circ} \pm 5^{\circ}C$ room for three hours at which time the a_w value is read directly from the dial. Abbeon recommends that the sensor system be recalibrated once a week using the saturated barium chloride solution.

b. The VPM Method

The details of using the vapor pressure manometer are given in Section VA. This method can be used between 0.0 to 0.98 a_w with a high degree of accuracy and precision ($\pm$ 0.01 a_w).

c. The Fett Voss – Isopeistic Method

This method uses the absorption of water from the food onto a known dry absorbing material. In this method as published by Vos and Labuza (1974) microcrystalline cellulose is used as the absorbent. The VPM method should only be used between about a_w 0.78 to 0.95.

3. Calibration check of Abbeon device

The purpose of this project was to evaluate the a_w analyzer for use in the food industry especially for intermediate moisture foods. To do this the general calibration accuracy of the a_w analyzer was first tested using saturated salt solutions. The a_w value measured by the a_w analyzer was compared to both the literature values for these salt solutions and to values measured by the vapor pressure manometer using the same stock solutions used in the a_w analyzer. The results of this comparison for duplicate determinations done two or three times, are shown in Table 1. The results indicate that values for salts do not compare exactly to literature values for every test nor to the VPM measurements. Duplicates are close but the averages in retesting both increase or decrease. Given that the tester can be used in quality control the deviation observed is $\pm$ 0.02 units.

4. Food samples tested

Table 2 lists the foods tested by all three methods. Testing was done by two technicians with duplicate or quadruplicate measurements being made.

Table 1

Check of a_W Analyzer on Standard Solutions

Saturated Salt Solution	Literature Value	VPM	Abbeon Analyzer Test I	Test II	Test III
NaCl	0.75	0.745	0.765	0.760	–
		0.749	0.769	0.744	–
AVG		0.75	0.77	0.75	–
K_2CRO_4	0.88	0.850	0.850	0.872	0.866
		0.855	0.850	0.871	0.862
AVG		0.85	0.85	0.87	ʼ0.86
$BaCl_2$	0.91	0.902	0.903	0.904	–
		0.891	0.903	0.904	–
AVG		0.90	0.90	0.90	–
$LiSO_4$	0.85	0.839	0.854	0.850	–
		0.830	0.852	0.846	–
		0.831	–	–	–
AVG		0.83	0.85	0.85	–

Table 2

Food Samples Tested

Parmesan cheese (grated)

Swedish whey cheese

Bongaard's Colby cheese

Special Cuts (IMF dog food Ken L Rations)

Tender Vittles - Fish flavored (IMF cat food Ralston Purina)

Hard salami - Swift Premium

Beef Tricks (beef sausage sticks Smokecraft)

Thuringer - Schweigert

5. Results and discussion of preliminary study

The results of the first part of this study are shown in Table 3. As seen, variabilities exist between each method, between both technicians, and within each method. The difference between the cup analyzer and the other methods is shown in the last columns. The greatest difference exists for the high a_w colby cheese where the a_w measured by the cup analyzer was far below the values found by the other two methods. In addition, Table 4 shows the results for meat samples which are usually at high a_w. In each case the cup analyzer showed lower readings. This could be due to incomplete equilibration in the three hours specified for the method. The difference for other samples of intermediate a_w between methods however, was always within 0.02 a_w units.

In Table 3 it can be seen that the difference between technicians was always within 0.02 a_w units except for the VPM method for Swedish whey (0.03) and for the beef tricks measured by the Fett Vos method. The latter could be expected since it was out of the range of use whereas the former deviation for the VPM could have been an experimental error.

Based on these results one could use the cup analyzer as a quality control tool since the difference that exists between it and the other methods is within the experimental error found within methods as well as between technicians.

Table 3

a_w Values for Food Items - In Comparative Study

Technician	Fett - Vos		VPM		Abbeon Analyzer		Average Difference of Analyzer From	
	A	B	A	B	A	B	F/V	VPM
Parmesan	.777	.762	.724	.757	.778	.778		
	.757	.742	.748	.747	.768	.768		
	.752	.762	–	–	.762	.768		
	.780	.730	–	–	.770	.768		
AVG	0.77	0.75	0.74	0.75	0.77	0.77	+0.01	+0.02
Swedish Whey (cheese)	.887	.890	.885	.900	.868	.876		
	.902	.890	.862	.903	.864	.880		
	.898	.890	–	–	–	.910		
	.892	.890	–	–	–	.910		
	.877	–	–	–	–	–		
	.880	–	–	–	–	–		
	.875	–	–	–	–	–		
	.875	–	–	–	–	–		
AVG	0.89	0.89	0.87	0.90	0.87	0.89	– 0.01	+0.01
Colby Cheese	.970	.990	.962	.992	.922	.918		
	.965	.990	.973	.992	.922	.915		
	–	.998	–	–	–	.913		
	–	.998	–	–	–	.922		
AVG	0.97	0.99	0.97	0.99	0.92	0.92	– 0.06	– 0.06
Special Cuts	.885	.848	.876	.836	.873	.838		
	.877	.842	.835	.844	.873	.838		
	.873	.862	–	–	–	.860		
	.872	.858	–	–	–	.868		
AVG	0.88	0.85	0.86	0.84	0.87	0.85	– 0.01	+0.01

Table 3 - a_w Values for Food Items - In Comparative Study

Technician	Fett - Vos		VPM		Abbeon Analyzer		Average Difference of Analyzer From	
	A	B	A	B	A	B	F/V	VPM
Tender Vittles	.875	.912	.900	.888	.888	.901		
	.874	.902	.861	.892	.884	.901		
	.902	.908	.861	–	.928	.896		
	.903	.920	–	–	.926	.896		
AVG	0.89	0.91	0.88	0.89	0.91	0.90	+0.01	+0.02
	*	*						
Beef Tricks	.675	.624	.703	.745	.730	.708		
	.652	.624	.735	.710	.650	.678		
	.730	.614	–	–	–	.686		
	.718	.614	–	–	–	.694		
AVG	0.69	0.62	0.72	0.73	0.69	0.69	+0.02	-0.03

* out of range

Table 4

Measurement of a_W of Meat Samples

	Fett-Vos	a_W VPM	a_W Analyzer
Thuringer	.962	.987	.934
(Brand A)	.937	.999	.926
	.960	–	.924
	.952	–	–
AVG	0.95	0.99	0.93
Hard Salami	.822	.851	.782
(Brand A)	.840	.833	.788
	.840	–	.790
	.835	–	–
AVG	0.83	0.84	0.79
Thuringer	.998	.993	.898
(Brand B)	.984	.993	.898
	.990	–	.916
	.982	–	.912
AVG	0.99	0.99	0.91
Hard Salami	.860	.939	.852
(Brand B)	.848	.879	.836
	.858	–	.845
	.862	–	.845
AVG	0.86	0.91	0.84

The following part of this study was designed to statistically determine this error further between the different methods. In any case the following points should be taken into consideration in using the cup a_w analyzer.

(1) <u>Sensitivity to temperature</u>. If the sample to be measured is cooler than room temperature it will take longer than three hours for the indicator to come to equilibrium. The literature is based on testing at constant temperature for the full three hour equilibration period.

(2) <u>Length of equilibration period</u>. At high a_w's a longer period than three hours is needed for meat products. This may be because it takes longer for the water to diffuse out of foods as compared to salt solutions.

(3) Leaving the sensor in contact with vapor containing volatile glycols for long equilibration periods may damage its sensitivity if any are permanently adsorbed. This is not known.

(4) <u>Calibration</u>. Calibration once a week is recommended by the manufacturer. However, when using the analyzer once or twice every day the readings were found to be significantly higher before the end of one week. In this study, the analyzer was recalibrated in between every food sample. Some standard calibration period between sampling must be established.

(5) Any material (salt solution or food matter, even when completely dry) allowed to remain on the metal plate between the sensor and the sample rendered the analyzer completely inoperable.

However, simply cleaning this plate made the analyzer operable again. Extreme care must be taken that nothing is allowed to enter the holes and come in contact with the filament itself or any part inside that could not be cleaned.

6. Statistical comparison of methods

In this statistical comparison, parmesan cheese and Quaker Oats Special Cuts dog food were measured at least 10 times each by each of the three methods. The data are shown in Table 1 and the F values and statistical comparisons are shown in Table 2.

As seen in this study the VPM gave lower values than either the Fett/Vos or the cup device. The latter two methods were not significantly different from each other but were statistically significantly different and read higher than the VPM. The reason that the VPM read lower is not known since in the preliminary study all three seemed to give similar readings. One problem that could have caused the difference is that although standard ASTM thermometers were used, they all read differently from eachother $\pm 0.8^\circ F$ at the same temperature. The original values for the VPM were about 0.02 a_w units higher before this was found out. The standard deviations for all methods show that a_w's for these methods best be reported as ± 0.015.

Table 1

Results of Multiple Analysis for a_W Determination by Three Methods

PARMESAN CHEESE

	Fett/Vos	Abbeon Cup Device	VPM
	.742	.738	.676
	.730	.732	.669
	.715	.737	.680
	.710	.728	.680
	.730	.748	.680
	.725	.742	.683
	.739	.698	.679
	.739	.698	.684
	.724	.737	.677
	.730	.734	.681
	.730	.733	–
	.730	.739	–
	.730	–	–
	.718	–	–
	.728	–	–
	.739	–	–
	.718	–	–
	.710	–	–
AVG	.727	.730	.679
	SD = ± 0.010	SD = ± 0.016	SD = ± 0.004

SPECIAL CUTS

	Fett/Vos	Abbeon Cup Device	VPM
	.840	.844	.801
	.838	.846	.787
	.858	.838	.779
	.848	.840	.780
	.855	.838	.791
	.855	.838	.793
	.842	.858	.800
	.844	.858	.795
	.838	.840	.780
	.844	.838	.788
	.825	–	–
	.822	–	–
	.840	–	–
	.844	–	–
	.808	–	–
	.802	–	–
	.792	–	–
	.788	–	–
	.818	–	–
	.825	–	–
AVG	.831	.844	.789
	SD = ± 0.021	SD = ± 0.008	SD = ± 0.008

Table 2

Statistical Comparison of Methods

	Fett-Vos vs. VPM		Fett-Vos vs. Cup Device		Cup Device vs. VPM	
	Sp. Cuts	Parmesan	Sp. Cuts	Parmesan	Sp. Cuts	Parmesan
Total degrees of freedom	29	28	29	30	19	21
F Value	37.648	232.148	3.356	0.505	230.002	97.774
Probability of significant difference using F Value	99.5%	99.5%	90%	95%	99.5%	99.5%
t Value	6.136	−15.236	1.832	0.711	−15.166	−9.88
Probability of significant difference using t Value	99.9%	99.9%	90%	95%	99.9%	99.9%

C. Humectant Water Sorption Isotherms

1. Introduction

Many hygroscopic chemical compounds are employed by the food industry to bind water in food products. These humectants are particularly important in the production of intermediate moisture foods (IMF). Various humectants such as polyols, sugars and salts are used in IMF to lower the water activity (a_W) into the intermediate range ($a_W = 0.60 - 0.90$), (Bone, 1973; 1974). The increased shelf stability of these nonrefrigerated IMF products is based on the principles that added solutes increase the viscosity of the liquid phase, which slows reactant diffusion rates, and lowers the availability of water by binding it, thereby making it unavailable chemically and biologically. The addition of solutes causes reactions to decrease in rate as a_W is lowered. At some lowered water availability, the reactive solutes may crystallize out, thus stopping a reaction completely. In addition to the ability to bind water, some humectants also exhibit other desirable effects in a food system as a result of their antimicrobial properties, texturizing characteristics, sweetening capacity and caloric value. No direct comparison of the water binding properties of the possible humectant compounds has ever been undertaken in the literature.

At present, due to increased cost and limited availability of commonly used humectants, alternatives must be found for the food industry. In recent years the price of sucrose and propylene glycol

(the main humectants used) has increased because of the world energy crisis and widespread food shortages. It may be necessary to combine several alternative compounds in order to achieve the desired water sorption and other properties which would produce the proper a_w lowering effect. In order to successfully replace sucrose and propylene glycol, it is imperative that an accurate determination of the water sorption properties of all the possible substitutes be made.

The direct measurement of the vapor space surrounding the sample by manometric techniques is one of the best methods of a_w measurement (Labuza et al., 1975). As a result, humectant isotherms were prepare using the vapor pressure manometric technique. Manometric devices have been described by Taylor (1961), Karel and Nickerson (1964), and Labuza (1974).

As shown by Sloan and Labuza, (1974), the order of mixing of humectants into an IMF food, added in a dry state or as a solution, has no effect on the resultant a_w, even though the pure humectant water sorption isotherm may exhibit a hysteresis effect. Also, no significant difference in the a_w lowering ability of amorphous vs anhydrous sugars was found in the IMF range. Thus in a dry mix system, the crystalline form of a humectant must be rapidly dissolved in the available water and changed into a solution or amorphous form so that the a_w is the same as in the wet mix system. As a result, the desorption curves are of major importance in choosing alternative humectants for foods.

As a result of the current interest in finding alternative humectants for use in foods, we have incorporated our most recent water sorption data collected since our last publication, into a table of Humectant Isotherms.

2. Procedure

Desorption curves were prepared by measuring the a_w of solutions of different humectant/water ratios by the vapor pressure manometer method at 23°C. For solid humectants (sugars and salts), the portion of the curve below the solubility point was prepared by equilibrating a high moisture content sample over saturated salt solutions at lower a_w's. A minimum of 10 points were collected per curve. The best curve was drawn through these points. Points were chosen from the curve at various a_w intervals.

3. Results

The humectant water sorption isotherms are listed in Table 1. It should be noted that this data differs slightly from the literature based data presented in our previous article (Sloan and Labuza, 1975), which was a summary of the literature data. We feel this data is more accurate.

4. Conclusions

As stated in our recent articles, the polyols are the most desirable humectants over the entire range of a_w's studied from a water holding standpoint. Although, at very high a_w's (0.95), the salts are

the most desirable humectants, crystallization limits their use in foods at lower a_w's.

In order to meet the criteria for a good replacement for propylene glycol or sucrose and to minimize the cost, a combination of the humectants presented here will be necessary to achieve the desired goal.

When choosing a humectant or combination of humectants for use in food properties, organoleptic qualities, nutritional implications and microbial stability must also be carefully considered.

This table of data has been published in Food Product Development, December, 1975 (article included). Since that time many processors have used the information successfully in IMF formulation.

Table 1

HUMECTANT ISOTHERMS — VPM 23°C

Moisture Content $(g H_2O/100g$ solids)

a_w	Propylene Glycol	Glycerol	1,3 Butylene Glycol	Polyethylene Glycol 400		Sorbitol	Sucrose	Glucose	Fructose	Corn Syrup Solids	Lactose		NaCl	KCl		
.95	650	625	615	310		485	133	207	380	90	240		1668	1320		
.90	270	215	205	127		135	77	114	160	64	~3		950	620		
.85	168	145	124	81		90	56	81	92	47	~3		560	320		
.80	113	108	80	61		67	56	63	55	36	~3		380	130		
.75	84	82	57	48		55	47	54	44	27	~3		270	~2		
.70	64	64	46	39		46	38	12	33	22	~3		16	~2		
.60	40	41	30	26		30	~3	11	18	15	~3		~15	~2		
.50	25	27	18	17		~3	~3	9	13	11	~3		~15	~2		
.40	16	18	12	10		~2	~2	~4	6	7	~2		~15	~2		
.30	10	12	10	6		~2	~2	~4	4	5	~2		~15	~2		
.20	6	10	8	4		~2	~2	~3	3	4	~2		~15	~2		
.10	4	5	5	2		~2	~2	~3	~3	~3	~2		~15	~2		

humectant water sorption isotherms

A. E. Sloan and T. P. Labuza/Dept. of Food Science, Nutrition/University of Minnesota/St. Paul

BECAUSE OF CURRENT interest in finding alternative humectants for use in foods, we have incorporated our most recent water sorption data (collected since the article appearing in FOOD PRODUCT DEVELOPMENT, Sept. '75, p. 75) in this table of humectant isotherms.

These desorption curves were prepared by measuring the a_w of solutions of different humectant/water ratios by the vapor pressure manometer method at 23°C. For solid humectants (sugars and salts), the portion of the curve below the solubility point was prepared by equilibrating a high-moisture content sample over saturated salt solutions at lower a_w's. A minimum of ten points were collected per curve. The best curve was drawn through these points. Points were chosen from the curve at various a_w intervals and listed in the table. It should be noted that these data differ slightly from the literature-based data presented in the article cited above, but we feel these data are more accurate. ⑤

Isotherms for several commonly used water-controlling substances

a_w	PRO-PYLENE GLYCOL	GLYC-EROL	1,3 BU-TYLENE GLYCOL	POLYETH-YLENE GLYCOL 400	SORBI-TOL	SU-CROSE	GLU-COSE	FRUC-TOSE	CORN SYRUP SOLIDS DE 42	LAC-TOSE	NaCl	KCl
.95	650	625	615	310	485	188	207	380	90	210	1668	1320
.90	270	215	205	127	135	77	114	160	64	~3	950	620
.85	168	145	124	81	90	66	81	92	47	~3	560	320
.80	113	108	80	61	67	56	63	55	36	~3	380	130
.75	84	82	57	48	55	47	54	44	27	~3	270	~2
.70	64	64	46	39	46	38	12	33	22	~3	16	~2
.60	40	41	30	26	30	~3	11	18	15	~3	~15	~2
.50	25	27	18	17	~3	~3	9	13	11	~3	~15	~2
.40	16	18	12	10	~2	~2	~4	6	7	~2	~15	~2
.30	10	12	10	6	~2	~2	~4	4	5	~2	~15	~2
.20	6	10	8	4	~2	~2	~3	3	4	~2	~15	~2
.10	4	5	5	2	~2	~2	~3	~3	~3	~2	~15	~2

MOISTURE CONTENT (gH₂O/100 g SOLIDS)

D. Prediction of the Effect of Sequence and Method of Addition

of Humectants and Water on a_w Lowering in an IMF System

1. Introduction

The successful introduction of intermediate moisture food processing into both the world pet food and human food markets has resulted in an increased interest in this technology (Rhodes, 1975; and Labuza, 1975). Various humectants or water binding agents such as polyols, sugars and salts are incorporated into these products to lower the a_w into the intermediate moisture range, (Bone, 1973; and Bone et al., 1974). These humectants can be added into a food system in a dry state or as a solution. However, it was found in Phase III, that for single humectant systems, the order of mixing i.e. dry vs wet mix, has no effect on the resultant a_w (Sloan and Labuza, 1975).

However, Bone et al., (1974), suggests that differences in order-of-mixing or sequence of mixing in multi-humectant systems, can result in significant differences in the resultant a_w. Bone prepared solutions of sucrose and collagen by three different methods. He found that a lower a_w was obtained when collagen was allowed to hydrate first without the competition of sucrose rather than the reverse order-of-mixing with sucrose added first. Bone also demonstrated differences in the final a_w as a result of the order of mixing of KCl with either sucrose or dextrose. However, the differences were in the third decimal place using an electric hygrometer. Acott et al., (1975), has shown this a_w technique as being poor.

This study is designed to investigate the effects of differences in the sequence and method of addition of humectants and water on the final a_W in an IMF model system and test our previous prediction equations.

2. Paper

The following is the paper accepted for publication which summarizes the findings of this part of the study. It will appear in the *Journal of Food Science*.

EFFECT OF SEQUENCE AND METHOD OF ADDITION OF HUMECTANTS AND WATER ON A_W LOWERING ABILITY IN AN IMF SYSTEM

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ABSTRACT

The sequence and method of addition of multiple humectants and
water to a food system has been reported to have an effect on the resultant
water activity (a_w). These effects using three major classes of water
binding agents were investigated in a model intermediate moisture dog food
system. Both the sequence of addition of each humectant and water and the
method of addition by either adding directly to the food or initially
dissolving the humectants in the water were varied. Water activity was
measured by the vapor pressure manometric technique. This study has shown
that neither the sequence or the method of addition of multiple humectants
to an IMF food system has an effect on the final a_w. The Ross equation
can be used to predict the final a_w in multi-humectant systems with a
high degree of accuracy.

Semi-moist pet foods represent the majority of IMF products now in the marketplace. They account for 40% of the U.S. pet food market (Rhodes, 1975). The humectants most frequently used in IMF pet foods are sucrose, propylene glycol, sorbitol and sodium chloride.

Humectants can be added into a food system either in a dry state or as a solution by previously dissolving the humectant in water. As a result of the method of addition a hysteresis effect may occur giving a different adsorption or desorption isotherm (Rao, 1941; Bettleheim and Erlich, 1963; Berlin et al., 1969; Mackenzie and Luyet, 1971).

The reasons for hysteresis have been discussed in detail by Labuza, 1974; Labuza, 1968; Labuza and Rutman, 1968; and Gregg and Sing, 1967. The structural effect of pores as described by Rao's theory (Rao, 1941; Labuza and Rutman, 1968), the effect of super saturation and the degree of hygroscopicity of crystalline vs. amorphous material are all factors contributing to this hysteresis effect. As shown by Sloan and Labuza (1975), a true sorption hysteresis occurs for crystalline humectants which is dependent on the initial crystalline form of the material. The amount of water that can be held in a humectant/water solution at any one a_w value thus depends on the method of addition of water to the system, with the desorption procedure or amorphous/solution form binding more water. This would be an advantage in lowering the need for added humectant to a food system.

Sloan and Labuza (1976a) investigated the effect of this method of mixing or hysteresis phenomenon in a semi-moist model dog food system for

the four humectants commonly used. It was found that for single humectant systems, the method-of-mixing (i.e. dry vs. solution mix) had no effect on the resultant a_w. Also, no significant difference in the a_w-lowering ability of amorphous vs. crystalline sugars was found in the IMF range. It was concluded that above the monolayer value, enough water is available for the crystalline form to be rapidly dissolved and changed into a solution or amorphous form so that the a_w is the same as in the solution mix system.

Bone et al., (1974, 1975) suggest that differences in the sequence of mixing of multi-humectant systems, can result in significant differences in the resultant a_w. They prepared solutions of 5.43 molal sucrose and 1.72% collagen by weight by using three different methods. They found that a lower a_w was obtained when collagen was allowed to hydrate first without the competition of sucrose rather than the reverse sequence with sucrose added first as seen in Table 1. Bone et al., (1975) also demonstrated differences of about 0.005 in the final a_w as a result of the sequence of mixing of KCl with either sucrose or dextrose or with two salts. An electric relative humidity hygrometer was used to measure the final a_w's in both studies. Measurements were made at 21-22°C after a sixteen hour equilibration period. Labuza et al., (1976) have shown this technique to be inaccurate and readings in the third decimal place were considered to be useless. They reported accuracy of $\pm$ 0.02 for the hygrometer method. Therefore, the differences in a_w shown in Table 1 may well be due to the inaccuracy inherent in the method of measurement.

This study investigated both the sequence and the method of addition of multiple humectants and water on the final a_w in an IMF model system. Available a_w prediction equations were also tested to see if the final a_w

in multi-humectant systems can be accurately predicted. The Ross equation

and the linear slope equation were the only equations shown to accurately

predict the final a_w in an IMF model system containing a single humectant

(Sloan and Labuza, 1976b). By using these equations, processors could

accurately predetermine the amount of a particular humectant or combination

of humectants necessary to add to a particular food system to obtain a

desired a_w. This would eliminate the current "trial and error" procedures

and prevent unnecessary over-addition of expensive water-binding agents.

The Ross equation (1) is based on the Gibbs-Duhem relationship (Ross, 1975).

where

$$a_f = a_i \cdot a_{H_1} \cdot a_{H_2} \cdots\cdots a_{H_N} \qquad\qquad (1)$$

a_f = final a_w of system + humectant

a_i = initial a_w of food

     (no humectant added)

a_{H_1} = a_w of specific humectant/H_2O solution based on total water content

a_{H_2}, a_{H_3} = a_w of humectant/H_2O solution based on total water content for components 2 and 3, respectively

This equation assumes that in a food system, each a_w-lowering component

behaves independently. The final a_w is a product of each component a_w

based on its being dissolved in all of the water in the system. Ross has

discussed in detail the problems concerning his prediction equation and

presents methods for coping with non-ideality for simple solutions. However,

these data have not been applied to most solid food systems.

For the linear slope method, the food at the initial a_w is assumed

to have a theoretical humectant concentration at that a_w from the humectant

isotherm (Sloan and Labuza, 1976b). Water activity lowering is then based

solely on the increase in weight of humectant above this concentration.

The predicted value can be calculated from the slope of the curve, as shown

by equation (2) in which an a_w vs. weight fraction humectant curve is used.

$$a_f = a_i - \frac{z \delta W_H}{W_d} \tag{2}$$

where

a_f = final a_w

a_i = initial a_w of food

δ = slope of humectant isotherm curve at initial a_w (a_w vs. weight fraction humectant)

z = g humectant added

W_H = moisture content (g H_2O/g humectant system) at initial a_w

W_d = initial moisture content of food system (g H_2O/g food)

For a triple humectant system, a series of calculations must be performed assuming the initial a_w (a_i) to be the a_w after the addition of each humectant. The slope must be calculated at the resultant a_w and the moisture content adjusted after the addition of each humectant.

MATERIALS AND METHODS

A meat-soy flour model system was employed. The composition and ingredient suppliers are listed in Table 2. Ground beef, soy flour, citric acid and potassium sorbate were ground in 50 g portions in an Osterizer for 2 minutes at high speed. These portions were combined in a large stainless steel bowl, hand mixed and equilibrated for 24 hours at room temperature. After equilibration, nineteen 50 g portions were prepared. The amount of water necessary to give an initial $a_w = 0.94$ was determined from the isotherm of the model system. Based on this, 24 ml H_2O/50 g of model system was added. The amount of each humectant added was that equivalent to a solution at an $a_w = 0.94$ based on the total water content in the mixed system.

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The humectants tested are listed in Table 2. A combination of three humectants was used for each experiment. These were added as described below with the water to a 50 g portion of the food system and mixed for five minutes in a Brabender Farinograph bowl at high speed after each addition was made.

Three different methods of mixing were evaluated also incorporating changes in humectant sequence. In Test I, the first of the three humectants was added dry; the other two were added in sequence into the added water, then mixed into the food. The sequence of each humectant was varied. In Test II, the humectants were added dry. The water was previously added into the food. The humectant sequence was also varied. In Test III, all three humectants were added into the water with the sequence varied. After mixing, the sample was divided into two portions and sealed in 202 x 214 cans. These were stored at room temperature for 24 hours and then the a_w was measured by the vapor pressure manometric technique ($\pm$ 0.01 a_w).

RESULTS AND DISCUSSION

The results of the first experiment using propylene glycol, sucrose and NaCl are shown in Table 3. The measured a_w was close to 0.78 in all cases.

Therefore, all of these humectants must be rapidly dissolved in the aqueous phase and must behave independently such that the a_w is the same regardless of the sequence or method of addition of water and humectant to the food.

The results of the second experiment using only crystalline humectants are shown in Table 4. The measured a_w was close to 0.79. These data also

demonstrate that there is no effect of either sequence or method of addition using crystalline humectants.

Since neither sequence, method of addition or crystalline state had any effect on the resultant a_w in this IMF system, a processor can add these ingredients in the manner that is most convenient or least costly.

The results as predicted by both the linear slope and the Ross Equation are compared to the measured a_w value in Tables 3 and 4. The linear slope equation does not provide an accurate a_w prediction, probably because estimation of the slope of the isotherm can lead to error. The Ross Equation, however, gives a good prediction and is recommended.

In conclusion, the sequence and method of addition of humectants and water has no effect on the resultant a_w of an IMF food system.

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TABLE 1.
WATER ACTIVITIES OF COLLAGEN – SUCROSE
SOLUTIONS PREPARED BY THREE DIFFERENT METHODS
(Bone et al., 1975)

I Collagen added to 45°C water followed by sucrose at 2 h a_w	II Collagen added to 45°C water followed by sucrose immediately a_w	III Sucrose added to 45°C water followed by collagen at 2 h a_w
0.861	0.878	0.877
0.865	0.873	0.877
0.862	0.870	0.867
0.866	0.879	0.864
0.857	0.859	0.861
0.862	0.864	
0.862	0.866	0.866
0.862	0.867	0.864
0.869	0.870	0.869
0.874	0.874	0.869
0.872	0.876	0.875
0.865*	0.871	0.870

* 95% sig.

TABLE 2
MEAT – SOY FLOUR MODEL IMF SYSTEM

	% By Weight
Meat (lean ground hamburger)	19.5
Soy Flour (PDI-20) (Cargill, Inc.)	78.2
Citric Acid (J.T. Baker Chemical Co.)	2.0
Potassium Sorbate (Anheuser-Busch, Inc.)	0.3

Humectants	g Humectant Added/74 g Model System
Experiment A –	
Propylene glycol (Dow Chemicals)	6.7
Sucrose (Mallinckrodt)	22.5
NaCl (Mallinckrodt)	2.2
Experiment B –	
Sorbitol-Crystalline (ICI, United States)	8.5
Fructose (J.T. Baker Chemical Co.)	11.3
NaCl (Mallinckrodt)	2.2

TABLE 3

EFFECT OF ORDER OF ADDITION ON A_W WITH PROPYLENE GLYCOL, SUCROSE AND NACL

Test # I – One humectant added dry – 2 in solution

	Dry	Solution		Measured a_w
Humectant	1	2	3	
	PG	S	N	0.77
	PG	N	S	0.78
	S	N	PG	0.78
	N	S	PG	0.78
	S	PG	N	0.78
	N	PG	S	0.78

Test # II – H_2O pre-mixed in food – add humectants dry

	Dry		-	Measured a_w
Humectant	1	2	3	
	PG	S	N	0.78
	PG	N	S	0.78
	S	PG	N	0.78
	S	N	PG	0.79
	N	PG	S	0.78
	N	S	PG	0.78

Test # III – Add all in solution

	Solution			Measured a_w
Humectant	1	2	3	
	PG	S	N	0.79
	PG	N	S	0.78
	S	PG	N	0.78
	S	N	PG	0.78
	N	PG	S	0.79
	N	S	PG	0.78

Predicted a_w

Ross	0.78
Linear Slope	0.71
Overall Measured Average	0.78

TABLE 4

EFFECT OF ORDER OF ADDITION ON A_w WITH SORBITOL, FRUCTOSE AND NACL

Test #I - One humectant added dry - 2 in solution

Humectant	Dry	Solution		Measured a_w
	1	2	3	
	S	F	N	0.79
	S	N	F	0.79
	F	N	S	0.79
	N	F	S	0.79
	F	S	N	0.79
	N	S	F	0.79

Test # II- H_2O pre-mixed in food - add humectants dry

Humectant	Dry			Measured a_w
	1	2	3	
	S	F	N	0.79
	S	N	F	0.79
	F	S	N	0.79
	F	N	S	0.79
	N	S	F	0.78
	N	F	S	0.79

Test #III-Add as solution

Humectant	Solution			Measured a_w
	1	2	3	
	S	F	N	0.78
	S	N	F	0.79
	F	S	N	0.79
	F	N	S	0.79
	N	S	F	0.78
	N	F	S	0.79

Predicted a_w

Ross	0.78
Linear Slope	0.77
Overall Measured value	0.79

3. Details of the calculations presented in the paper.

    a. Determination of the amount of humectant to add,

       using the Ross Equation.

       <u>Experiment I</u>

       a.) Moisture content of soy-meat system (measured)
= 85.7 g H_2O/100 g solids or 46.1% H_2O. See Figure 1 at a_w 0.94.
Original moisture 19.5 g H_2O/100 g system.

       b.) Humectant added to 74 g of meat-soy flour system
(50 g meat-soy system + 24 g H_2O) therefore, g H_2O/74 g system =
74 g of system X .461 = 34.11 g H_2O in 74 g system.

       c.) If each term in the Ross Equation is equal to
a_w = 0.94, then from humectant water sorption isotherm at 0.94.

       d.) Calculate g H_2O/g humectant.

       (1) <u>Propylene glycol</u> - 6.7 g added/74 g system

$$\frac{34.11 \text{ g } H_2O/74 \text{ g system}}{6.7 \text{ g PG}/74 \text{ g system}} = 5.09 \text{ g } H_2O/\text{g propylene glycol}$$

$$= 509 \text{ g } H_2O/100 \text{ g humectant}$$

       (2) <u>Sucrose</u> - 22.5 g added/74 g system

$$\frac{34.11 \text{ g } H_2O/74 \text{ g system}}{22.5 \text{ g sucrose}/74 \text{ g system}} = 1.516 \text{ g } H_2O/\text{g sucrose}$$

$$= 151.6 \text{ g } H_2O/100 \text{ g sucrose}$$

       (3) <u>NaCl</u> - 2.2 g added/74 g system

$$\frac{34.11 \text{ g } H_2O/74 \text{ g system}}{2.2 \text{ g NaCl}/74 \text{ g system}} = 15.50 \text{ g } H_2O/\text{g NaCl}$$

$$= 1550 \text{ g } H_2O/100 \text{ g NaCl}$$

(4)   From Respective Moisture Sorption Isotherms

(Section VC).

HUMECTANT	MOISTURE CONTENT (g H_2O/100 g humectant)	A_w
propylene glycol	509	0.94
sucrose	152	0.94
NaCl	1550	0.94

e.) $a_f = a_i \cdot a_{PG} \cdot a_S \cdot a_N$

$= 0.94 \cdot 0.94 \cdot 0.94 \cdot 0.94$

$= 0.78$ expected a_w of system

Experiment II

a.)   Moisture content (measured) = 77.7 g H_2O/100 g solids

or 43.7% H_2O.

b.)   Moisture content of model meat-soy flour system

at $a_w = 0.94$ is same as in Experiment 1 -

moisture content = 0.771 g H_2O/g solid

or   % water = 45.6%

or   total water = 67.5 g H_2O/148 g meat-soy system

c.)   If each term in Ross equation is equal to $a_w = 0.94$,

then from humectant water sorption isotherm at 0.94:

Humectant	g H_2O/100 g Humectant
sorbitol	380
fructose	286
NaCl	1470

Sorbitol

g H_2O in 148 g meat-soy system = 67.5 g H_2O

$$\frac{67.5 \text{ g } H_2O/148 \text{ g system}}{X \text{ g humectant}/148 \text{ g system}} = 4.00 \text{ g } H_2O/\text{g humectant}$$

$= 16.9$ g sorbitol added to 100 g meat-soy system

Fructose

g H_2O in 148 g meat-soy system = 67.5 g H_2O

$$\frac{67.5 \text{ g } H_2O/148 \text{ g system}}{X \text{ g humectant}/148 \text{ g system}} = 3.00 \text{ g } H_2O/\text{g humectant}$$

$= 22.5$ g fructose added to 100 g meat-soy system

NaCl

g H_2O in 148 g meat-soy system = 67.5 g H_2O

$$\frac{67.5 \text{ g } H_2O/148 \text{ g system}}{X \text{ g humectant}/148 \text{ g system}} = 15.10 \text{ g } H_2O/\text{g humectant}$$

$= 4.47$ g NaCl added to 100 g meat-soy system

d.) Amount of Humectant added to 50 g of meat-soy system

Sorbitol	8.5 g
Fructose	11.3 g
NaCl	2.2 g
Water	24.0 g

FIGURE 1

Effect of the Addition of Increments of Water

on the a_w of Meat – Soy Flour

System
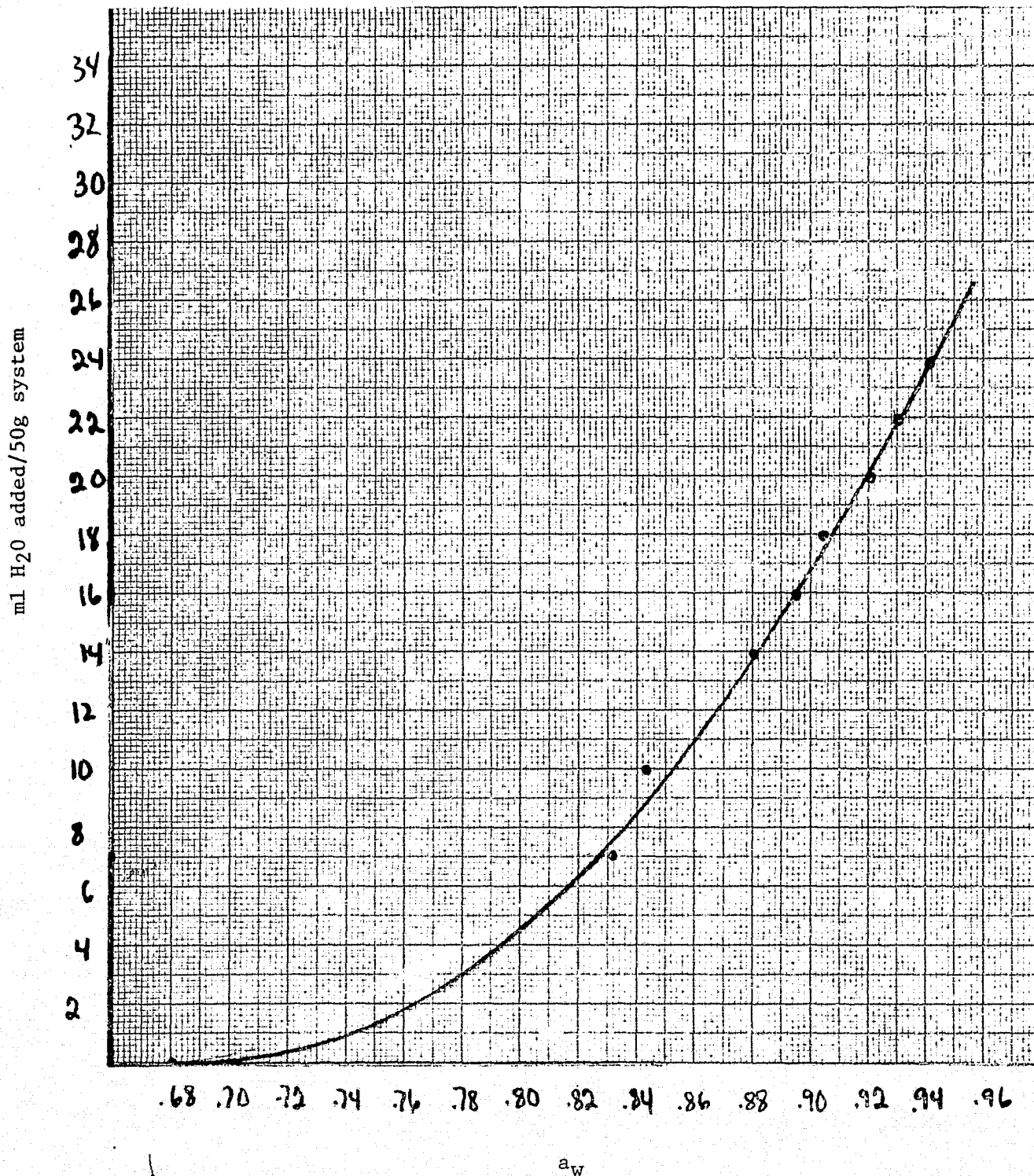
E. Summary and Conclusions

The control and determination of water activity is one of
the most important principles in the development of shelf stable
intermediate moisture foods. In terms of this:

1. The details of the VPM procedure and the equipment diagram
have been included so that food processors may duplicate the method.

2. A statistical comparison of several a_w determination
methods has been made. It has been found that the Abbeon a_w cup
analyzer can be used as a quality control device for a_w measurement
as it is reproducible to within $\pm$ 0.02 a_w units but reads significantly
lower than either the VPM and Fett-Vos techniques. The method should
only be used between 0.75 to 0.90 a_w which is within the IMF region.

3. The true water holding capacities for thirteen humectants
have been determined and published.

4. Processors can use the published humectant isotherms along
with the Ross equation to accurately predict the a_w of food formulations
using multiple humectants.

5. The sequence of addition of multiple humectants into a food
formulation does not affect their water activity lowering ability.

6. In the IMF range the method of addition of humectants,
either dry or in solution has no effect on a_w lowering ability.

VI.   SHELF LIFE STUDY OF INTERMEDIATE MOISTURE PROCESSED CHEESE

A.   Introduction

In Phase III of this contract techniques were developed for the
determination of methods to prepare a shelf stable intermediate moisture
cheese.   This information was used to prepare several cheeses which were
evaluated after processing.   The data were published in the following
publication.   In Phase IV of this contract six cheeses were prepared
at different a_w's and were shelf life tested for both microbial and
chemical deterioration.

Development of an Intermediate-Moisture Processed Cheese Food Product

H. LEUNG, H.A. MORRIS, A.E. SLOAN, and T.P. LABUZA

☐ MOST CHEESES can have a relatively long shelf life at refrigerated temperature if they are properly manufactured and packaged. The heat treatment given the milk and the acids produced during the fermentation process are primarily responsible for stability. However, microbial spoilage may occur, with undesirable mold growth being the most common problem. If present, most food pathogens can also grow in the cheese.

During the fall of 1965, there was an outbreak of staphylococcal food poisoning in the U.S., involving Cheddar, Monterey, and Kuminost cheeses (Zehren and Zehren, 1968). Early in 1974, Argentine authorities confirmed an outbreak of botulism caused by a commercial cheese spread (personal communication). These cheese-related public health problems have led the Food and Drug Administration, as well as other food regulatory agencies around the world, to consider setting an upper limit on the water activity (a_w) of certain processed cheeses and cheese foods.

Water activity has an important effect on controlling the growth of microorganisms (Scott, 1957; Troller, 1974). Information on the a_w of cheeses would be valuable in assessing microbial stability of the products and the potential for food poisoning. Unfortunately, no extensive information is presently available in the literature. One of the objectives of this study, therefore, was to determine the a_w and pH of various kinds of commercial cheeses to assess their stability.

Although processed cheese can usually be stored for a prolonged period with no refrigeration, it is not sterile. Heat-resistant spores, if present, could germinate under favorable conditions. If the wrapper is damaged, not properly sealed, or left unsealed after opening, contamination by undesirable bacteria and molds can occur. Therefore, the second objective of this study was to produce a shelf-stable processed cheese food product in the intermediate-moisture range by the addition of milk solids, humectants, and antimycotic agents (Bone, 1969; Brockmann, 1970). The product should retain the basic characteristics of regular processed cheese and withstand adverse storage conditions, including high ambient temperature and faulty packaging. No attempt was made to conform to the standards for processed cheese; however, only FDA-approved ingredients and additives were used.

COMMERCIAL CHEESES ANALYZED

Thirty assorted cheeses were purchased from a supermarket. The rinds were removed and an internal sample was taken. The a_w of duplicate samples was determined at room temperature (23°C) by the Fett-Vos method (Fett, 1974; Vos and Labuza, 1974) and the vapor-pressure manometer (VPM) technique (Labuza, 1974); these methods, if done properly, measure a_w to 0.01 units. The pH of all cheese samples except Parmesan was determined by direct measure-ment (Hausler, 1972) using a Beckman non-aqueous electrode No. 39142 together with a Copenhagen radiometer (type PHM276). The dilution method (Thomas and Hyde, 1972) was used for the Parmesan cheese. Moisture and fat contents of the cheese samples were determined according to AOAC (1965) methods.

The a_w and pH values of the 30 commercial cheeses are listed in Table 1. With the exception of Parmesan and whey cheese, all the samples tested showed an a_w greater than 0.94. Many cheeses had an a_w very close to 1.0. The a_w values determined by the two different techniques are within 0.02 a_w units in most cases. Labuza et al. (1975) compared several different methods of a_w determination, and found that a_w measurements by different methods varied considerably for products in the high-a_w range of 0.96-1.0. They concluded that the true value in the high-a_w range is very difficult to measure unless the VPM method is used.

However, gas formation from the product sometimes prevents use of this method. Thus, for a few samples (Table 1), a_w measurement using the VPM technique was not possible, since an equilibrium condition could not be attained. The gradual, continuous increase of the manometer reading was due to the respiration of microorganisms or release of volatiies from these cheeses (Labuza et al., 1975). As shown in Table 1, most cheeses had very high a_w and medium acid pH. The pH of most of the cheeses ranged from 4.7 to 5.8 in most cases. However, Camembert and Brie cheeses exhibited a high pH (6.1, 7.0, 7.4); this is due to the greater enzymatic degradation of proteins during ripening. Therefore, these cheeses could be subject to microbial spoilage and growth of *Staphylococcus aureus*, especially if not held at refrigerated temperature. The inhibitory effect of the acids in cheese is only partially responsible for retarding the growth of any microorganism present if the cheese is held at refrigerated temperature.

The moisture and fat contents of the cheeses are also summarized in Table 1. The cheese foods have a lower a_w than do the natural cheeses, even at higher moisture levels, because of added low-molecular-weight ingredients which control the a_w through water binding. For example, Muenster cheese has a moisture content of about 1.2 g water/g nonfat solids, and shows an a_w of approximately 1.0, while Ye Old Tavern cheese food and Swiss-American spread, both with higher water contents (1.6 and 1.5 g water/g nonfat solids, respectively), have an a_w of 0.95.

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Table 1—ANALYSIS of commercial cheeses and processed cheeses

Cheese	Manufacturer	a_w Fett-Vos	VPM	pH	% H₂O	% Fat	g H₂O/g dry matter (fat-free basis)
Cracker Barrel (Cheddar)	Kraft	0.97	0.98	5.3	36.3	32.7	1.156
Canadian Cheddar	Purity	0.95	0.95	5.2	34.5	32.4	1.042
Longhorn Colby	Byerly's	0.99	0.98	5.3	38.1	34.0	1.365
Bongard's Colby	Bongard's	0.99	1.00	5.0	44.4	23.4	1.380
Monterey Jack	Purity	0.99	1.00	5.8	43.5	29.0	1.685
Mozzarella	Kraft	1.00	1.00	5.8	45.2	20.4	1.314
Parmesan (grated)	Kraft	0.76	0.75	5.4	17.3	27.1	0.311
Romano	Kraft	0.97	1.00	5.3	38.7	22.3	0.992
Provolone	Kraft	0.98	1.00	5.4	43.1	27.5	1.468
Swiss	Kraft	1.00	0.97	5.7	39.8	27.3	1.211
Gouda	Purity	0.99	1.00	4.7	43.6	27.3	1.499
Edam	Purity	1.00	—ᵃ	5.3	43.1	26.1	1.399
Muenster	Purity	1.00	0.99	5.4	39.4	27.1	1.175
Farmer Cheese	Purity	0.99	0.96	5.8	40.5	30.3	1.387
May Bella	Purity	0.98	—ᵃ	5.0	47.8	26.5	1.858
Jackie	Denmark	0.99	—ᵃ	5.4	38.3	38.3	1.638
Whey	Sweden	0.91	0.88	5.8	24.1	8.8	0.359
American (processed cheese)	Kraft	0.98	0.97	5.8	38.9	36.1	1.298
Hoffman's processed cheese	Anderson Clayton	0.97	0.97	5.8	40.0	31.1	1.386
Pot (processed cottage cheese)	Milwaukee	1.00	1.00	5.1	72.9	0.4	3.151
Fondue Swiss Knight	Switzerland	1.00	—ᵃ	5.5	61.3	15.4	2.521
Kaukauna Klub (cold-pack Cheddar)	International Multifoods	0.96	1.00	4.8	35.2	32.0	1.073
Ye Old Tavern (Cheddar)	Cheese food	0.96	0.95	5.1	47.1	22.9	1.570
Swiss-American spread	Kraft	0.96	0.93	5.6	47.2	21.3	1.499
Velveeta (spread)	Kraft	0.96	0.99	5.8	52.9	21.0	2.029
Brie	Fromagerie Bongrain Inc.	1.00	1.00	7.4	50.4	28.9	2.436
Camembert	France	1.00	—ᵃ	6.1	56.7	21.6	2.611
Camembert	Denmark	0.99	—ᵃ	7.0	53.0	23.0	2.206
Blue	Treasure Cave	0.94	—ᵃ	5.1	44.2	27.2	1.551
Blue Stilton	England	0.94	—ᵃ	5.8	39.5	30.0	1.294

ᵃNot adaptable to a_w measurement by VPM

IM CHEESE FOODS PREPARED

Nine-month-old cheddar cheese was obtained from Land O'Lakes, Inc. (Minneapolis, Minn.). The cheese was weighed, cut into pieces, then milled using a Hobart meat grinder. Four different batches were prepared; the formulations are shown in Table 2.

A Damrow steam-cooker (Fond Du Lac, Wis.) equipped with agitator, steam jacket, and direct steam injection was used for processing. The cooker was preheated to 120°F, and the cheese was added with agitation. The other ingredients were then blended into the cheese. The mixture was heated to 180°F, and the product was removed from the cooker. The total processing time was approximately 20 min. The cooked cheese was filled into 2-lb boxes containing a cellophane liner. The products were then cooled at 4°C and subsequently stored at room temperature.

A 9-point hedonic scale was used for sensory evaluation. The panel consisted of 10 members from the University of Minnesota Food Science Department, including three experts in the cheese area. Samples were evaluated one week after manufacture for flavor, texture, appearance, aroma, and overall acceptance. The taste panel results for the four processed cheese food products are summarized in Table 3. The first product—which was obtained by blending 30 lb of cheese and 10 lb of nonfat dry milk (NFDM)—was hard and dry. The inferior texture was reflected by the low texture score of 4.2 ("dislike"). Furthermore, some of the NFDM solids did not dissolve during

Table 2—FORMULATIONS for intermediate-moisture processed cheese food products

Ingredient	Weight (lb) Product 1	2	3	4
Cheddar cheese	30	30	30	30
Nonfat dry milk	10	10	10	11
Disodium phosphate	1	1	1	1
Propylene glycol	—	2	1	1.5
Butter	—	1	0.5	0.5
Salt	—	0.5	1	1
Sodium citrate	—	0.1	0.1	0.2
Potassium sorbate	—	0.044	0.044	0.044

cooking and formed clumps in the cheese, rendering it undesirable. Clumps probably occurred because not enough liquid phase was present to dissolve the solids, especially the protein.

Different levels of propylene glycol, together with salt and potassium sorbate/citrate as the antimycotic agents, were incorporated into the other three products. Besides acting as an effective water-binder (Brockmann, 1970; Sloan and Labuza, 1975), propylene glycol also helps to solubilize the milk solids, thus preventing the clumping—the NFDM solids completely dissolved in these samples during cooking. The glycol also increases the plasticity of the product and has specific antimicrobial action (Acott and

Product	Flavor	Off-flavor	Texture	Appearance	Aroma	Overall	Comments
1	6.5	5.8	4.2	6.2	6.8	5.5	Hard, dry
2	6.4	6.2	6.9	7.2	7.8	7.1	Off-flavor, sweet
3	7.2	5.6	6.5	7.6	7.5	7.0	Sweet, salty
4	6.4	4.7	6.6	5.9	7.0	5.9	Dark, salty off-flavor

[a]Scale: 9 = like extremely; 5 = undecided; 1 = dislike extremely

Labuza, 1975; Boylan et al., 1976). Also, the texture of the product was improved significantly by adding the glycol (Table 3).

Products 2 and 3 were rated substantially better than the other two, with overall acceptance scores of 7 ("like"). Considerable browning occurred in product 4; this is probably a result of the increased glycol content and milk solids content, which is high in lactose. This cheese also tended to crumble and had poor sliceability. All four products showed a high resistance to melting when heated, making them unsuitable for use in foods where melting is desired.

An off-flavor from propylene glycol was quite noticeable when the cheese was hot. However, as the cheese cooled down, the off-flavor became hardly discernable. Most panelists were able to pick up the more-intense salty flavor in products 2 and 3. However, this did not have much effect on the flavor scores. The salt was added as both a preservative and a water-binder. Judging from the taste panel results, products 2 and 3 appear to be quite satisfactory and should have market potential.

STABLE AT ROOM TEMPERATURE

The a_w and pH values of the four processed cheese food products were determined using the VPM technique and the direct-measurement procedure, respectively. Fat and moisture contents were determined according to AOAC (1965) methods.

The a_w and pH values are shown in Table 4. The first product had an a_w of 0.85. The incorporation of propylene glycol and salt reduced the a_w by 0.02 and 0.03 units. The pH of products 3 and 4 was below the optimum range of 5.4-5.7 for texture stability; however, this is desirable from an antimicrobial standpoint.

The products were also examined for physical changes after 4 mo of storage at room temperature. No fat separation, browning, or significant change in flavor occurred. However, the products did become harder and drier. When the products were stored at refrigerated temperature (40°F), some salt crystallization occurred on the surface of the samples.

According to Acott and Labuza (1975) and Boylan et al. (1976), the combination of propylene glycol, potassium sorbate, and sodium citrate should inhibit

the growth of S. aureus and molds at room temperature in intermediate-moisture foods, especially at a_w of 0.82 and 0.83. Therefore, it is envisioned that products 2 and 3 should be shelf-stable to growth of these microorganisms. In addition, the work of Warmbier et al. (1976) indicates that propylene glycol may help to retard non-enzymatic browning in the intermediate-moisture cheese foods.

Although further studies on the microbiological and chemical stabilities of the products would be necessary to determine actual shelf life, this study indicates that—unlike most commercial cheeses, which have a_w's above 0.95 and therefore are subject to spoilage—intermediate-moisture processed cheese food products with an a_w of 0.83, prepared by the addition of NFDM solids, salt, and propylene glycol, should be shelf-stable at room temperature.

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Table 4—ANALYSIS of intermediate-moisture processed cheese food products

Product	a_w (VPM)	pH	% H_2O	% Fat	g H_2O/g dry matter (fat-free basis)
1	0.85	5.4	32.1	24.8	0.745
2	0.83	5.4	33.8	23.3	0.788
3	0.82	5.2	35.4	22.0	0.831
4	0.82	5.2	37.0	20.4	0.869

B. Study of the Shelf Life of an Intermediate Moisture Process

Cheese Food Product

1. Introduction

Most cheeses have a relatively high water activity, a_w,
(Leung et al., 1976) and provide a suitable substrate for microbial
growth if contaminated under normal conditions, undesirable mold growth
is the most common cause of cheese spoilage. However, food pathogens
can also grow in cheeses. Staphylococcus aureus growth and enterotoxin A
production in many different cheeses have been documented (Allen and
Stovall, 1960; Hauster, Burge, Scarborough and Hendricks, 1960; Hendricks,
Belknap, and Hausler, 1959; and Tatini, Wesala, Jezeski and Morris, 1973).
Cheese contaminated with Staphylococcus aureus has caused outbreaks of
food poisoning (Zehren and Zehren, 1968; and Keogh, 1970). Likewise,
cheese contaminated with Salmonella has caused cases of food poisoning
(Food Chemical News, August 30, 1976; and Keogh, 1970). Laboratory
research has shown the survival time of salmonella in cheese contaminated
during production to extend well into the shelf life of the cheese
(Tzannetis and Papavassilou, 1975; Park, Marth and Olson, 1970; and
White and Custer, 1976).

It should be possible to eliminate growth of all pathogenic
bacteria and most molds from cheese by a combination of lowering the
water activity and adding microbial inhibitors. Scott (1957) and
Troller (1973) have reviewed the effect of a_w on the growth of
microorganisms.

Leung et al., 1976 developed four intermediate moisture
process cheeses and tested them for product acceptability. The present
study was designed to test the microbial resistance and storage life
of process cheese formulated at several water activities ranging from
0.81 to 0.94. Five different intermediate moisture food (IMF) cheeses
were prepared according to the formulations in Table 1. They differed
only in the amount of water in the formulation. Several different
tests were performed on these cheeses to determine their stability over
storage time with respect to textural changes, color, taste and melta-
bility, as well as their resistance to microbial growth. These
properties were compared with a commercially available process cheese.

2. Preparation of the cheeses

The cheeses were processed in a Damrow steam-cooker
(Fond Du Lac, Wis.) equipped with agitator, steam jacket and direct
steam injection. Steam injection was not used in this test. The
cooker was preheated to 120°F. The milled American process cheese was
added first and then the other ingredients were added in the following
order: disodium phosphate, sodium citrate, potassium sorbate, butter,
salt, propylene glycol and last, the nonfat dry milk (NFDM) alternately
with the water. The temperature was kept high (∿146°F) while the
NFDM and water were added to keep the cheese molten and prevent excess
stress in the agitator motor. There is no added water in cheese D
so the NFDM was first mixed with the milled American cheese and they
were added together before the other ingredients. This procedure

Table 1

IMF Cheese Formulation

	A	B	C	D	E
American process cheese	22.5 lb	22.5 lb	22.5 lb	22.5 lb	22.5 lb
Disodium phosphate	.75	.75	.75	.75	.75
Butter	.375	.375	.375	.375	.375
Salt	.75	.75	.75	.75	.75
Sodium citrate	.075	.075	.075	.075	.075
Propylene glycol	.75	.75	.75	.75	.75
NFDM	7.5	7.5	7.5	7.5	7.5
Potassium sorbate	.033	.033	.033	.033	.033
Water	10.0	7.5	5.0	0.00	2.0

C .4

would have also worked better for cheese E which has only 2 lb of water. Cheese E contains some undissolved NFDM since the ingredients were added in the same order as cheeses A, B, and C.

The cheese was taken out of the cooker when the temperature reached 176°F. The temperature rose to ~184°F before all of the cheese was removed. All but 10 lb of cheese in the cooker was poured into 2 lb boxes with cellophane liners. The cellophane liners have a waxy intercoating which melts onto the hot cheese and seals the package. Then, .0454 g of triphenyl terzolium chloride (TTC) dissolved in 5 ml of water was added to the mixer and mixed in the remaining cheese. This last cheese with TTC was then poured into large (150 x 15 mm) disposable sterile petri dishes.

Cheeses A, B, C and D were prepared in one day in the order listed. Cheese E was prepared eight days later in order to obtain a cheese with an a_w between .81 and .90.

As each cheese was poured it was placed in a 4°C environmental room until it cooled to room temperature. Cooling time was two to three hours. All of the cheeses were stored at room temperature (varying from 68°F to 78°F). The cheese in the petri plates was kept in covered fish tanks kept at constant relative humidity. The cheese in the 2 lb boxes was stored in the general laboratory with no special humidity precautions.

3. Analysis of physical properties of the cheese

    a. Moisture content

        The water content of the cheese was measured in two
ways: (1) by a USDA (1955) method whereby the cheese is dried for 16 hr
at 100 - 103°C at atmospheric pressure and then for 1 hr at 100 - 103°C
in a 25" vacuum and (2) by the GLC thermal conductivity detection
procedure on a Hewlett-Packard gas-liquid chromatograph Model #7620A
equipped with a Model #3380A integrator (Labuza, Tsuyuki and Karil, 1969).

    b. Fat content

        The percent fat was assayed by a modified Mojonnier
USDA method.

    c. pH

        The pH's of the cheeses were measured directly using
a Beckman nonaqueous electrode #39142 with a Copenhagen radiometer
(Type PHM 276).

    d. Water activity

        The a_w's of the five cheeses were measured in dupli-
cate by the vapor pressure manometer technique (Labuza et al., 1976).

    e. The results of the physical properties as measured
for the five cheeses are shown in Table 2. As expected the vacuum oven
gave a higher moisture content due to glycol loss during drying. All
other values fell in the expected range.

Table 2

Physical Analysis of Intermediate Moisture Cheese

	A	B	C	D	E
Water content g H_2O/g solid:					
Gas-liquid chromatograph	.78	.67	.60	.35	.46
Vacuum oven drying	.90	.77	.66	.42	.48
Percent fat	16.84	17.83	19.17	21.74	21.08
a_w	.94	.91	.90	.81	.86
pH	5.67	5.79	5.76	5.58	5.77

4. Methods for stability testing

    a. Microbiological stability test.

        Each of the five cheese and also Land O' Lakes
American process cheese were challenged with five different microorganisms:
three molds (Aspergillus glaucus, Aspergillus niger, Penicillium
roqueforti) and two bacteria (Staphylococcus aureus and Salmonella anatum).
Five plates of each cheese were spread with each of the microorganisms.
Staphylococcus aureus grows at a minimum a_W of 0.86 whereas Salmonella
spp. generally need an a_W of 0.95 to grow (Scott, 1957). Penicillium spp.
need an a_W of 0.84 - 0.86 for growth (Leistner, 1973 and Snow, 1949).
Snow (1949) reports the minimum a_W for growth of A. niger to be 0.84.
But Hubbard, Earle and Senti (1957) and Acott and Labuza (1975) have
found it to be considerably lower, around 0.79. A. glaucus can grow
at an a_W as low as 0.73 - 0.75 (Christensen and Meronuck, 1974).

        (1) Preparatory studies.

        Before the cheese was prepared preliminary
studies were carried out in order to: (a) Develop standard curves of
turbidity vs. cfu/ml for estimating the cfu/ml of the active bacterial
cultures and freshly harvested mold suspensions without having to
store them while plates of dilutions grow up and are counted. (b) Test
compounds are to be added to the cheese to change the color of the
growing bacterial colonies so they can be identified on the surface
of the cheese.

In order to perform the test properly, the number of colony-forming units initially spread on the surface of the cheese must be known so the number that did not grow can be known and reported. At the same time, the cheese is not an ideal growth medium for the microorganisms and to estimate the maximum resistance of the cheese it must be challenged with healthy organisms that have been subjected to a minimum of stresses. Thus it was necessary to develop a method of estimating the number of cfu/ml of actively growing (log phase) bacterial cultures and freshly harvested mold suspensions.

Mold colonies grow with a texture and color significantly different from the cheese. The three species used in this study also have distinctive colors very different from each other. However, the texture and color of the Staphylococcus and Salmonella colonies are too similar to that of the cheese to be readily visible. A compound had to be chosen that would make the growing colonies visible.

(a) Method of estimating cfu/ml.

The quickest and most reliable method of determining cfu/ml is to calibrate the turbidity of cultures and mold suspensions (as measured on a colorimeter) against plate counts of several dilutions. Once this relationship has been determined, the results of any new plate counts can be estimated by quickly taking a turbidity reading of the growing culture or freshly harvested mold. The turbidity can be accurately measured only in the range of 10^5 to 10^9 cfu/ml so in the data presented below only the turbidity of the

undiluted culture suspensions and two decimal dilutions was measured. The turbidity was read as percent transmittance at a wavelength of 450 nm on a Beckman Spectrophotometer 20. The percent transmittance of the undiluted bacterial cultures was read against a TSYB blank. The percent transmittance of the mold suspensions and of all dilutions was read against a sterile buffered water blank. For accuracy, only 20-300 colonies on a petri plate can be counted. Dilutions of 10^{-4} to 10^{-6} were extrapolated back to the optical density of the culture suspension.

The bacterial cultures measured were grown for 18 hours at varying temperatures (in an attempt to obtain varying initial densities) in 100 ml of TSY broth. The Penicillium roqueforti grew best on Sabouraud's medium; Aspergillus niger on Czapek's medium with 3% sucrose; and Aspergillus glaucus on Czapek's medium with 20% sucrose (it has a low a_w requirement).

The mold cultures were all grown at room temperature. Bacterial plates were all grown at 37^oC and mold plates at room temperature.

The molds were harvested by rinsing the surface of a culture growing for 5-7 days on an agar slant in a dilution bottle with a 3 x 10 cm surface. The molds did not go into suspension but simply floated to the top of the dilution blank unless a small amount of a mild detergent was added. A 10^{-5} w/w solution of sodium lauryl sulfate (SLS) was used. With the SLS a more even suspension was

obtained giving more accurate dilutions and turbidity readings. SLS is not inhibitory to the mold at this concentration.

The results are shown in Figures 1-5 for all the organisms. Figure 1 for S. aureus indicates that a culture with a percent transmittance reading of 10-15 should be diluted to 10^{-4} to give an initial inoculant level of $1 \pm .5 \times 10^3$ cfu in 0.1 ml. The variation between runs could be due to clumping but is not large enough to cause significant error. The runs for Salmonella anatum are shown in Figure 2. The results are similar to that for the S. aureus.

In the study with Penicillium roqueforti the initial runs without SLS did not give reliable results because of poor suspension. The results presented in Figure 3 show the data with SLS. As seen, at high concentration there is greater variability, but at 50-60% transmission good results can be obtained.

Similar problems occurred with Aspergillus niger and Aspergillus glaucus. The results with SLS present are shown in Figures 4 and 5 respectively. Fairly good standard curves are shown. It should be noted that for A. glaucus two cultures were grown on Czapek's medium with 20% sucrose. The high concentration of sucrose seemed to lower the % transmittance of these suspensions and the readings did not fall in the range of the other runs.

FIGURE 1

Staphylococcus aureus Transmission Results

cfu/ml

% Transmittance
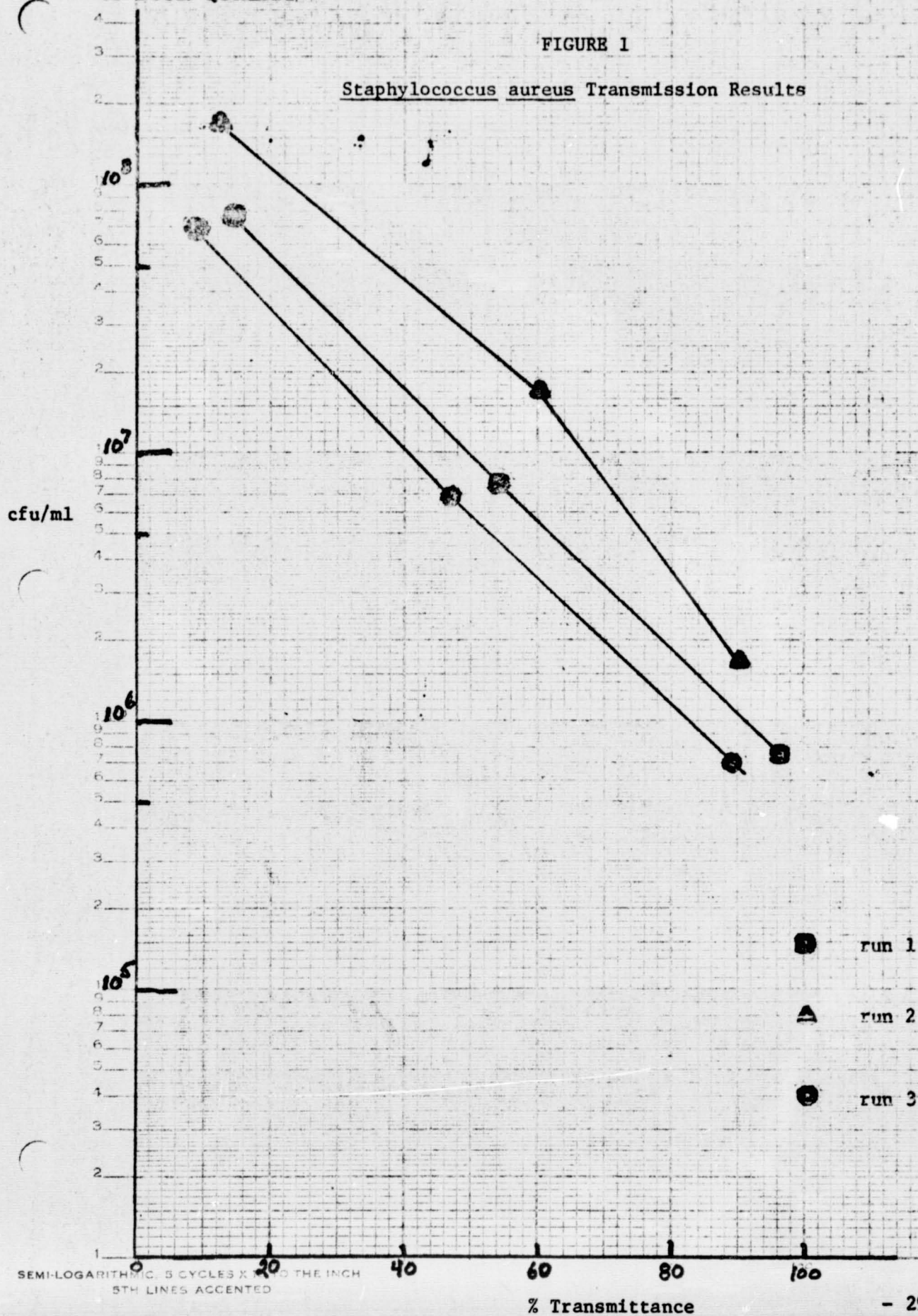
Figure 2

Salmonella anatum Transmission Results
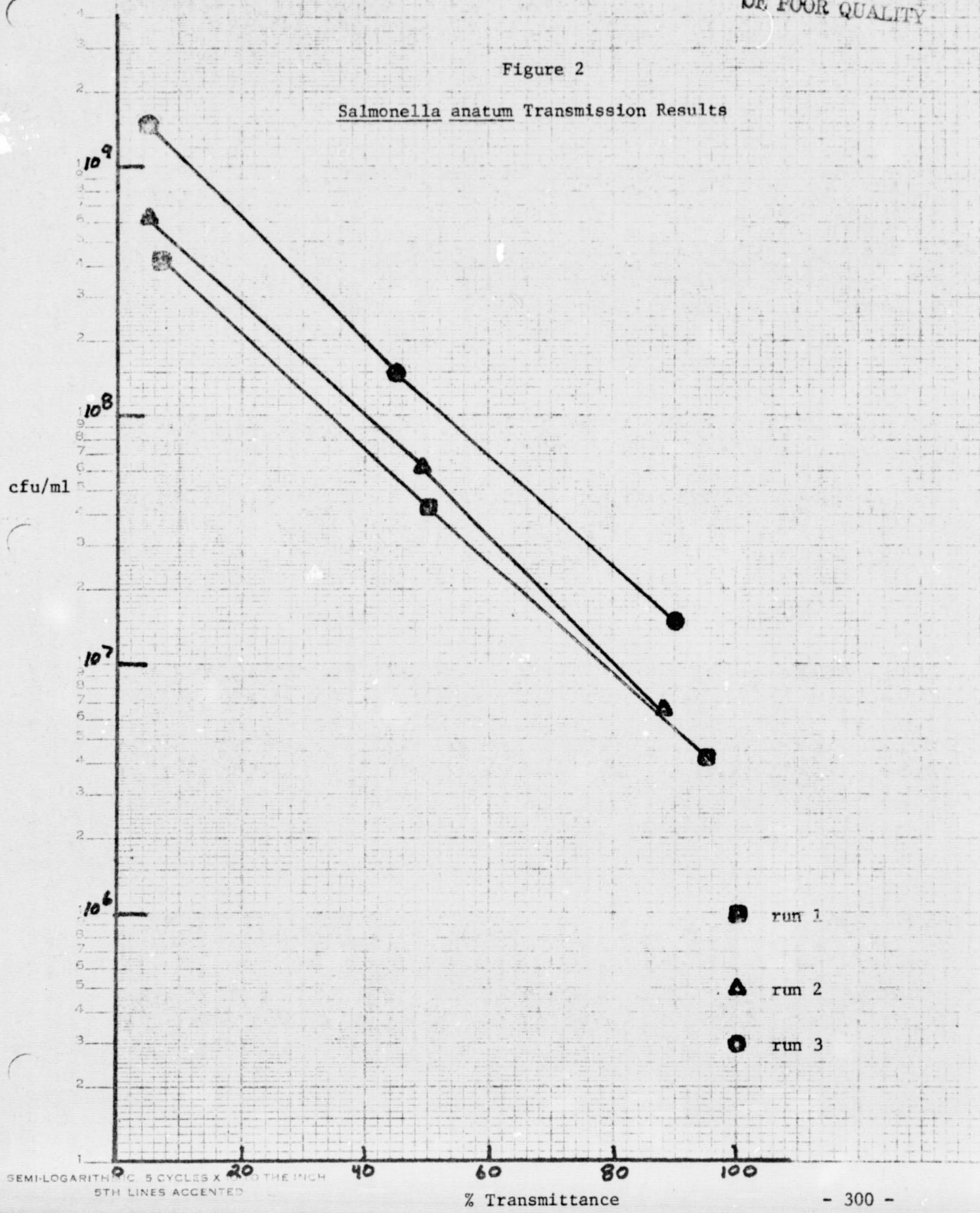
Figure 3

Penicillium roqueforti Transmission Results

Figure 4

Aspergillus niger Transmission Results

cfu/ml

□ run 1
△ run 2
◐ run 3
● run 4
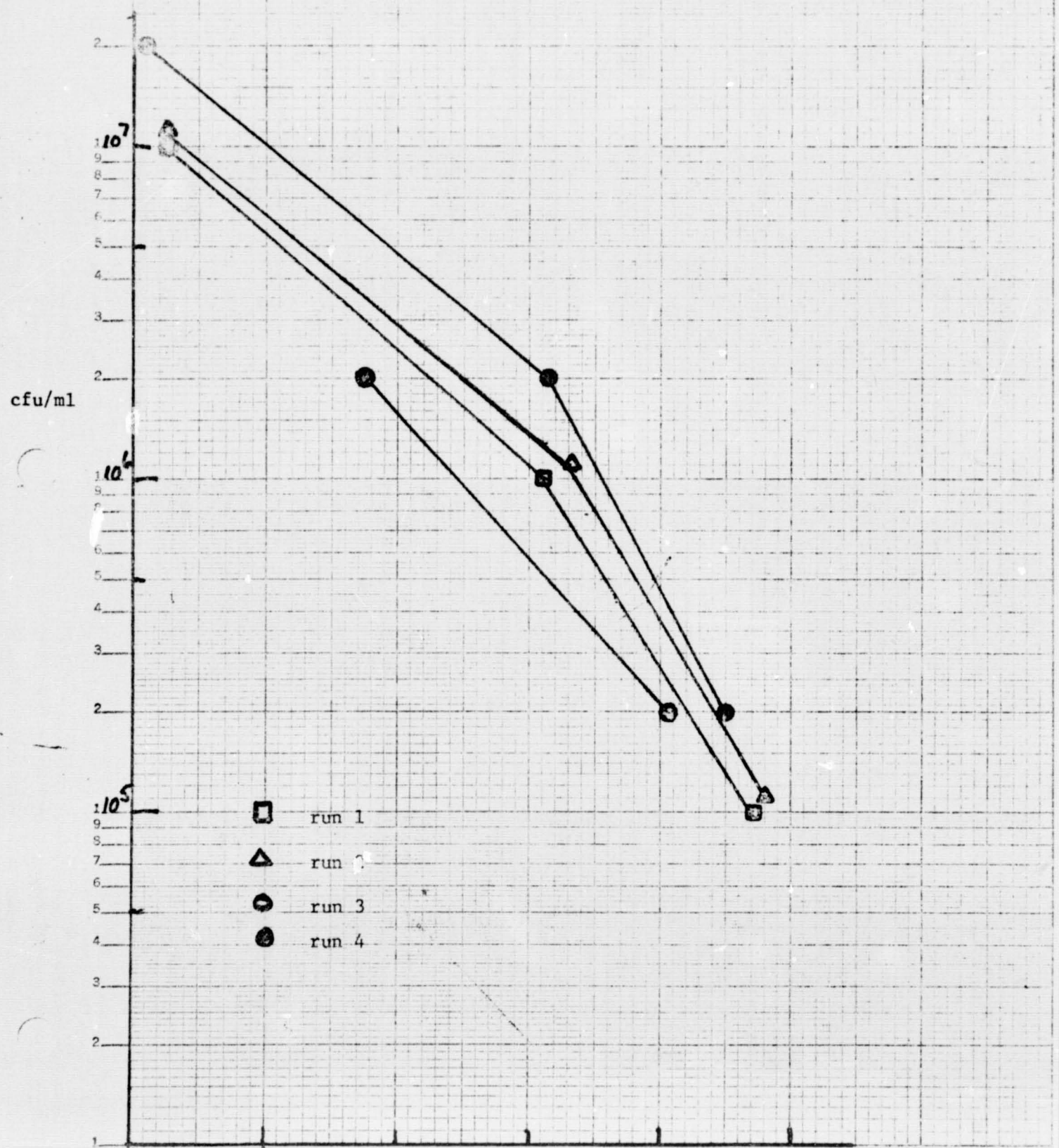
% Transmittance

Figure 5

<u>Aspergillus</u> <u>glaucus</u> Transmission Results
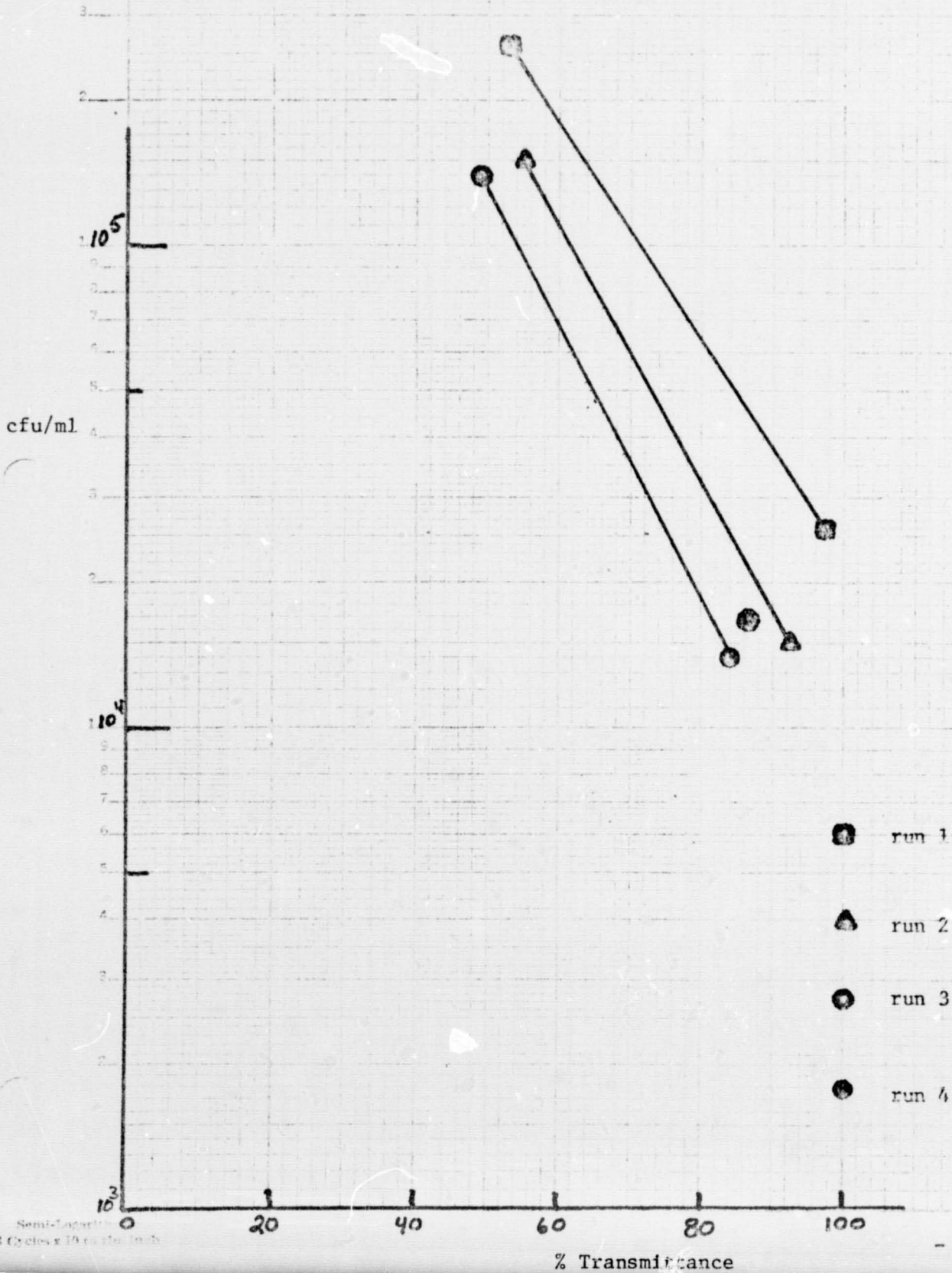
(b) <u>Method of bacterial colony identification</u>.

Numerous compounds are available that can be metabolically converted to a different color during growth of a microorganism. Three of these compounds were tested. The criterion used was that the compounds must not be inhibitory to the microbes and they must give a highly visible color on the surface of the cheese upon growth of a colony. The three compounds tested were:

2,3,5 - Triphenyltetrazolium chloride (TTC)

Potassium tellurite (K_2TeO_3)

Brilliant green (BG)

The color of TTC changes from clear to red as it is reduced by the growing colony, K_2TeO_3 changes from clear to black as it is reduced, and BG changes from green to pink upon growth of bacterial colonies.

In order to test the visibility of colonies growing on cheddar cheese 0.1% of each of the three compounds was added to separate portions of cheddar cheese. The cheese was blended, melted at 100°C in a steamer and poured into small (15 x 50 mm) petri dishes. Two petri plates of each of the mixtures were then inoculated with 0.1 ml of a growing <u>Staphylococcus aureus 196 E</u> culture and two were inoculated with 0.1 ml of a growing <u>Salmonella anatum</u> culture. The plates were incubated at 37°C. None of these plates showed any microbial growth. The BG changed the color of the cheese so much that it was eliminated from use.

In a second trial run only TTC and K Tell were used. The concentration was lowered to 0.001%. Since K Tellurite can be inactivated by excessive heat, unmelted portions of the cheese were plated as well as portions melted over hot water with a minimum of heat.

To test for inhibition, growth studies of Staphylococcus aureus and Salmonella anatum were done on plates of TSYA, TSYA with 10^{-5} TTC and TSYA with 10^{-5} $K_2T_eO_3$.

The results of the use of dyes for bacteria identification are shown in Table 3. The Salmonella did not grow at all on the cheese while the Staphylococcus grew on almost all of the plates with both K Tellurite and TTC.

The red pigment of the reduced TTC was more visible against the cheese than was the black of the reduced K Tellurite.

The results of the plate counts using TSYA, TSYA with 10^{-5} TTC and TSYA with 10^{-5} K Tellurite are shown in Table 4. The Staphylococcus grew equally well on all three types of media. The Salmonella grew as well on TSYA with 10^{-5} as it did on TSYA but was completely inhibited by the K Tellurite. TTC was chosen to be used for identification of bacterial colonies on the surface of the cheese.

Table 3

Comparison of Bacterial Colony Visibility
On Cheddar Cheese Using TTC and K Tellurite

	Cheese with 10^{-5} K Tellurite	Cheese with 10^{-5} TTC
	# of plates growth vs total plates	# of plates growth vs total plates
Staphylococcus aureus	5/6	6/6
Salmonella anatum	0/6	0/6

Table 4

Results of Plate Counts Testing Inhibition of S. aureus 196E and Salmonella anatum by K Tellurite and TTC

	TSYA	TSYA + 10^{-5} TTC	TSYA + 10^{-5} K_2Te0_3
Staphylococcus aureus	4.8×10^8 cfu/ml	4.7×10^8 cfu/ml	4.5×10^8 cfu/ml
Salmonella anatum	2.3×10^9 cfu/ml	2.4×10^9 cfu/ml	none

(2) Inoculation of the cheeses.

Cultures were grown as in the preliminary study
(NASA Phase IV, 3rd Quarterly Report). The percent transmittance at
450 nm was read on a Beckman spectrophotometer 20 and the cfu/ml
estimated using the data collected in the preliminary study. Table 5
shows the percent transmittance of each culture or spore suspension,
the estimated cfu/ml, the dilution used to inoculate the cheese, and
the counts from plates spread at the time of inoculation. The plate
count results of the molds are low for cheeses A, B, C and D since
TSYA plates are not well suited for mold growth. The counts from
cheese E, where each microorganism was grown on a suitable media, are
as predicted. No growth occurred on the Salmonella plates for the
American process cheese. The culture was clumpy and may not have been
diluted properly. This inoculation should be repeated with a new
culture. 0.1 ml of each of the inoculants was spread on the surface
of each cheese. Cheeses C, D and E were too thick when poured to
cool with a smooth surface. On these cheeses the inoculant was deposited
in ~10 drops around the surface and was spread with a slightly moist
sterile cotton swab. The American process cheese was sliced with a
sterilized knife in a laminated flow hood under a germicidal u.v. light.
The slices were placed in small (100 x 50 mm) disposable plastic
petri plates under the hood. The 10^{-5} TTC solution was added to the
inoculating dilution of the S. aureus and Salmonella anatum cultures
immediately before pipetting onto the surface of the American cheese
slice.

Table 5

Initial Inoculation Values for IMF Cheeses and American Process Cheese

	A. glaucus	A. niger	P. roqueforti	S. aureus	Salmonella anatum
O.D. of culture or spore suspension:					
A, B, C and D	55	$0.0 \dfrac{10^{-1} \text{ dilution}}{40}$	5	4	5
E	70	0.8	11	3	8
American	$6 \dfrac{10^{-1} \text{ dilution}}{76}$	8.0	48	5.5	71.0
Dilution used:					
A, B, C and D	10^{-1}	10^{-4}	10^{-4}	10^{-4}	10^{-5}
E	10^{0}	10^{-3}	10^{-3}	10^{-5}	10^{-5}
American	10^{-2}	10^{-2}	10^{-3}	10^{-4}	10^{-4}
Plate count; from 0.1 ml of inoculating dilution:					
A, B, C and D	NG	>200	>200	5×10^{3}	1.5×10^{3}
E	overgrown	4×10^{3}	2.5×10^{3}	2×10^{3}	3×10^{3}
American	3.8×10^{3}	5×10^{3}	7×10^{2}	3.8×10^{3}	NG

(3) Storage of the inoculated cheeses.

The inoculated cheeses in petri plates were stored in covered fish tanks with a saturated salt solution to keep each cheese close to its initial water activity. The different saturated salt solutions used and the a_w values are given in Table 6. The fish tanks were kept at room temperature. The temperature was recorded with a Bacharach Instrument Co., Tempscribe, with 24 hr movement. The temperature varied between 68°F and 72°F except for day 41 when the temperature reached 78°F. For the first week the fish tanks were placed on the floor of the lab and condensation formed on the inside walls of the tanks allowing mold growth on the outside of the petri plates. The tanks were then raised off the floor and the condensation disappeared.

(a) Color study.

Color change with storage time was measured on a Gardner Color Meter using a white standard.

On the colorimeter, the b scale indicates yellow (+) to blue (-), the a scale indicates red (+) to green (-) and the L scale indicates white (100) to black (0). Fresh slices were cut each time and measured in duplicate.

(b) Meltability.

Samples of each cheese were prepared by cutting a cylinder from the center of a cheese block with a #13 cork

Table 6

Water Activity Chambers for Storage of IMF Cheese

	a_w of cheese	saturated salt solution used	a_w of saturated salt solution used
A	.94	KNO_3	.93 - .94
B	.91	$BaCl_2$	.90 - .91
C	.90	$BaCl_2$	.90 - .91
D	.81	$CdCl_2$	.82
E	.86	Li_2SO_4	.85

borer. This cylinder was then sliced with a kitchen-type egg slicer. The resulting slices were 2 cm in diameter, 0.6 cm thick and weighed about 2.5 grams. The duplicate samples for each cheese were taken from different cylinders. The samples were placed in the center of a glass petri dish with a cover. A piece of graph paper was taped to the bottom of the plate, the outline of the cheese sample traced before and after melting, and the diameter after melting was recorded. All samples were done in duplicate.

A Stabil Therm ^{R} gravity oven (Blue M Electric Co.) was preheated to 155°C. When the door was opened to place the samples in it the temperature went down to ~135°C. The samples were left in the oven for 15 minutes. It took about 10 minutes for the temperature to reach 155°C again.

In order to obtain a measure of the acceptability of the meltability of the IM process cheeses, a comparison was made between their meltability and the meltability of Land O' Lakes American process cheese at different temperature. Duplicate samples (prepared as described above) of each cheese were placed in the oven for 15 minutes, using four different temperature ranges (as described above): 1) 132°C to 152°C; 2) 114°C to 142°C; 3) 110°C to 132°C; and 4) 110°C to 127°C.

(c) Toughness.

Duplicate samples of each cheese were compressed on an Instron Universal tester using an eight-wire grid,

at various time periods during storage. The samples were 5.8 x 2.8 x 3.0 cm
and were deformed 75% of the 5.8 cm dimension at a rate of 5 cm/min.

At day 65 a package of Land O' Lakes
American process cheese was purchased off the shelf of a local store
and duplicate samples of it were tested on the Instron along with the
IM process cheeses. The force vs. distance trace was then used to
determine toughening during storage.

(d) Organoleptic evaluation.

A taste panel was conducted periodically
during the study. The panel consisted of 12 members of the University
of Minnesota Department of Food Science and Nutrition. The panel was
asked to rate the cheese on a 9-point hedonic scale. The evaluation
sheet used by the panel is shown in Table 7.

C. Results and Discussion

1. Microbial growth on inoculated plates.

Mold growth as observed on inoculated plates (five were
prepared for each sample) is shown in Table 8.

Cheese A has shown very little resistance to A. glaucus
or P. roqueforti. In seven weeks all of the A plates had P. roqueforti
growth, even those inoculated with the other microorga_sms. Cheeses
B and C have shown greater resistance to A. glaucus and thus far have
been resistant to P. roqueforti.

Table 7

IMF Process Cheese Form

Indicate rating from:

9 – like extremely
8 – like very much
7 – like moderately
6 – like slightly
5 – neither like nor dislike
4 – dislike slightly
3 – dislike moderately
2 – dislike very much
1 – extremely dislike

SCORE

Attribute	A	B	C	D	E
Texture					
Flavor/Aroma					
Off Flavor (1 – very intense)					
Color					
Overall Acceptability					

Table 8

Mold Growth on IMF and American Cheese

Mold	Cheese	Weeks	# of Plates [b]	Weeks to Sporulation
A. niger	A	2	@	NS
	B	14	4	NS
	C	14	2	NS
	D	14	NG	NS
	E	14	NG	NS
	American	0.5	5	0.5
A. glaucus	A	1	2	14
		2	5	
	B	4	4	NS
		14	4	NS
	C	7	2	NS
		14	2	NS
	D	14	NG	NS
	E	14	NG	NS
	American	0.5	5	0.5
P. roqueforti	A	5	3	NS
		6	5	NS
	B	11	1	NS
		14	2	NS
	C	14	NG	NS
	D	14	NG	NS
	E	14	NG	NS
	American	0.5	5	0.5

@   overgrown with P. roqueforti; hard to differentiate.
(b)   number of positive plates out of 5
NS   no spores
NG   no growth

A. glaucus will grow at an a_w as low as 0.73, but in the IMF cheeses the combination of propylene glycol and potassium sorbate is an effective inhibitor at a_w's below 0.90 (Acott et al., 1976). This combination is probably also inhibiting the P. roqueforti in the a_w 0.90 and a_w 0.91 cheeses. However, this combination of antimycotics did not prevent A. niger growth at high a_w.

The other IMF cheeses have been much more resistant to A. niger as well as the other molds, probably because of the combination of propylene glycol, potassium sorbate and sodium citrate (Acott and Labuza, 1975). In contrast to the IM cheeses, American process cheese has proven as good a substrate for all three molds as the laboratory media used.

Due to the thickness of the molten cheese, the small amount of TTC added, and the inability of the Damrow cooker to mix a small amount of cheese well, the TTC may not have been evenly distributed throughout the cheese in the petri plates. Therefore, as a check on the bacterial growth a most probable number (MPN) procedure for Staphylococcus aureus (AOAC, 11th Ed., 1970) was performed after 5 weeks of storage. The results are shown in Table 9. As seen, the number of organisms increased in Cheese A (a_w 0.94) although TTC reduction was not visible until after nine weeks.

In cheeses B and C (a_w's 0.91 and 0.90) the level of organisms has remained at about the inoculation level, allowing for uneven inoculation on the uneven surface. In Cheeses D and E

Table 9

MPN of _Staphylococcus aureus_ in IMF Cheese
After 5 Weeks Storage at Room Temperature

	MPN/g	MPN/plate	inoculation level cfu/plate
A	>1100	$>10^5$	5×10^3
B	23	3×10^3	5×10^3
C	43	6×10^3	5×10^3
D	<3	$<5 \times 10^2$	5×10^3
E	<3	$<5 \times 10^2$	2×10^3

(a_w's 0.81 and 0.86) the level of Staphylococcus organisms has declined

below the sensitivity of the test. These results are in accord with

the growth minimum a_w of 0.85 for Staphylococcus under ideal conditions

and with the findings of Boylan et al., (1976) that 0.3% potassium

sorbate is an effective inhibitor of S. aureus at a_w 0.90 and pH 5.2.

The level of potassium sorbate in the IMF cheeses ranges from 0.1%

to 0.07% depending on the amount of water in the cheese.

There was no visible sign of Salmonella anatum growth

but no confirming procedure has been performed. According to White

and Custer (1976) the critical parameters for Salmonella growth in

cheese include a NaCl level below 8% and a pH above 5.5. The cheeses

in this study have a NaCl level from 1.9% to 2.5% and pH's slightly

above 5.5. However, the addition of sorbate and the use of propylene

glycol and NFDM to lower the water activity of the cheeses below the

critical level of 0.95-0.96 for Salmonella should prevent Salmonella

growth. There was no visible sign of either Staphylococcus aureus

or Salmonella anatum growth on the American process cheese after one

week incubation.

2. Color.

The readings for the L, a and b scales on the Garnder

Color Meter obtained at two-week intervals are shown in Table 10.

All of the cheeses became slightly less yellow (except E), less red

(except D), and darker during storage. Cheeses A, B, and C are fairly

close to each other in all three paramters. Although D has less

Table 10

Color Change in IMF Cheese During Storage

COLOR METER DATA

Cheese	Day:	13	28	42
A color parameter				
L		73.5	73.4	73.1
a		–	8.6	7.8
b		33.8	27.0	26.4
B color parameter				
L		66.5	67.6	65.9
a		–	8.1	7.4
b		31.9	27.1	26.4
C color parameter				
L		60.4	65.6	62.8
a		–	8.2	7.9
b		27.3	26.8	26.2
D color parameter				
L		63.9	58.3	56.3
a		–	8.5	8.9
b		30.9	23.8	22.9
E color parameter				
L		52.3	50.8	49.5
a		–	7.1	6.8
b		25.2	20.2	20.5

water than E the colorimeter showed it to be lighter and more yellow-red. This is partly caused by the dryness of the C cheese - the cut edge tends to crumble, thus appearing lighter. However, the color changes that have occurred during storage are small for all the five cheeses.

Scofield's equation for estimation of color differences (Scofield, 1943) as outlined by MacKinney and Little, (1962), was used to evaluate the data. The Scofield equation states that color difference $\Delta E = (\overline{\Delta L^2} + \overline{\Delta a^2} + \overline{\Delta b^2})^{1/2}$. Table 11 lists the ΔE values at each time of color measurements. The first readings taken, those at Day 13, were used as the original values except for the a values which could not be measured at day 13 because of instrument malfunction. Further measurements will be necessary to see the overall pattern of color change but, as seen, the changes thus far are slight and probably negligible.

3. Meltability.

The diameters of the cheese samples after melting at different times in the storage period are listed in Table 12. All cheese samples were 2 cm in diameter before melting.

The large amount of lactose added to the cheeses via the NFDM causes them to brown extensively at the temperature ($135^{\circ}C - 155^{\circ}C$) used in the test (Warmbier et al., 1976). Consistently, Cheeses A, B and C melted most. Cheese D did not change shape at all. Cheese E bubbled upwards as much or more than it melted outwards.

Table 11

Total Color Change in IMF Cheese During Storage

Cheese	ΔE Day 13 → 28	ΔE Day 13 → 42	ΔE Day 13 → 74
A	6.8	7.5	6.2
B	4.9	5.6	4.7
C	5.2	2.7	2.5
D	9.0	11.0	10.7
D	5.2	5.5	6.6

Table 12
Change in
Meltability of IM Process Cheese After Storage
(diameter in cm after melting)

Cheese	Day 7	Day 23	Day 37	Day 51	Day 99
A	3.5	3.2	3.2	3.7	5.0
	3.5	3.7	3.7	3.4	3.5
B	3.8	3.6	4.5	3.6	3.7
	3.7	4.0	3.9	3.8	3.8
C	3.7	3.9	3.5	4.3	3.2
	3.8	3.7	3.7	4.0	2.9
D	2.0	2.0	2.0	2.0	2.0
	2.0	2.0	2.0	2.0	2.0

Cheese	Day 1	Day 15	Day 29	Day 43	Day 91
E	2.2	2.5	2.5	2.3	2.0
	2.3	2.7	2.7	2.3	2.0

Storage for 14 weeks has not significantly altered the meltability of any of the cheeses. The diameters of the IMF cheese samples, as well as similar samples of American process cheese, melted at four different temperatures are shown in Table 13. As can be seen, the meltability of all of the IMF process cheeses at high a_w varied directly with the temperature. Temperature differences in the 152°C to 127°C range seemed to have little effect on the meltability of American process cheese or the lower a_w cheeses (D and E).

At the 152°C temperature all of the IMF process cheeses were browned whereas the American cheese was not. IMF process cheeses A, B and C melted more than the American cheese at this temperature. The American cheese was crisper than the IMF cheses, which retained their pliability even when brown. The American cheese thinned out at the melting edges whereas the IMF cheeses did not.

At the 142°C temperature IMF cheese A was not browned and was still soft. IMF cheeses B, C, D and E were all browned and firm. IMF cheeses A and B melted about the same amount as the American cheese and IMF cheese C still melted more. American process cheese was not browned and was only crisp at the thinner, melted edges.

At 132°C IMF cheese A, B and C were not browned. Cheeses D and E were only slightly browned. IMF cheese C melted the same amount as the American process cheese and all the other IMF cheeses melted less. The American process cheese was not browned and was not even crisp at the edges.

Table 13

Meltability of Process Cheeses at Different Temperatures
(cm after melting)

Cheese	132°C to 152°C	114°C to 142°C	110°C to 132°C	110°C to 127°C
A	4.0	3.5	3.0	3.0
	3.9	3.3	3.2	3.0
B	4.0	3.6	3.1	3.1
	4.0	3.4	3.3	2.7
C	4.0	4.4	3.4	2.6
	4.0	4.2	3.6	2.6
D	2.0	2.0	2.0	2.0
	2.0	2.0	2.0	2.0
E	2.3	2.2	2.1	2.0
	2.3	2.1	2.0	2.0
American Process	3.2	3.5	3.6	3.8
	3.9	3.6	4.0	3.4

At the 127°C temperature IMF cheeses A, B and C were not browned, were still soft and had smooth surfaces. IMF cheeses D and E were only slightly darker and firmer than before heating. All of the IMF cheeses melted less than the American cheese at this temperature. The American cheese was not browned, was firm but not crisp and had bubbled on the surface.

4. Toughness.

Figure 6 is a representative Instron curve of the force (calibrated in kilograms) applied vs. the distance compressed (in cm) of an IMF cheese. Table 14 shows the maximum force needed to deform the samples, i.e., the highest point reached on the curve of duplicate samples. Table 15 shows the kilgrams needed to deform the first one-half centimeter of the sample, as a measure of the compressability of the cheese. Cheeses A, B, and C increased only slightly in toughness over the fourteen week period. They increased from about 1 to 3 kg force. Cheese E increased in toughness to an extent equal to a change of 17 kg force and in fact started out eight times as tough. Cheese D seemed to get slightly tougher but the readings varied greatly. Duplicate readings taken on the same day were also very different from each other, indicating a lack of uniformity in cheese D. In fact, it was the toughest of all the cheeses even on day 6. The American process cheese was between IMF cheeses B and C in texture even though its a_w is higher than IMF cheese A.

Figure 6

Force (grams) vs. Distance Curve for Compression and Shearing of IMF Cheese
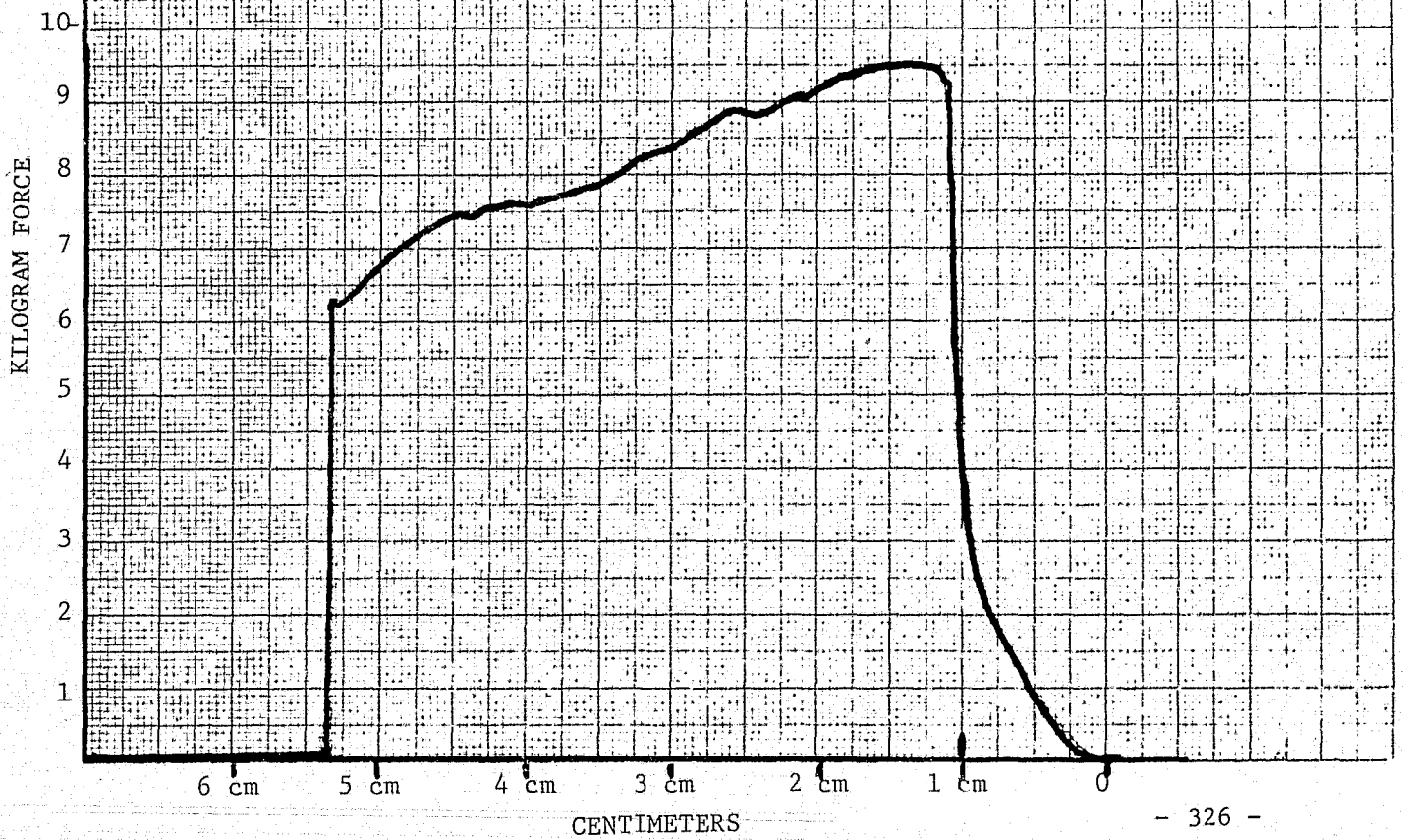
Table 14

Measurement of Toughness of IMF Process Cheese During Storage at 70°F

(toughness units in kilograms force)

Cheese	Days Storage					
	6	21	35	49	65	96
A	3.8	3.5	4.2	4.8	4.5	4.2
	3.8	4.0	3.9	4.7	4.7	5.6
B	6.5	7.1	7.2	6.4	7.9	9.0
		5.5				
	6.2	6.6	6.6	7.8	6.3	7.4
		7.1				
C	11.4	11.0	9.7	11.6	15.4	16.2
	12.0	9.5	13.7	13.2	15.7	13.9
D	52.8	45.5	70.2	60.0	66.8	72.5
	68.8	52.5	64.5	62.0	66.0	69.9

Cheese	Days Storage				
	13	27	41	57	88
E	34.7	35.2	45.0	32.0	49.5
	32.6	35.1	46.0	37.2	49.8
	31.4				

Cheese	Days Storage
	1
Land O' Lakes	9.8
American Process	10.0

5. Organoleptic evaluation.

The taste panel ratings of the cheeses during storage
are given in Table 15. Cheeses A, B and C maintained their acceptability
very well, showing no significant change in ratings during storage
for eleven weeks. Cheese E however, decreased significantly in accepta-
bility after three weeks storage. Cheese D always received a low rating
but got significantly worse during storage time. The texture of D
became very grainy after the fourth week. Comments from the taste
panel members indicate that the cheeses are salty with textures ranging
from soft and moist through rubbery and waxy to hard and grainy.

D. Conclusions

Cheeses A, B and C are very similar in taste, texture, color
and melting qualities. They have also been very stable in these qualities
during fourteen weeks of storage. Cheese C ranks a little better in
taste acceptability than A and B and has more resistance to microbial
growth.

Cheese E is significantly but not unacceptably tougher, darker
and less meltable than the higher water content cheeses. Although
ranked lower by the taste panel it was still acceptable and thus
far has proven resistant to microbial growth. However, it seems to
be aging more quickly than cheeses A, B and C so its shelf life may
not be as long as the shelf life of C.

Table 15

Organoleptic Evaluation of IMF Process Cheese During Storage at 70°F

Hedonic Scale (1 = extreme dislike - 9 = extreme like)

Cheese	Days	Texture	Flavor/aroma	Off-flavor	Color	Overall Acceptability
A	15	6.7	6.9	6.9	6.3	6.7
	29	6.8	6.2	6.8	5.2	6.0
	44	6.3	6.5	7.4	5.9	6.2
	75	6.4	7.1	6.8	6.1	6.8
B	15	6.5	6.4	6.9	7.3	6.3
	29	5.4	5.7	5.8	6.0	5.7
	44	7.0	6.2	6.7	6.8	6.5
	75	7.3	6.9	6.6	7.5	6.9
C	15	6.5	6.5	6.2	7.5	6.5
	29	6.6	6.6	7.1	7.6	6.8
	44	6.9	6.3	7.1	7.3	6.4
	75	6.9	6.6	6.8	6.8	6.3
D	15	2.7	4.6	5.3	5.6	3.3
	29	3.8	4.0	5.1	5.8	3.7
	44	2.0	3.1	5.0	4.9	2.8
	75	1.6	3.6	4.1	2.8	2.0
E	7	4.0	5.7	6.2	5.5	6.0
	21	5.0	6.0	6.5	6.2	6.1
	36	5.3	5.3	5.8	5.7	4.9
	67	4.6	4.6	4.3	4.9	3.9

Cheese D has also been resistant to microbial growth but its taste, texture, color and melting properties are all unacceptable.

Cheeses C and E are the two cheeses with the best combination of qualities thus far. The study will be continued on the cheeses over the next several months.

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F. Summary and Recommendations

A series of IMF cheese food products were made utilizing nonfat milk solids and propylene glycol to lower the a_W. The stability of these cheeses were studied over a four month period at room temperature. The high a_W cheeses (0.91 to 0.94) supported the growth of three molds and Staphylococcus aureus whereas the lower a_W cheeses were more stable. The lowest a_W cheese was stable microbiologically but was not acceptable in terms of color, flavor, texture and meltability. It is recommended that processors can make a stable IMF cheese food in the a_W range of 0.9 to 0.86 by using milk solids, glycol and sorbate.

VII. PREDICTION OF MICROBIAL DEATH DURING DRYING OF A MACARONI PRODUCT

A. Introduction

In Phase III of this contract studies were done to determine the thermal resistance of two pathogens Staphylococcus aureus and Salmonellae anatum as a function of water activity. It was found that they showed maximum heat resistance in the IMF a_w region. Data were collected both as a function of temperature and a_w. In this further study these results were used to predict the death that a pathogen would undergo in real time unsteady state processes, namely pasta processing in which a macaroni dough is dried from an initial a_w of 0.93 to a final a_w of 0.55. The prediction methods are similar to that discussed for vitamin C degradation in Section III.

B. Prediction of Microbial Death During Drying of a Macaroni Product

The following is the paper accepted for publication which summarizes the findings of this part of the study.

Prediction of Microbial Death During Drying of a Macaroni Product

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ABSTRACT

Death of *Staphylococcus aureus* 196E in a semolina-egg dough was studied during a variable temperature simulated drying test. Data for the death rate constant of the organism collected under steady state conditions of constant temperature and water activity were used to predict the amount of death occurring in the unsteady state test. Very good agreement was found. Utilizing the steady state information it was predicted that over 5-log cycles of kill would occur for *S. aureus* 196E and *Salmonellae anatum* NF₃ during the pasta drying process. This indicates that kill caused by the process itself may not be enough if high levels of these pathogens occur initially in the dough.

Recently it was found that among 89 pasta plants manufacturing noodles and macaroni products, only 46% were judged sanitary, 26% needed voluntary clean-up action, and 28% had serious violations [FDA memorandum, July 19, 1974 (AP)]. Lee et al. (8) also reported that routine surveillance by the Food and Drug Administration showed *Staphylococcus aureus* contamination of some pasta products manufactured in the United States. This pathogen and salmonellae pose a danger because of the nature of flour (4), and eggs (1). More importantly, macaroni products are usually not subjected to heating during manufacturing and the drying temperatures are not high. Because of this, some microorganisms will be found in even the most carefully processed macaroni products (10).

The presence of 10^3-10^6 of *S. aureus* per gram in pasta products has been reported by Lee et al. (8). The widespread consumption of these products and the increased number of seizures and recalls which involved microbiologically contaminated products, suggest that extensive investigation is needed to determine the effect of pasta processing on the viability of microorganisms (10).

Extrusion and drying are the major operations in the macaroni industry. In spaghetti extrusion, Walsh et al. (10) found that less. than two log cycles death of *Salmonella typhimurium* resulted at 35 C. At higher temperature (up to 55 C), however, survival was greater probably because of a faster flow rate in the extruder.

Less than one log cycle reduction in *S. aureus* at 35 C extrusion temperature was also reported (11). Only a slight decrease in population was observed when the temperature was increased to 55 C which was the upper temperature limit for extruding spaghetti (10). This shows that microbial death during the extrusion process of pasta making is not significant. Using an experimental dryer in which the temperature was kept at 35 C and the relative humidity was lowered from 95 to 61% in a linear manner over an 18 h period, Walsh et al. (10) found 95% of *S. typhimurium* were destroyed. It should be noted that growth of microorganisms during the early stages of drying was possible because of the optimal temperature (35 C) and the high initial relative humidity (95%) in the drying cabinet. Walsh and Funke (11) recently confirmed that a sevenfold increase in *S. aureus* resulted during a pasta drying operation under the same conditions. Although no enterotoxin was detected, the hazard potential is apparent. Lee et al. (8) showed the potential for growth and enterotoxin formation by *S. aureus* in pasta dough, as well as their persistence in the dried finished products.

The purpose of this study was to predict the death of *S. aureus* for the conditions of an actual drying operation used in a local pasta company. Since it is not feasible to inoculate food pathogens in the large commercial dryer, a simulated process was developed and the experimental results were used to compare with the theoretical prediction.

MATERIALS AND METHODS

Theoretical considerations

Generally, in real food processes one or more of the physical-chemical characteristics, such as temperature and a_w of foods will undergo continuous changes. If temperature and a_w are the only two parameters that change with time during processing and if the heat resistance or death rate as a function of a_w and temperature is known for a certain microorganism, then prediction of death for this organism during processing is possible for food processes in which changes in both temperature and a_w are known.

Prediction of the microbial death is based on the assumption that the microbial death follows a first order reaction, i.e.

$$\frac{dN}{dt} = -kN \tag{1}$$

or

$$\frac{d\ln N}{dt} = -k \tag{2}$$

where

t = time of heating
N = number of survivors at time t
k = death rate constant

The death rate constant k, can be determined experimentally at certain levels of a_w and temperature, which are of interest, as was shown by Hsieh et al. (3). For other a_w values and temperatures incurred in real food processes, k must be estimated from these experimental data. One way to do it through the use of the Arrhenius equation,

$$\frac{d\ln k}{dT} = \frac{E_a}{RT^2} \tag{3}$$

where

T = absolute temperature
E_a = activation energy of microbial death
R = universal gas constant

$$\frac{d\ln k(a_w, T)}{dT} = \frac{E_a(a_w)}{RT^2} \tag{4}$$

Equation [4] stresses the fact that k is a function of both a_w and temperature while E_a is a function of a_w which is true only within a narrow range of temperature. Upon integration, equation [4] gives,

$$K(a_w, T) = k_0(a_w) \exp [-E_a(a_w)/RT] \tag{5}$$

The constants k_0 and E_a for a certain a_w can be calculated when the death rate constant k, is known for more than two different temperatures. This has been assessed for *Staphylococcus aureus* 196E and *Salmonella anatum* NF³ in semolina-egg medium (5). These constants are shown in Table 1 and Table 2 for *S. aureus* 196E and *S. anatum* NF_3, respectively. Substituting equation [5] into equation [2] leads to:

$$\frac{d\ln N}{dt} = -k_0(a_w) \exp [-E_a(a_w)/RT] \tag{6}$$

An integration of equation [6] gives,

$$\log (N_t/N_0) = \frac{-1}{2.3} \int_0^t k_0(a_w) \exp [-E_a(a_w)/RT] \, dt \tag{7}$$

N_0 = initial number of cells

Nt = number of survivors at time t

This is a general equation in which the amount of organisms surviving a process can be predicted if the following information is available (a) a_w of food versus time and (b) product-temperature versus time.

Cultures

S. aureus 196E was obtained from Sita R. Tatini, Tepartment of Food Science and Nutrition, University of Minnesota. *S. anatum* NF_3 was selected from the Culture Collection of the same Department. They were grown in 100 ml of TSYB [Trypticase Soy Broth (BBL) in which 0.5% of Yeast Extract (BBL) was added], with glass beads and incubated at 37 c for 24 h with shaking.

Solid media

The solid media was composed of semolina (Como No. 1 Semolina,

Capitol Durum Division, International Multifoods Corp., Minneapolis, MN), whole egg solids (A. J. Pietrus & Sons Co., Sleepy Eye, MN 56085) and distilled water. Semolina and whole egg solids were mixed first in the mixing bowl of a Brabender Faringraph (C. W. Brabender Instruments, Inc., South Hackensack, NJ) and various amounts of water were added such that a_w values of the solid medium varied from 0.5 to 0.92. The a_w was measured by the Vapor Pressure Manometer technique as described by Labuza (7). The solid medium was not sterilized so that its characteristics would not be changed. Counts made on the TSYB agar used in the actual study showed less than 10 organisms per gram. Since a large inoculum was used for the death study, it was felt that interference would not be a problem.

Simulated food process

Because of the difficulty in predicting the a_w during drying, a simulated process was developed in which temperature was the only variable. This was done through use of the mixer bowl of a Brabender Faringraph heated by a Haake FK2 Constant Temperature Circulator (Haake Instruments, Inc., Saddle Brook, NJ). The solid medium of a certain a_w value was prepared and placed into the mixer bowl. The temperature of the medium was monitored with thermocouple probes spaced throughout the bowl and was continuously recorded. The mixer was started at high speed and water at 40 C was circulated through the jacket of the mixer bowl. One ml of organisms from TSYB was then inoculated into 99 g of solid medium to give an initial population of 10^6 to 10^7 CFU/g. When 5 to 6 min had elapsed which allowed uniform distribution of cells to about 10^5 per gram, two steps were done simultaneously: (a) Approximately 1 g of the sample was taken out to estimate the initial population of organisms of TSYB agar. All counts were done at 37 C after 24 h. (b) The setting of temperature of the water bath circulator was changed to 75 C so that the product would now heat up linearly. At appropriate time intervals, about 1 to 2 g of sample was removed with a sterile tongue depressor and weighed into a blender jar which contained 99 ml of sterile 0.1% peptone water (DIFCO). The exact weight of the sample was recorded. The sample was then blended well and plated immediately by the surface spread technique on TSYB agar. The survivors were estimated by enumerating these plates after 24 h incubation at 37 C. It should be noted that prolonged incubation up to 48 h did not affect the number of survivors estimated.

RESULTS AND DISCUSSION

Simulated food process

A solid medium (semolina-egg) of a_w 0.87 was chosen. Figure 1 shows how the temperature of this medium in the mixer bowl of the Brabender Faringraph increased with time when the temperature regulator of the circulating water bath was changed from 40 to 75 C. A linear increase in temperature was observed after about 3 min for the center or 2 min for the wall of the mixer bowl. After 21 min, the temperature rise was no longer linear. The increase in temperature for the center and the wall from 3 to 21 min can be approximated by,

$$T_c = (73 + 2.5 \times (t-3)) \times 5/9 \tag{8}$$

$$T_w = (77 + 2.5 \times (t-3)) \times 5/9 \tag{9}$$

where

T_c = center temperature in C

T_w = wall temperature in C

t = time of heating in minutes

It should be noted that the mean average temperature of the solid medium is not uniform but falls between T_c and T_w. Thus, both temperatures were used to calculate the upper and lower limits of death. To predict death,
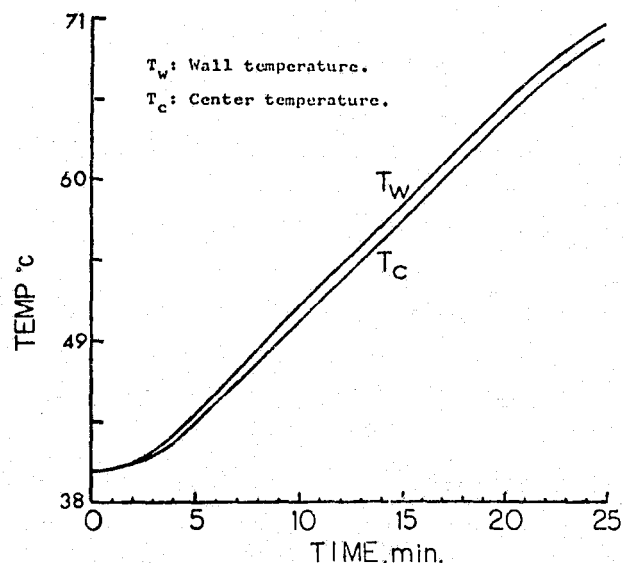
Figure 1. *Center and wall temperature of semolina-egg dough mix as a function of time during heating and mixing.*

equation [7] was integrated numerically using k_0 from Table 1 in the case of *S. aureus* 196E or k_0 from Table 2 for *S. anatum* NF$_3$. Figure 2 shows the prediction of the
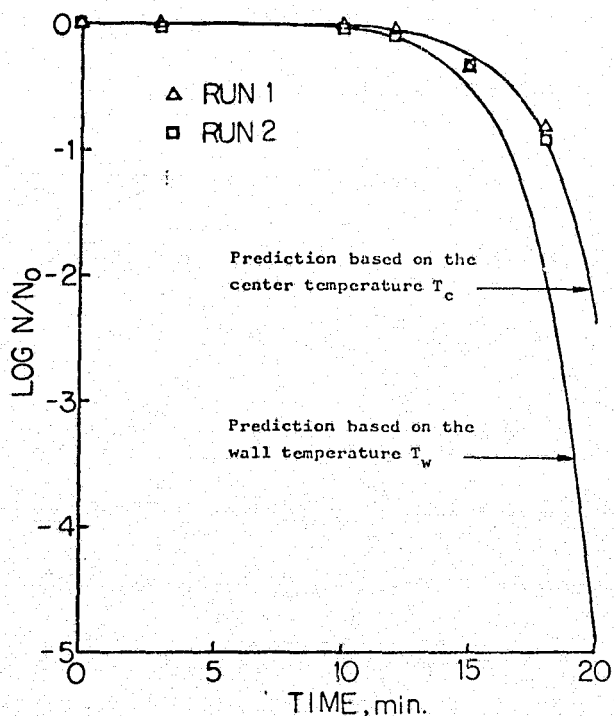

Figure 2. *Predicted (lines) and actual death ($\triangle$, $\square$) of S. aureus 196E during unsteady state heating process in semolina-egg dough.*

death of *S. aureus* 196E in a solid medium of a_w 0.87. The upper curve was estimated from the lowest temperature T_c. Experimental results are also shown in this figure. In general, they are within the upper and lower limits as expected. Similar results for *S. anatum* NF$_3$ are shown in Fig. 3. It should be noted that most of the destruction

TABLE 1. *Kinetic constants for death rates of* S. aureus *196E in semolina-egg at various* a_w *values* (3)

$$\frac{2.3}{D} = k = k_0 e^{E_a/RT}$$

a_w	Death rate absolute constant k_0 min^{-1}	Activation energy E_a kcal/mole
0.61	7.56×10^{30}	48.5
0.76	3.74×10^{31}	49.9
9.80	2.67×10^{34}	54.6
0.83	3.72×10^{33}	52.2
0.85	8.26×10^{42}	66.1
0.87	6.04×10^{47}	73.2
0.92	1.52×10^{50}	76.6

TABLE 2. *Kinetic constants for death rates of* S. anatum *NF$_3$ in semolina-egg at various* a_w *values* (3)

$$\frac{2.3}{D} = k = k_0 e^{-E_a/RT}$$

a_w	Death rate absolute constant k_0 min^{-1}	Activation energy E_a kcal/mole
0.61	4.52×10^{42}	65.3
0.76	3.69×10^{44}	68.2
0.80	9.04×10^{43}	67.4
0.83	7.30×10^{51}	78.9
0.85	5.97×10^{46}	71.2
0.87	1.07×10^{48}	72.9
0.92	7.50×10^{56}	85.6

k = death rate constant
D = min for 90% destruction
T = temperature in °K
R = 1.986 cal/mole °K

occurred during the final stage of heating. This is not surprising since a temperature at which death starts to occur is not reached until 10 to 12 min have elapsed as shown in Fig. 1. An examination of Fig. 2 and Fig. 3 also reveals that the experimental results are better fitted by the prediction based on the center temperature of the medium. This is probably because the center temperature is a better representation of the median temperature of the medium. Analysis was not done beyond 1 to 2 log cycles since at that point death was so rapid, counts could not be determined accurately with the amount of sample available.

Microbial death during macaroni drying process

Table 3 shows the drying conditions utilized for various pasta prodcuts produced at the Creamette Company, Minneapolis, Minnesota. The change in a_w of the product can be determined by direct measurement of the product, before and after each drying stage. The first product, G.M. Macaroni, listed in Table 3, was chosen for prediction. Samples were secured and the a_w and moisture content were measured by the Vapor Pressure Manometer and vacuum oven method, respectively. It was assumed that the decrease in a_w during drying is linear. The temperature history of the product during drying, however, is very difficult to assess. Because of the thinness of the elbow macaroni, it was assumed that
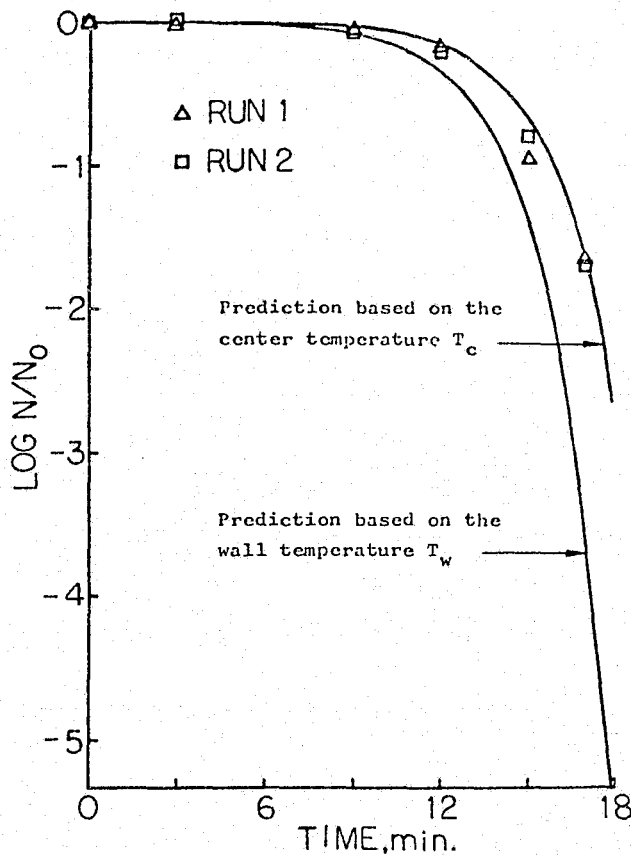
Figure 3. *Predicted (lines) and actual death* ($\triangle$, $\square$) *of* S. anatum NF_3 *during unsteady state heating process in semolina-egg dough.*

there is no temperature gradient within the product itself. In the beginning of preliminary drying, Charm (2) suggested that the surface of the product reaches the wet-bulb temperature of the drying air immediately. The temperature of the product will be maintained at this level till the end of the constant drying period (9). The product temperature will then increase when it enters into the falling rate drying period and gradually reaches the dry-bulb air temperature (2, 6, 9). It was assumed that the increase in temperature is linear and that the temperature reaches the dry-bulb air temperature at the end of preliminary drying. It was further assumed that the product will be maintained at the lower dry-bulb air temperature throughout the finishing drying stage. Both dry-bulb and wet-bulb measurements were taken for each stage.

Husain et al. (6) observed that for the entire drying process of potato tuber slices, 5 cm in diameter and 0.25 cm thick, only the falling rate period occurred with no constant rate period. Earle and Rogers (3) reported, however, that a constant rate period took place when the free moisture content of sheet macaroni was above 15% With respect to elbow macaroni, they found that the falling rate prevailed when the moisture content of the product was lower than 32% (dry basis). The moisture content of our product was decreased from 42.0% (dry basis) to 18.9% in the preliminary drying stage. This was further lowered to 11.3% after finishing drying. It was assumed that the decrease in moisture was also linear with drying time and the constant drying rate period

TABLE 3. *Drying conditions for various macaroni products in a typical finishing dryer*

Creamettes Company product names®	Preliminary dryer (0.5 h)			Finishing dryer (5 h)		
	Air temperature, C		%RH[a]	Air temperature, C		%RH
	Dry bulb	Wet bulb		Dry bulb	Wet bulb	
G. M. Macaroni	60.6	53.9	70	55.7	51.1	79
G. M. Spaghetti	63.9	49.4	49	54.4	50.8	80
Creamettes	63.3	55.8	70	55.7	51.4	80
R. C. Spaghetti	62.8	52.2	60	54.4	50.8	80

[a] % relative humidity = $a_w \times 100$

TABLE 4. *Prediction of the destruction of* S. aureus *196E in G. M. Macaroni during drying from the projected* a_w *temperature profile*

Time t min.	Water activity of product a_w	Temperature of product T C	Death rate constant k min^{-1}	kΔt	ΣkΔt	Log cycles death $\log_{10} \frac{N_t}{N_0}$
0	0.93	53.9	0.11	0	0	0
6	0.91	53.9	0.096	0.576	0.576	-0.250
12	0.88	53.9	0.083	0.498	1.074	-0.467
15	0.87	54.7	0.095	0.285	1.359	-0.591
18	0.86	55.9	0.125	0.375	1.734	-0.754
21	0.85	57.0	0.148	0.444	2.178	-0.947
24	0.84	58.2	0.165	0.495	2.673	-1.162
27	0.83	59.4	0.170	0.510	3.183	-1.384
30	0.82	60.6	0.135	0.405	3.588	-1.560
45	0.805	55.7	0.020	0.30	3.888	-1.690
60	0.79	55.7	0.016	0.24	4.128	-1.795
90	0.765	55.7	0.023	0.69	4.818	-2.095
120	0.74	55.7	0.026	0.78	5.598	-2.434
180	0.68	55.7	0.031	1.86	7.458	-3.243
240	0.63	55.7	0.036	2.16	9.618	-4.182
300	0.58	55.7	0.040	2.40	12.018	-5.225
330	0.55	55.7	0.043	1.29	13.308	-5.786

takes place when the mosisture content of the product decreases from 42% to 32%. After that, the falling rate prevails. The projected changes based on initial and final values in a_w moisture content and temperature of the product are summarized in Fig. 4.
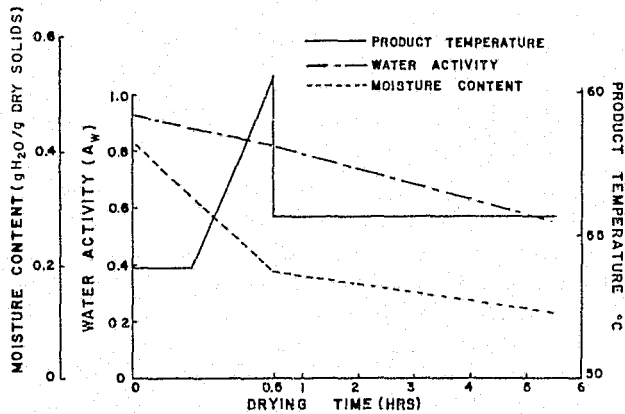

Figure 4. *Conditions of water activity, moisture content, and temperature of a typical pasta product during drying based on predicted and actual measurements.*

To predict the decrease in viable cells occurring, the procedure was as follows:

(a) The a_w and temperature of the product at increments of the drying time Δt is found from Figure 4.

(b) The death constant k corresponding to the particular a_w and temperature was derived from Table 1 or 2 (or a plot of k vs. a_w and T).

(c) Equation [7] was solved with this data for the small time interval Δt and the population decrease was found.

(d) The procedure was repeated for the next Δt and the death was summed.

The results are given in Table 4 for *S. aureus* 196E. As seen, about one and a half lograithmic cycles of death will occur within the first half hour of preliminary drying. Four more log cycles of death will result from the subsequent finishing drying. Based on the constants for *S. anatum* NF_3 there would be about six log cycles decrease in this same time period. This suggests that if

initial populations are high enough pathogens can survive the process and might result in product seizure. Although these calculations are based on the measured a_w and temperature at the start and finish of each stage, assuming that the engineering parameters hold as discussed above the death predictions should be valid and would indicate the safety of the process. Thus if counts are initially high normal pasta drying will not be sufficient to make the product safe.

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C. Summary and Recommendations

This study shows that use of steady state kinetic data for death of microorganisms can be used successfully to predict death during an unsteady state heating process in which both temperature and a_w change. Based on this six log cycles of death would occur for $\underline{S}$. $\underline{aureus}$ and $\underline{S}$. $\underline{anatum}$ NF_3 during pasta processing.

VIII.   WATER BINDING OF MACROMOLECULAR HUMECTANTS

A.   Introduction

The ability of macromolecular humectant systems to hold water is a well known phenomenon. However, when compared to the water binding properties of low molecular humectants (salts, polyols and sugars) which were studied in the early phases of this investigation (Sloan and Labuza, 1975, 1976a, 1976b), it is a very poorly understood effect.

Traditionally this effect is known as water binding capacity or water holding capacity. The ill-defined term "bound" water is usually referred to as the portion of water in a system which exhibits properties different than "bulk" or "free" water. Some physical characteristics of this "bound" fraction include: lower vapor pressure, high binding energy as measured by dehydration, reduced rotational mobility as measured by dielectric experiments, reduced mobility as seen by nuclear magnetic resonance (NMR), unfreezability even at temperatures below $-40^\circ C$, unavailability as a solvent and water which moves with a macromolecule in experiments dealing with viscosity, sedimentation or diffusion.

Each of these characteristics has been used to define bound water. However, each criteria specifies water which is held with a discrete maximum energy which is different from all other criteria.

Therefore, quantitation of a bound water fraction varies greatly from one worker to another depending on which defining parameter is used. Due to the complexities of the binding forces involved no universal definition of bound water has been adopted.

Water may be associated with macromolecular systems (such as food materials) as (1) strongly chemically bound water, such as water of hydration salts, (2) water adsorbed on hydrophilic sites by weak hydrogen bonding forces, (3) water imbibed or held by capillary forces between surface or in a gel network, (4) structured water in solution or suspension held by long range physical forces close to macromolecular surfaces, and (5) imbibed water trapped within the macromolecular amorphous network due to super saturation of dissolved species. Among these types of forces the most unusual and ill-defined one is the water held in gel systems by capillarity. Some gels can be formed with less than 0.1% of solids forming a plastic solid material. Little or no water leaks out of these solids upon standing, except by evaporation or upon collapse of the material. The nature of the attractive forces involved has not been extensively explored and is difficult to isolate due to the other water binding forces.

In the earlier studies on water binding in gels, determination of bound water was based mainly on its unfreezability (Kuprianoff, 1958). The freezing point depression method (Briggs, 1931, 1932) and dilatometry, which measures the volume change upon freezing (Moran, 1926; Jones and Gortner, 1932), have been used to measure the amount of unfrozen water below the normal freezing temperatures.

In recent years, more sophisticated techniques have been employed to measure the bound water. Among these are the measurement of latent heat of fusion (Mrevlishvili, Privaloa, and Moy et al., 1971), freeze-drying (Gentzler and Schmidt, 1975), differential thermal analysis (DTA) (Duckworth, 1971; Parducci and Duckworth, 1972), and differential scanning calorimetry (DSC) (Berlin et al., 1976). DTA and DSC involve measuring the thermal energy of a sample with respect to an inert reference substance. As the temperature is scanned through the freezing point, the frozen sample begins to melt and consequently liberates latent heat of fusion. Appearance of an energy peak at the freezing point indicates the thawing or melting process. The amount of unfrozen water can be determined using samples of various moisture content (Duckworth, 1971).

Rockland (1969) and Labuza (1974, 1975) have used the measurement of vapor pressure lowering as defined by water activity (a_W) to quantify water binding. In this case the vapor pressure above a macromolecular system is measured directly. Both have correlated the a_W lowering with effects on reduction of chemical reaction rates in the adsorbed water phase. At very low a_W the water is tightly bound as measured by the other previously metnioned techniques, and reaction rates are negligible. At high a_W the rates are comparable to those in bulk water. What is interesting as pointed out by Labuza (1976) is that at high a_W (>0.99), although the water behaves as a bulk solution, it does not leak out of the gel but is held by other forces, probably related to capillarity.

Sussman and Chin (1966), and Toledo et al., (1968), used NMR to quantify the amount of bound water in cod muscle and wheat flour respectively. Their methods are based on the fact that ice does not contribute to the NMR signal because of its very broad resonance line. The abrupt change in signal at the freezing temperature can be used to determine the amount of liquid water that remained in the system. However, these authors did not take into account the temperature dependence of the NMR signal, resulting in erroneous interpretation of the results (Steinberg and Leung, 1975). Recently Belton et al., (1972, 1973), used the pulsed NMR technique to determine the amount of unfrozen water by measuring the loss of signal as free water is converted to ice. They found that 20% of the water in frog muscle remained mobile down to $-80^\circ C$.

Hanson (1975) and Okamura (1973) used NMR, freezing point depression and water vapor pressure depression as measured by water activity (a_w) to quantify water binding at low moisture contents. They did not apply these techniques to higher moisture levels where capillary forces should be significant (Labuza, 1975).

Dielectric absorption measurement which reflects the rotational state of water has been applied to water binding studies recently. Bound water shows properties intermediate between ice and free water. Brey et al., (1969), reported that the bound water content of proteins ranged from 0.05 to 0.10 g/g solid. Roebuck et al., (1972), found that the dielectric constant and dielectric loss factor increased

sharply at 30% water in granular potato starch and at 17% water for
gelatinized starch. However, a recent article reported that the
dielectric properties of agar/water gels at 0.5, 1 and 2% solids are
similar (Roebuck and Goldblith, 1975). This indicates that the water
in the gels is largely free and mobile while at the same time is being
held within the system.

Infrared (IR) has also been applied to the study of water
binding. Falk et al., (1970) concluded, from the IR spectra of hydrated
and partially deuterated films of DNA, that the water in the hydration
shell is not "ice-like." Hansen and Yellin (1972) observed two bound
water fractions and a liquid-like fraction in hydrated human stratum
corneum by IR.

Among all the modern techniques used in water binding studies,
NMR appears to be the most promising method. In general, NMR can be
categorized into continuous wave (cw) and pulsed. The cw NMR detects
the water signal, while the pulsed NMR measures the relaxation rate
of the water protons.

Studies on agar gels (Hecther et al., 1960) and starch gels
(Collison and McDonald, 1960), by cw NMR have yielded resonance lines
significantly broader than that of pure water. The former authors
attributed the line broadening to an overall decrease in the mobility
of water molecules. The latter, however, explained the results in
terms of rapid exchange of water molecules between a large free water
fraction and one or more bound water fractions with restricted mobility.
Recent studies seem to favor the latter interpretation.

Using deuterated water Cope (1969) reported that the resonance peak height of D_2O in agar gel decreases significantly with agar concentration. In gelatin gels however, the change in peak height was small. The result seems to indicate that the mobility of water is restricted more in agar gels than in gelatin gels. Using wide-line NMR, Duckworth (1971) found that the calibration curve for agar gels relating a hydrogen resonance signal to water content was shifted further along the moisture axis from the origin than with those of starch and gelatin. This implied that the agar immobilized water to a greater extent compared with the other two colloids. Unfortunately the author did not give any explanation of the results in terms of NMR theory.

Pulsed NMR is useful in measuring such quantities as nuclear relaxation times and self-diffusion coefficients of water in different systems, which can be related to the molecular mobility of water. Several relaxation studies have been conducted on water in gels. Wossner and Snowden ((1970) found that the large relaxation effects, i.e., reduced T_1 and T_2 were due to a small fraction of the water molecules (<1% in a 10% agar gel) which interact strongly with the agar molecules. Based on the proton and deuteron magnetic relaxation data of water in agarose and carageenan gels, Child et al., (1970) concluded that the decrease in T_2 of water in the gels was due mainly to exchange between polymer hydroxyls, bound water and bulk water.

Recently the self-diffusion coefficient of water has been used to interpret the water mobility in biological systems. It has the advantage over the relaxation times in that it is a measure only of the translational motion of the water molecules and therefore appears to be a more direct parameter than T_1 and T_2 for studying the mobility of water. Wossner et al., (1970) reported that the self-diffusion coefficient of water in 8% agar gel and 10% gelatin gel was similar to pure water under the same experimental conditions. Therefore, the motion of most water molecules was unaffected by the macromolecules. However, the self-diffusion constant measurements of water in egg yolk, egg white and egg albumin solution indicated that a significant portion of the water has reduced translational mobility compared with bulk water (James and Gillen, 1972).

The problem of how water is held by macromolecules is one of great interest and importance. This is especially true in the development of intermediate moisture fabricated foods since these molecules hold more water than ideally predicted by Raoult's Law. Despite the numerous studies conducted on the water binding properties of these systems, the exact nature of the forces involved in holding water in gels is still largely unknown. The most recent studies indicate that the water held inside a gel is very similar to free water and is largely unrestricted. However, there are measurable differences between bulk water and that held in gels.

Many of these macromolecules are being applied to the development of intermediate moisture foods. A more complete understanding of the mechanisms involved in water holding by gel systems will lead to new and more exact uses of the macromolecules.

This research will be directed toward defining some of the mechanisms involved in holding water in gels in the IMF water activity range. Three macromolecules, gelatin, agar and carageenan, will be employed in the investigation. These systems have been selected because they form plastic gels at low concentrations, they are thermally reversible and they are easily prepared. From these systems it will be quite simple to expand the work into the binding mechanisms of other macromolecules such as starches and other gums.

B. Methods

1. Gel preparation

a. Initial gels

Two methods were used to make standard gel concentrations. For gelatin and carageenan gels dry macromolecules were added to deionized distilled water at 55-60°C. The samples were held at that temperature and stirred with a magnetic stirrer until complete solubilization was achieved. After solubilization, the solutions were held at 60°C in a water bath to allow escape of entrapped air.

Agar was prepared using the standard microbiological technique of adding dry sample to cold deionized-distilled water then heating for 10 minutes in a steam atmosphere, mixing by swirling the solution and heating an additional 5 minutes. To insure a uniform cooling rate all samples were cooled in a water-ice bath at 0°C.

b. Special gel preparation procedures.

The greatest problem when dealing with gel systems is in maintaining the structural integrity of the system during measurements. Toward this end a number of methods were developed to adapt various measuring techniques for work with gels.

Two methods were investigated to make very concentrated gel systems for NMR and DTA/DSC analyses. The first was substitution of water. By serial substituion water in a gel may be replaced by carbon tetrachloride. The steps involved include:

1) Soaking the gel in absolute methanol thus replacing water with methanol.

2) Substituting benzene for methanol in a stepwise-fashion. This involves dilutions of methanol:benzene at 1:4, 1:1, 4:1 and pure benzene.

3) Substituting CCl_4 for benzene.

To insure equilibration, samples were held in each solution for 24-48 hours.

This stepwise substitution should lead to gels which still have the original structure but which contain only the most tightly held water. This water may then be detected by both NMR and DTA or DSC since CCl_4 does not contribute signal to NMR and has a freezing point of $-22.9°C$.

This type of study can be expanded to include substitutions with increasingly larger molecules, giving an indication of the permeability of the gel systems.

The second method for making very concentrated gels is being developed. It includes partial drying, reheating to liquify the gel then resetting the gel. With the exception of gelatin the systems under study cannot be made up from a dry powder at concentrations greater than about nine percent. To circumvent making them up from powder the gels will be made at 4% then dried for varying lengths of time in a freeze drier. After treatment the gels will be removed to evacuated chambers to equilibrate for 24 hours. They will then be melted and cooled to reform a uniform gel. The resulting product may then be used for subsequent analyses.

2. Isotherms

Desorption isotherms are being developed for the three gels mentioned as well as for xanthan gel. These isotherms will accent the high a_w levels, thus points will be developed at 0.98, 0.97, 0.95, 0.93, 0.91, 0.88, 0.75, 0.68, 0.52, 0.44, 0.33 and 0.11. The systems

are all microbially unstable. It is necessary therefore to handle the gels as aseptically as possible. Also to insure that the low a_w samples cannot spoil equilibration will be conducted under refrigeration at 4°C. The static isotherm points will be supplemented by VPM measurements. Points will be developed using fresh gels at concentrations ranging from those necessary to attain an a_w of 0.99 to concentrations as high as may be achieved when making the gels from dry powders (50-60% for gelatin and 8-9% for the polysaccharides).

3. Gel strength

Gel strengths are also being studied. Each of these substances form a stable gel at about 0.5% and up. They therefore readily lend themselves to analysis of apparent viscosity by the Brookfield rotational viscometer method. This method measures the force required to move a wire of given diameter and length through a gel at a given speed. These analyses will be complimented by shear and puncture force measurements using an Instron Universal tester. This is required since each gel exhibits fracturing at high concentrations when analyzed by the Brookfield technique.

4. Pulsed NMR measurements

A Praxis PR-102 pulsed NMR spectrometer equipped with a Tektronix type RM503 oscilloscope and a Tektronix oscilloscope camera will be used in this study. The instrument was modified to handle the Carr-Purcell pulse sequence (Farrar and Becker, 1971). For freezing

experiments, sample temperature will be controlled by gas flow passage around samples using a home-built variable temperature controller.

The gel sample will be transferred to a 10 mm NMR tube while it is in liquid form. Adequate time will be allowed for the gel to set before any measurement is taken.

For T_1 measurement, a repeated 180°, T, 90°, pulse sequence will be applied, where T is the time between the two pulses. The amplitude after the second pulse can be obtained from the digital readout. Alternatively, the signal may be measured directly from the oscilloscope. Readings will be taken for different variable delay (T) values. Log $(M_O - M_z)$ is plotted versus T, where M_O and M_z are the amplitudes immediately after the first and the second pulses, respectively. The slope of the straight line obtained is equal to

$$- \frac{1}{2.301 \ T_1} \quad \text{according to} \quad \ln(M_O - M_z) = \ln 2 \ M_O - \frac{T}{T_1}$$

For T_2 measurement, the Carr-Purcell technique will be employed. In this case, a 90°, T, 180°, 2T, 180°, 2T, sequence is used. The echoes formed at 4T, 6T, 8T, etc. can be recorded or photographed with the use of an oscilloscope and a camera. It has been shown by Carr and Purcell (1954) that the effect of diffusion can be virtually eliminated by choosing T short enough (e.g., 1 msec) and the intensity of the echo at time 2T is determined by:

$$\ln A \ (\text{echo at 2T}) = \frac{-2T}{T_2} + \ln M_O$$

where M_O is the amplitude after the first pulse. A plot of log A versus T yields T_2.

Since the pulsed NMR does not detect any signal coming from ice, it provides a useful tool for the determination of unfreezable water. The amount of liquid water in the sample at subfreezing temperatures can be determined from the free induction decay (FID) of the proton signal. However, the signal must be corrected for two important factors, namely, the contribution of the observed signal from the macromolecular protons and the temperature effect. The liquid water signal can be obtained by subtracting the signal of dried solid from the total observed signal. As for the temperature effect, the Boltzmann temperature correction will be approximated by dividing the signal by the absolute temperature (Burke et al., 1975). Based on the above discussion, the amount of unfrozen water (L_T) in the gel sample may then be obtained from:

$$L_T = \left(\frac{A_T}{A_O}\right) X \left(\frac{273}{T}\right) L_O$$

where

A_T = signal at subfreezing temperature T (corrected for dry solid signal)

A_O = signal of liquid water at $0^O C$

T = absolute temperature of the sample

L_O = water content of the gel sample

To avoid supercooling of the free water, liquid nitrogen may be used to induce the freezing process.

5. Gel porosity

In order to investigate the effect of temperature and
concentration on the porosity of gels migration studies will be
conducted. Initial studies will use Red Dye #2 in water and the
gels previously mentioned. The movement of dye through the gels will
be measured at 4, 22 and 35°C and at various gel concentrations depending
on the gel system.

If these preliminary studies show significant differences
in gel type, concentration and/or temperature the pore sizes will be
further characterized using solutes of differing molecular size.

C. Results and Discussion

1. Solvent substitution

Two experiments were conducted to test the effectiveness
of substituting carbon tetrachloride for water in gelatin gels.

In the first experiment the size of gelatin cubes were
measured as a function of solvent. Gelatin gel at 8.2% (dry basis)
was made up and cut into approximate one cm squares. Eight samples
were measured and the total volume of the samples compared after each
treatment.

The initial substitution of water with methanol produced
no measurable change in volume as seen in Table 1. The texture changed

Table 1

Gel Substitution - Volume Changes

Solvent	ΔV	Appearance
$H_2O \rightarrow$ methanol	no change	firm plastic, translucent
methanol $\rightarrow$ 1:4 benzene:methanol	+10%	firm plastic, translucent
1:4 $\rightarrow$ 1:1 benzene:methanol	+ 5%	firm plastic, translucent
1:1 $\rightarrow$ 4:1 benzene:methanol	−5.2%	rigid glassy, transparent
4:1 $\rightarrow$ benzene	−2.8%	rigid glassy, transparent
benzene $\rightarrow$ CCl_4	−1%	rigid glassy, transparent

however, from soft plastic to firm plastic. The change in texture
was not measured quantitatively but has been observed by other workers
who characterized water as a plasticizing agent in gels.

The second series of substitutions with a solvent
mixture of 1:1 methanol:benzene initially gave a volume increase
then a decrease in size. This was accompanied by another texture
change - from firm, plastic to brittle, glassy.

These phenomenon may be accounted for if the gel is
characterized as a cross-linked macromolecular structure with finite
bond lifetimes. When the gel is plastic and able to exchange inter-
molecular bonds it will swell and adapt to the large benzene molecules.
As the benzene concentration increases and the gel no longer exhibits
the exchange phenomenon it becomes a "solid-porous" type of material.
Increasing the benzene concentration past this point causes methanol
dehydration and collapse of any pores too small to accomodate benzene
molecules.

Since the molecular volume of carbon tetrachloride is very
similar to that of benzene very little volume change would be expected
during this substitution. The data of Table 1 supports this hypothesis.

Further work is planned to characterize the change in
plasticity of the gel upon substitution. This will be accomplished
by measuring the modulus of elasticity (with a Universal Instron Tester)

and by characterizing any chemical changes in the gels upon substitution.

The pore size of the glassy structure will also be characterized using dye migration studies or capillary suction potential studies.

The effectiveness of the solvent replacement procedure was also measured by NMR. Table 2 gives the peak height of the FID signal for samples dried by vacuum oven, hydrated and freeze-dried and hydrated then solvent dried. The data is given in arbitrary units (mm of maximum peak height on an oscilloscope) but it is evident that complete dehydration may be achieved while maintaining most of the gel structure.

The residual signal of the solvent dried sample may be attributed to hydrogen atoms of gelatin proteins. The difference between the solvent and freeze-dried samples may be due to either a more complete extraction or to the formation of macromolecular cross-links in the solvent sample giving less hydrogen mobility.

2. Isotherm data

Static isotherm data is being collected. This will continue with weighings at three week intervals until the samples attain a constant weight.

Table 2

FID Signal of Dried Gelatin and Agar

Sample	Treatment	Signal (mm)
gelatin	substituted with CCl_4	2
gelatin	not hydrated, vacuum oven dried	15
gelatin	hydrated, freeze dried	8
agar	hydrated, freeze dried	4

VPM data was collected for both gelatin and agar, using the VPM procedure in Section VA. The data is tabulated in Table 3 along with the approximate expected value for gelatin in Table 4. The expected values were derived using theories developed by Flory and Huggins (1942 a,b,c) for the effect of macromolecules on vapor pressure depression. This effect may be calculated for solutions using the equation:

$$\ln a_1 = -\theta_2 \left(\frac{V_i}{V_2}\right) - \left(\frac{\theta_2^2}{2}\right) - \left(\frac{\theta_2^3}{3}\right) \ldots$$

where a_1 is the activity of the solvent, θ_2 is the volume fraction of the solute, and V_i is the molar volume of the ith species. They found a good fit to macromolecular solutions at concentrations where formation of a uniform latice would be expected.

There is no pattern evident from the experimental data. This is due mainly to lack of adequate temperature control in the VPM. Fluctuations around the sample of $\pm 2^\circ F$ causes condensation inside the VPM during measurements at high a_w's. This problem is being solved by placing the manometer into an isothermal enclosure. When the VPM environment is controllable to within $\pm 0.1^\circ F$ the measurements will be repeated.

3. Viscosity data

Brookfield data has been developed for solutions of gelatin and agar. It is summarized in Figures 1, 2, 3 and 4 in which viscosity in centipoise has been plotted vs macromolecular solids concentration – dry basis (c) or concentration squared (c^2).

Table 3

Water Activity of Gelatin and Agar Gels

Sample	Concentration (d.b)	a_w
gelatin	45	0.9709
	36	0.993
	22.5	0.993
	13.5	0.984
Agar	8.23	0.995
	7.75	0.967
	7.21	0.994
	6.98	0.705
		0.989
		0.954
	6.62	1.003
	6.44	1.016
	6.27	0.967
		0.961
	5.53	0.995
		0.971
	4.79	1.010
		0.974
	4.03	0.970
	3.25	0.970

Table 4

Expected* Water Activity of Gelatin Gels

Concentration (d.b.)	a_W
5%	0.9993
10%	0.9969
20%	0.9864
30%	0.9660
40%	0.9327
50%	0.879
60%	0.8071

* Formula used − $\ln a_1 = -\theta_2 \left(\dfrac{V_1}{V_2} \right) - \dfrac{\theta_2^2}{2} - \dfrac{\theta_2^3}{3} - \ldots \ldots$

calculations based on an average molecular weight of 75,000 and partial molar volume of 0.74.

Both solutions exhibit a non-linear increase in viscosity vc C with the greatest change in slope occurring at the concentration where the gel begins to break (as opposed to cut) during the viscosity measurement, The gels exhibit breaking at 8-10% for gelatin and about 4% for agar (Figures 1 and 3).

When viscosity points below breaking concentration are plotted vs C^2 a straight line results (Figures 2 and 4). These results parallel work done by Ferry (1948) in determining the modulus of rigidity - the ratio of shearing stress to shearing strain. Ferry correlated rigidity values for gelatin with C^2 and found it proportional up to a concentration of 13% dry basis. He explained the phenomenon as due to crosslink formation (i.e. a dimerization equilibrium) which involves two molecules and is an indication of orientation entropy.

It may be noted that the agar data is not a particularly good fit to a straight line in Figure 4. This may be due to sample makeup differences. Further investigations are necessary to determine if agar gives a straight line fit or not, and why.

A typical Instron curve is reproduced in Figure 5. Measurements of the initial peak height (A) and the height of the final plateau (B) were plotted as a function of C and C^2. The best straight line fit resulted from a plot of initial peak height vs. C^2. These plots are shown in Figures 6 and 7. Again gelatin shows excellent fit and reproducability. The drastic slope change at about 10% as found by Brookfield data was not evident however.

Figure 1

Brookfield Data – Gelatin
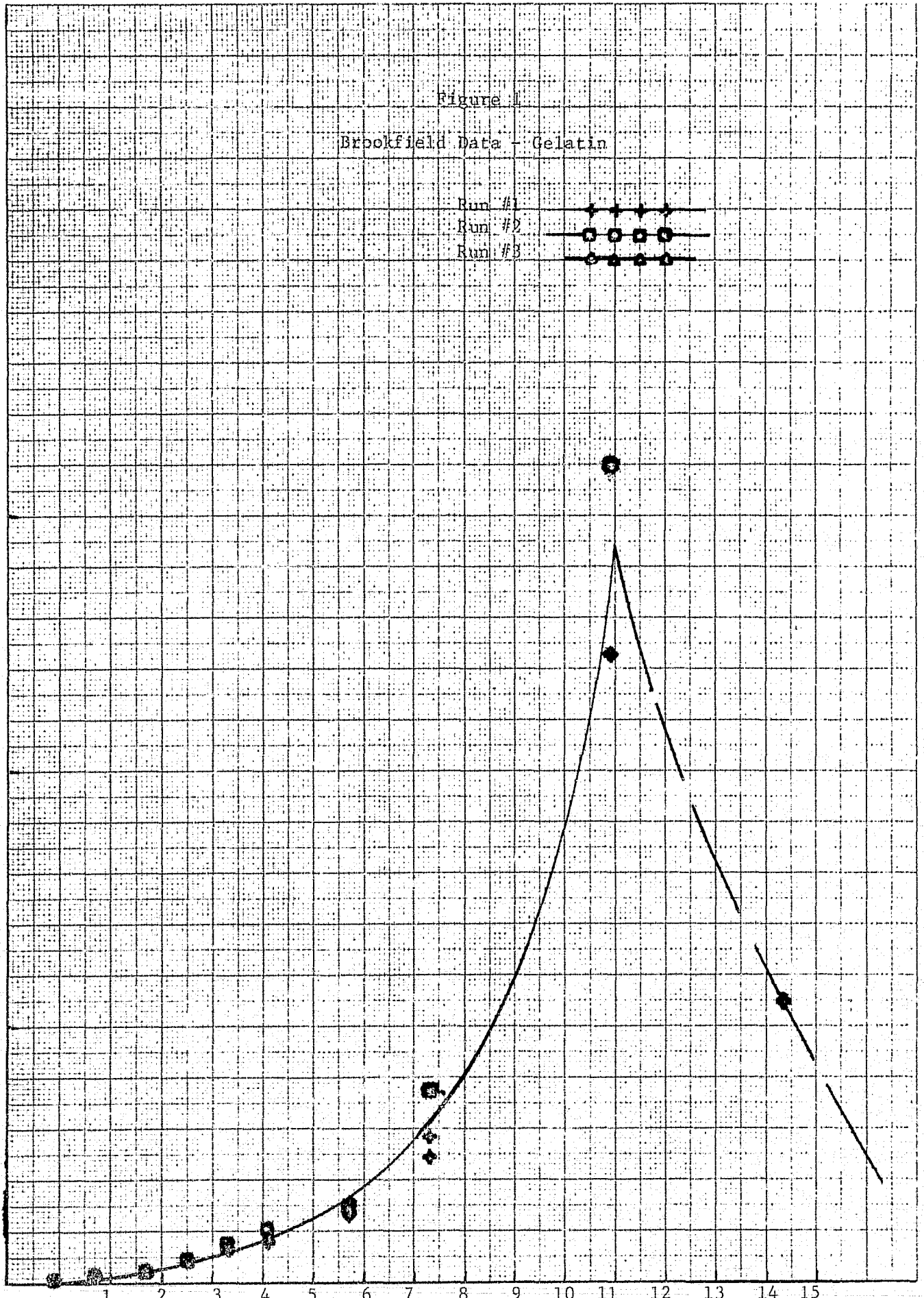
Run #1
Run #2
Run #3

Figure 2

Brookfield Data - Gelatin

Run #1
Run #2
Run #3

Viscosity (cp) x 10^{-5}

% (concentration)^2 (dry basis)

Figure 3

Brookfield Data – Agar
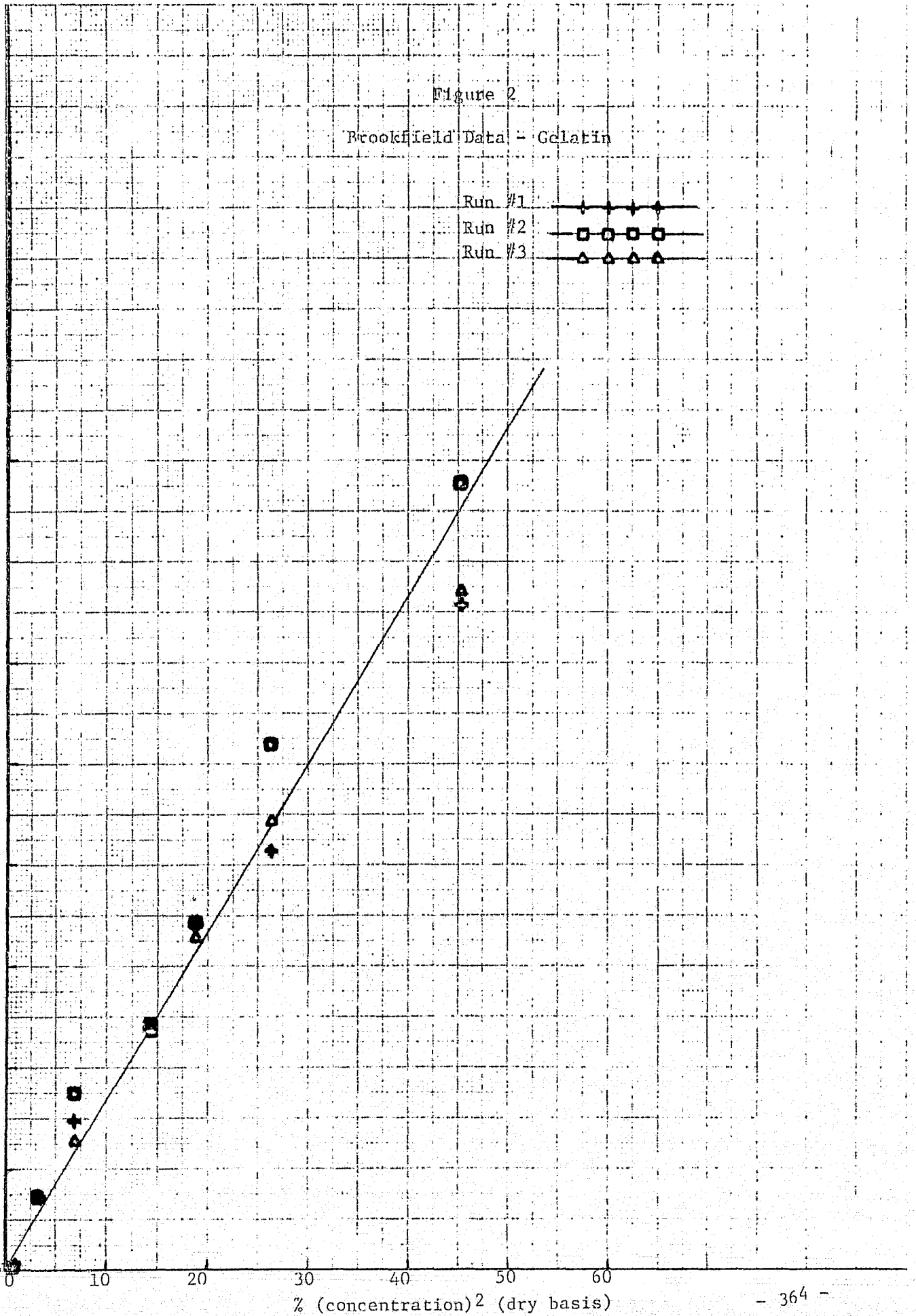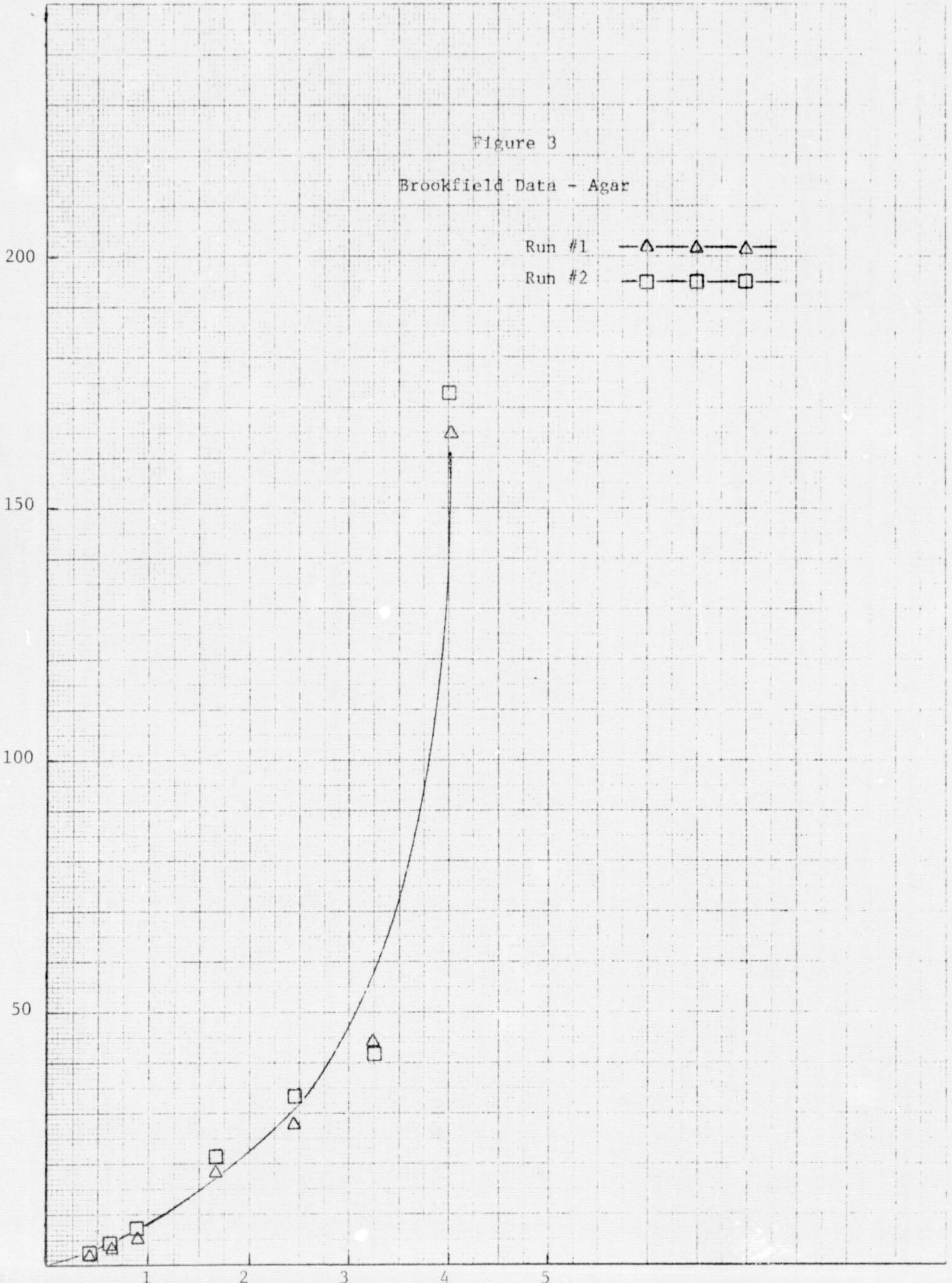
Figure 4

Brookfield Data — Agar
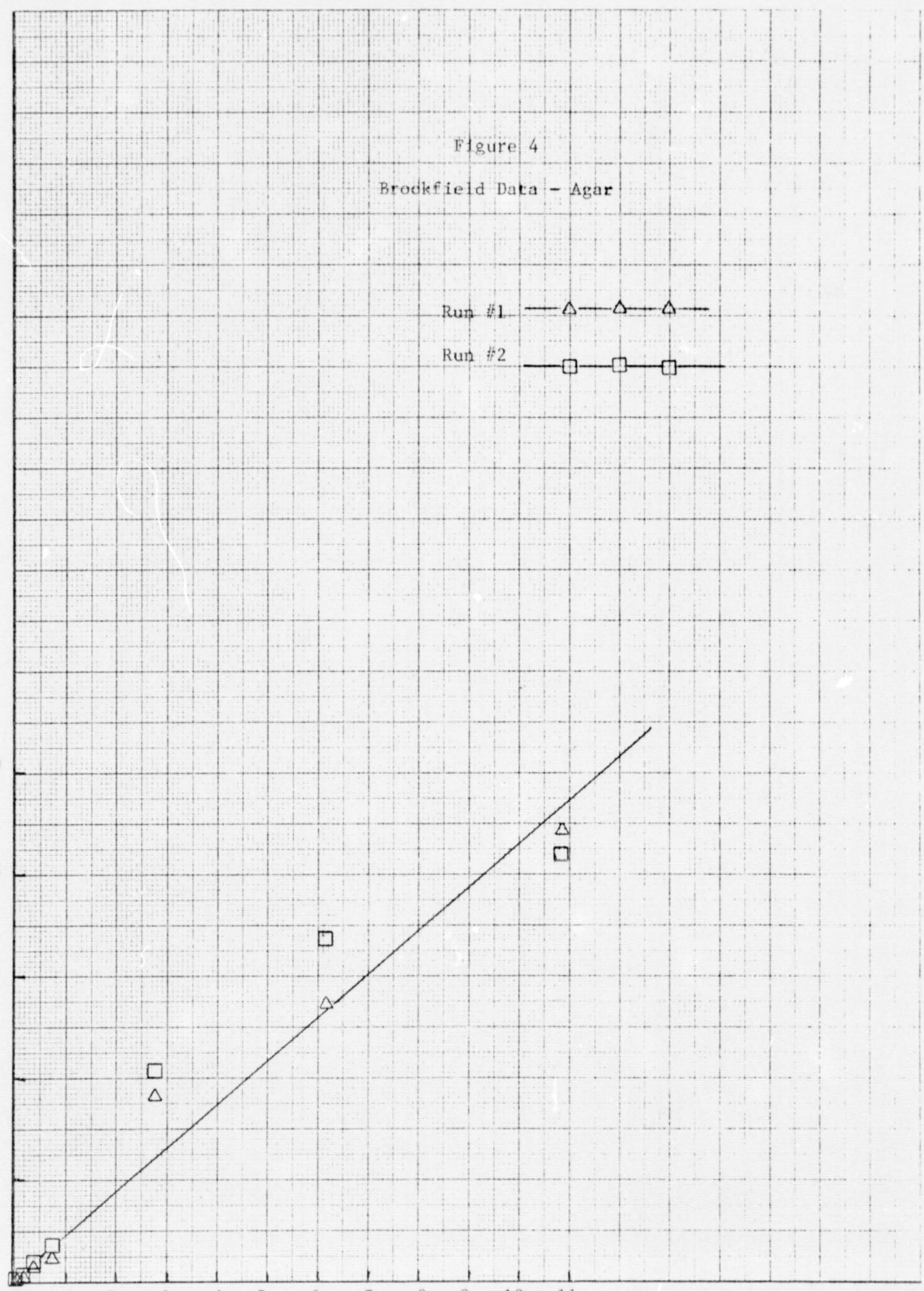
Figure 5

Example of Instron Curve for Gels

CTM System
Chart Speed 10
Crosshead Speed 5
18 Gauge Wire Dye
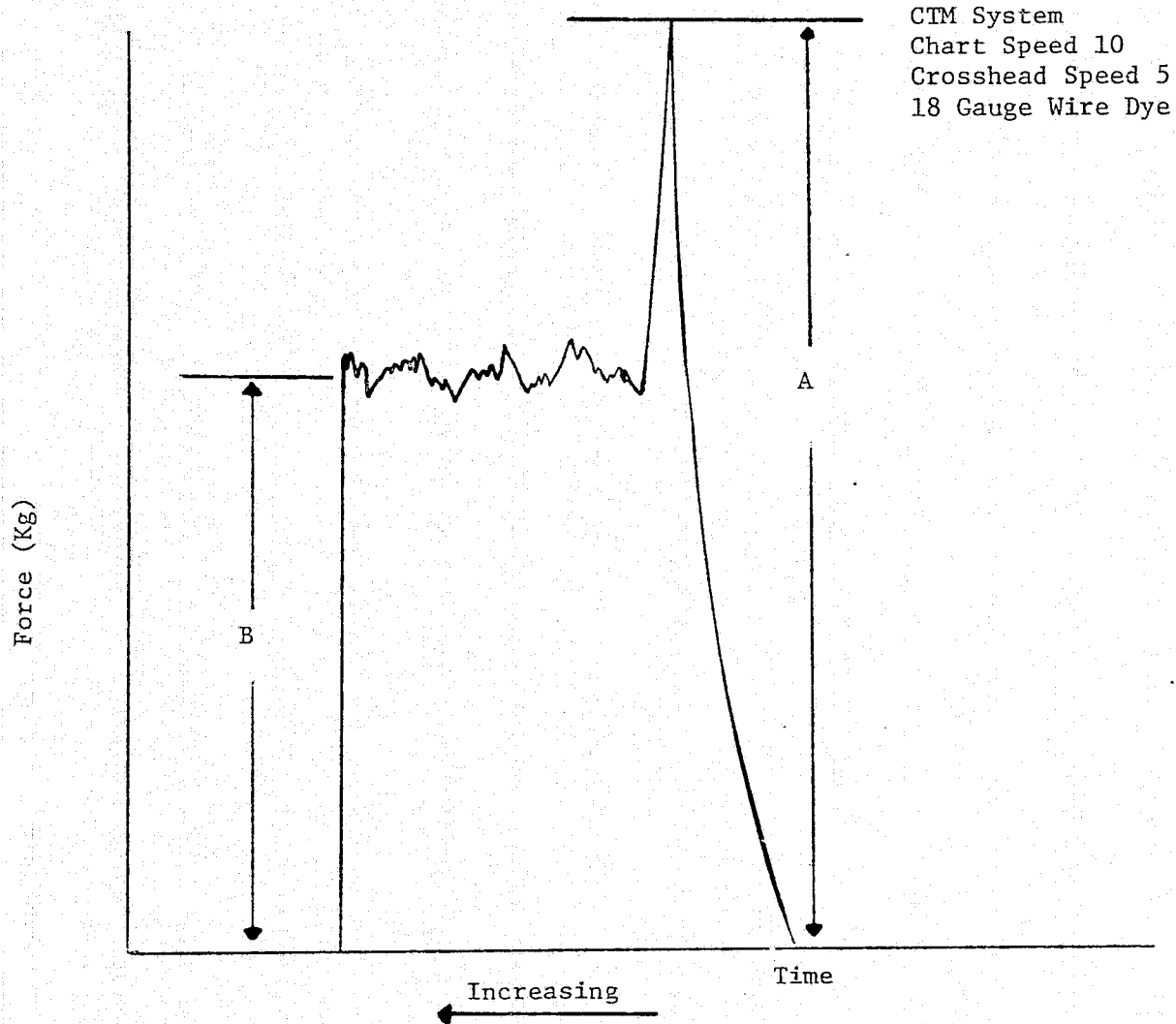
Figure 6

Instron Data - Gelatin

Figure 7

Instron Data — Agar

1st Peak

Run #1 —△——△——△—

Run #3 —□——□——□—

Force kg

%(Concentration)^2 (dry basis)
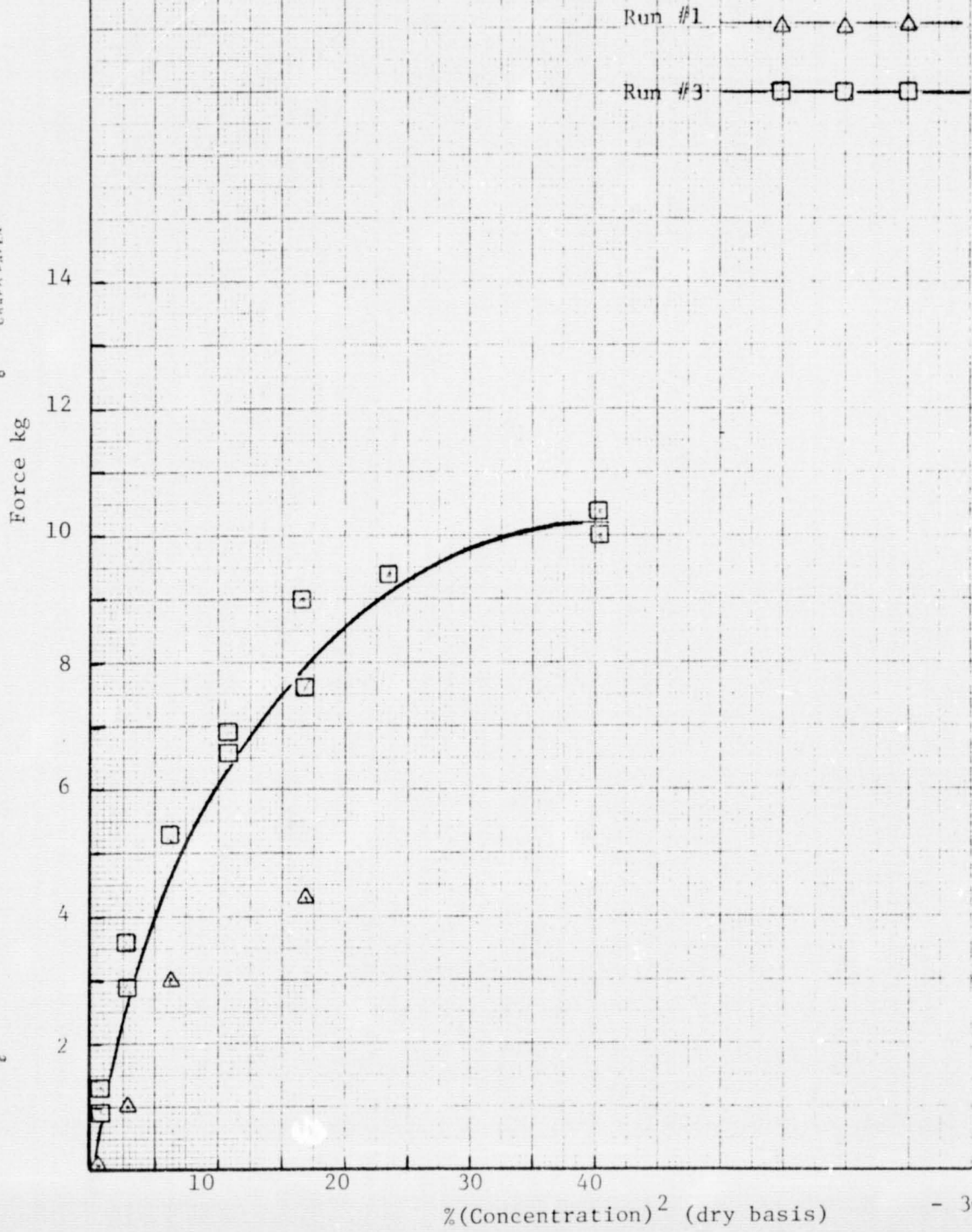
Agar gave a very poor fit and was not reproducible. The agar experiments must be repeated after differences due to the makeup procedure have been eliminated.

4. NMR data

Measurements of spin-latice (T_1) relaxation rates are summarized in Figures 8 and 9. Gelatin, agar and water relaxation rates have been measured as a function of concentration and are plotted as $1/T_1$ vs C.

Both gel systems show reduced T_1 times with increasing solute concentration indicating a more ordered water phase. For gelatin this order disappears (T_1 equal to that of water) at about 10% solids while for agar significantly reduced T_1's are noted at at 0.3% d.b.

Agar exhibits a more highly ordered water phase whether compared on a basis of solids content, Brookfield viscosity or Instron shear values. This would be expected since agar is a more highly charged molecule than gelatin.

Spin-spin (T_2) values have not yet been determined. The equipment previously described is not capable of determining this variable. The work will be pursued using a Bruker model mini-spec 20 currently housed in the Department of Horticulture and Landscape Architecture (University of Minnesota).

Figure 8

NMR Data – $1/T_1$

Gelatin
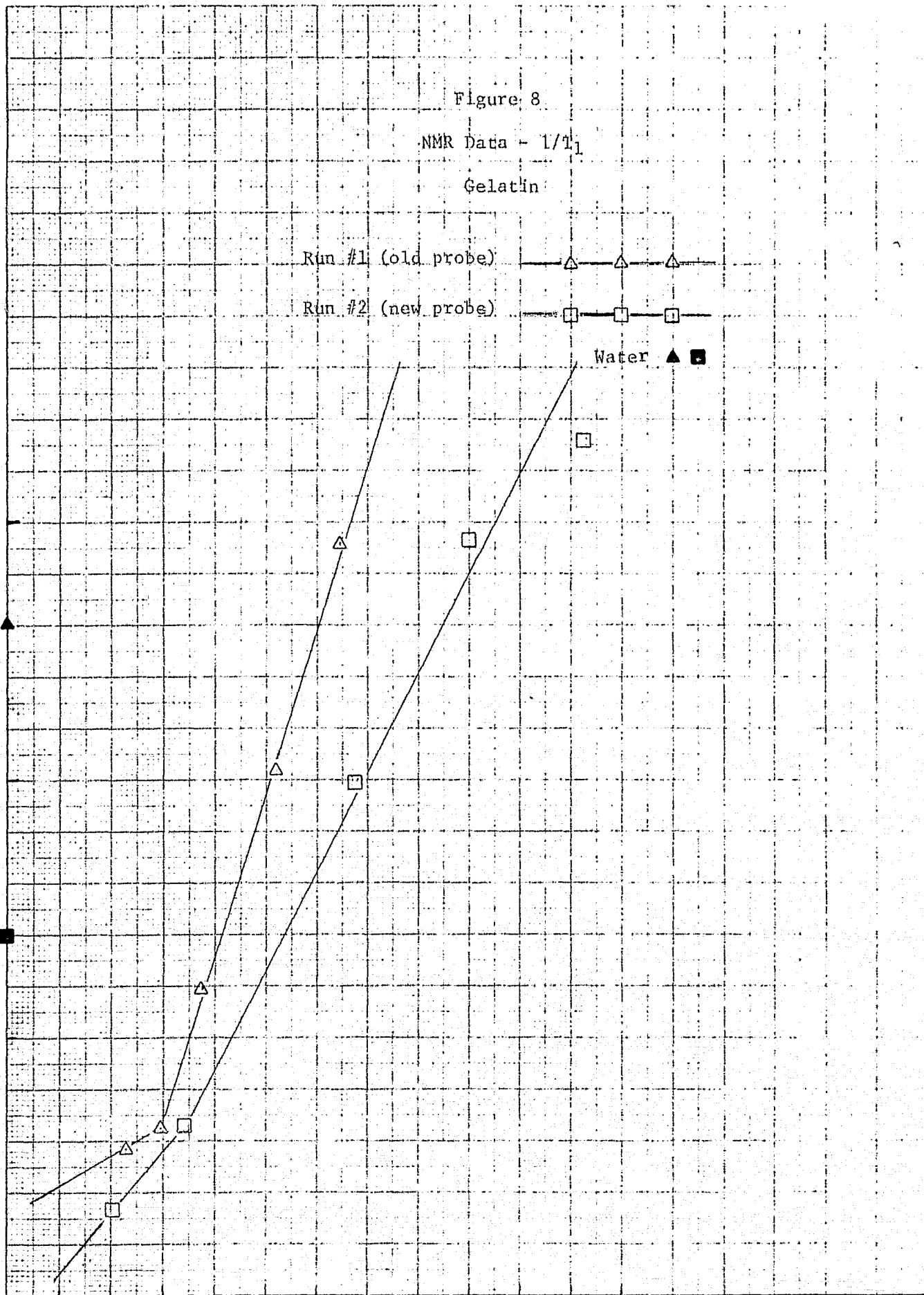
Run #1 (old probe) ———— △   △   △

Run #2 (new probe) ———— □ — □ — □

Water ▲ ■

$1/T_1$ $(\text{sec})^{-1}$

% Concentration (dry basis)

Figure 9

NMR Data — $1/T_1$

Agar

Agar

Water

$1/T_1 \ (\text{sec})^{-1}$

% Concentration (dry basis)

Clifford and Child (1971) have investigated the change in T_1 and T_2 as a function of concentration (0-8%) in both gelatin and agar. Using the fast exchange approximation that the residence time of a particular hydrogen at a binding site is very much less than the T_1 at that site, they used the following equations to explain the change in T vs C:

$$\frac{1}{T_{1obs}} = \frac{P_a}{T_{1a}} + \frac{P_b}{T_{1b}} + \ldots\ldots$$

where T_{obs} is the measured relaxation time 1, P_a and P_b are populations of exchangable hydrogens at sites "a" and "b" and T_a and T_b are the relaxtion times at sites a and b. In gel systems, site "a" corresponds to water-water interactions while site "b" corresponds to water-macromolecule interactions.

$$\text{And:} \quad \Delta\left(\frac{1}{T}\right) = \frac{1}{T} - \left(\frac{1}{T}\right)_O = \frac{C(MW)_O\ N_B}{N_S(MW)_S}\left(\frac{1}{T}\right)_B$$

where o refers to pure water, subscript s to solute, MW is molecular weight, N is the number of moles and C is in units of g/g solvent. The subscript B refers to exchangable protons.

Both of these equations apply to T_2 as well as T_1.

The change in T_1 with concentration is linear and assumes that water is structured with a single binding energy along the macromolecule.

The data of Clifford and Child shows gelatin has a single T_1 which varies linearly with C and has a single slope. They show agar has a single T_1 which varies linearly but has a change in slope at a concentration of about 3%. They explain the difference between gelatin and agar as being due to a higher degree of water structuring around the agarose molecule. The additional structure is a result of steric effects (i.e. placement of -OH groups) and the macromolecular charge. As the concentration increases molecular movement is more restricted allowing a stronger reinforcing effect on the bulk water structure.

Our data shows the change in slope may also be observed in gelatin at high concentrations. It is conceivable therefore, that the same phenomenon occurs with gelatin. At concentrations high enough to severely restrict macromolecular motion, the water structuring effects become quite pronounced.

It seems that an additional term should be added to the second equation above. A term that will account for the change in T due to water structuring by forces such as electrostatic and steric interactions. The exact nature of this term must be explored further.

5. Dye migration

These studies were initiated using gelatin at concentrations ranging from two to 43%. The experiment was performed in duplicate and at room temperature (22°C). Figure 10 gives the results in a plot

Figure 10

Dye Migration — Gelatin

of migration rate $\frac{dx}{dt}$ vs C. Two straight lines result with a break
at 8-10%. These data need verification by replication but, taken as is,
point to a structural change occurring in gelatin at a concentration
of about 10%. This change is at the same concentration as the change
in slope of the $1/T_1$ plot and the structural change determined by
Brookfield measurements.

From the preliminary data it seems as though at around
10% gelatin concentration there is a change in the characteristics
of the system, i.e., a change from liquid-like to porous-solid.
This must be documented more fully for gelatin and much more work
must be done with polysaccharides to see if they exhibit the same
characteristics.

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E. Summary and Recommendations

Many methods can be used to characterize the water binding capacity of macromolecular systems. In this phase of the study we have attempted to relate the many findings into a mechanism in which we can understand how water is bound in gels. By using this information we hope that in the future we can better formulate IMF systems to higher water contents and yet maintain an a_W which is microbiologically safe.

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