THE PERMEABILITY OF DEAD PLANT CELLS FOR SOME ENZYMES

*De permeabiliteit van dode
plantecellen voor enige enzymen*

N. GORIN

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(with a Summary in English, Dutch and Spanish)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS, DR. IR. F. HELLINGA,
HOOGLERAAR IN DE CULTUURTECHNIEK,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP DINSDAG 11 MAART 1969 TE 16.00 UUR

DOOR

N. GORIN

THEOREMS

I

In the study of niacin, more consideration should be given to the implications of overdosage of niacin.

GOLDSMITH, G. A. and O. N. MILLER. 1968. Niacin. In *The Vitamins, Chemistry, Physiology, Methods*. Vol. VII, second edition. Ed. by P. György and W. N. Pearson. Academic Press, New York, London. 137–167.

II

The influence of calcium ions on the stability of trypsin and chymotrypsin activities in rat pancreatic juice in the presence of sodium taurocholate deserves more attention than hitherto has been paid to it.

VAHOUNY, G. V. and A. S. BRECHER. 1968. Effect of bile salt on the digestive enzymes of rat pancreatic juice. *Arch. Biochem. Biophys.* 123, 247–254.

III

It should be made compulsory to have every newborn child tested for phenylketonuria in the second week of life.

FLEURY, P. 1968. Organizing screening programme for inborn metabolic errors in The Netherlands. In *Brain damage by inborn errors of metabolism. Symposium organised by the Interdisciplinary Society of Biological Psychiatry*. Amsterdam, 6 october 1967. De Erven F. Bohn N.V., Haarlem. 60–66.

IV

Intensive investigations should be carried out with a view to ascertaining a suitable method that would permit the determination of the karyotype of the human foetus before it is three months old. Should the karyotype be abnormal, abortion should be permitted.

BROWN, W. M. C. 1968. Criminals and chromosomes. *New Scientist* 40, 235–236.

V

Crates and boxes of vegetables that are brought into the various markets should bear a label indicating the producer.

N. GORIN
Wageningen, 11th March 1969

VI

In publications on the structure of yeast cell walls, more attention should be given to the possibility of the occurrence of metal bridges.

PHAFF, H. J. 1963. Cell wall of yeasts. *Ann. Rev. Microbiol.* **17**, 15–30.

LAMPEN, J. O. 1968. External enzymes of yeasts: their nature and formation. *Antonie van Leeuwenhoek, J. Microbiol. Serol.* **34**, 1–18.

ROGERS, H. J. and H. R. PERKINS. 1968. *Cell Walls and Membranes.* E and F. N. Spon Ltd., London. 135–152.

This thesis, p. 41.

VII

Some of the proteins present in the cementing material in plant tissues may be considered as proteins belonging to the plasmodesmata.

GINZBURG, B. Z. 1961. Evidence for a protein gel structure cross-linked by metal cations in the intercellular cement of plant tissues. *J. Exp. Botany* **12**, 85–107.

VIII

The Food Composition Table can be improved by giving coefficients of digestibility of the various foods based on digestion in vitro by appropriate enzymes under standardized conditions.

Nederlandse Voedingsmiddelen Tabel. 1967. 23e druk. Voorlichtingsbureau voor de Voeding. Den Haag.

IX

International standardization of immunological-fluorescent methods should be instituted, so as to render it possible for results to be compared from a quantitative point of view.

Twenty second Symposium held in October 1968 in London by the Permanent Section for Microbiological standardization. Round Table Conference on Standardization in Immunofluorescence. International Association of Medical Standards.

X

The assertion that the action of the Waring Blendor on soybean meal produces complete cell wall rupture of the cells is not entirely true.

VICKERY, H. B., as referred to by S. J. CIRCLE. 1950. Proteins and other nitrogenous constituents. In *Soybeans and Soybean Products.* Vol. 1. Ed. by K. S. Markley. Interscience Publishers, Inc. New York, p. 292.

XI

The South American countries Argentina, Brazil and Uruguay should take steps to encourage the greater consumption of the beverage "Mate" (Ilex paraguariensis) in the various industrial countries.

XII

The philosophical attitude of the research worker should not be dogmatic.

CONTENTS

1. INTRODUCTION

The digestibility of plant cells used as food for human beings or for animals, depends, among other factors, to a certain degree on the penetration of the digestive enzymes into them.

The first barrier that the enzymes must pass in order to enter the plant cells are the cell walls.

MITCHELL (1942) stated that the cell walls of plant tissues may be an obstacle to the digestion by enzymes of ruminants because they prevent the enzymes from reaching the contents of the cells from these tissues.

Furthermore, it is known that when old plants are fed to ruminants they are digested to a lesser extent than when younger plants of the same type are provided as feeding-stuff. In other words the age at which the plant is cut for feeding to animals is important. When the tissue is older it contains more lignin in its cell wall, this was mentioned by ROELOFSEN (1959). Strongly lignified cell wall is more difficult to degrade enzymically than when it is less lignified (BULL, 1967).

Recently MATELES and TANNENBAUM (1968) showed that the barrier formed by the cell wall of *Bacillus megaterium* must be considered as an important factor in the nutritional value of proteins originating from unicellular organisms. They did not study this problem with other unicellular organisms like yeast and algae.

LUND (1954) showed that several yeasts were still viable in the dung of horses and cattle. These yeasts, which must originate from fodder, passed through the alimentary canal in an undamaged state. *Saccharomyces cerevisiae* was not identified by LUND (loc. cit.) among the different species present.

RETTGER et al. (1924) fed baker's yeast to various animals and human beings, and looked to see whether viable cells could be found in the feces. They concluded that most of the yeast when administered orally died in the alimentary tract.

However, NESS et al. (1946, and also the writer (ANON., 1946) of an editorial of the *J. Am. Med. Assoc.*, concluded that live baker's yeast cannot be regarded as a satisfactory source of thiamine for human beings. In fact the reverse is the case, as live yeast does not provide its own thiamine, but on the contrary, it takes thiamine from the diet for its own metabolism. This implies indirectly that live baker's yeast is not degraded enzymically in the digestive system of human beings.

The previously mentioned authors stated that baker's yeast killed by heat is a good source of thiamine. They did not determine whether heating affected the permeability of its cell wall and cell membrane.

KING and SCHEFNER (1963) increased the nutritive value of an algal ration by treating these cells with enzyme systems derived from the snail *Helix pomatia* and the mould *Myrothecium verrucaria*. Pectinase was also effective.

So far we have seen that the cell wall is a barrier to the entry of digestion

enzymes. Since it is known that cooked foodstuffs are better digested than un-cooked the question arises whether heating alters the structure of the cell wall in order to render it more penetratable for enzymes.

GELDERMALSEN-DE JONG (1963) and DOESBURG (1962/63) studied the changes in the cell wall brought about by heating. They did not however directly de-monstrate whether digestive enzymes would penetrate to a larger degree through an altered cell wall than through an intact one.

REEVE (1954) points out that when a potato is heated the swelling of the gell-ed starch causes rupturing, which appears to begin in the weaker primary-pit[1] regions of the cell walls.

There is no further reference in literature to the influence of pits on the nu-tritive value of plant cells for food objectives.

REEVE (1967) reviews the cellular structures, starch and textural qualities of processed potatoes, but he adds nothing new to what he said in 1954 about pits.

Neither he nor other investigators – as far as we know – considered plasmo-desmata (pits) a place for the entry of big molecules (digesting enzymes).

ESAU et al. (1967) published electron micrographs showing a large number of particles of beet-yellow virus in the act of passing through plasmodesmata. As this study was made with living plants the mechanism of transport *across plasmodesmata* probably depended on a cytoplasmatic flow from one cell to another.

Be that as it may, in heated plant tissues there is no flow of protoplasm. This is because the protoplasm proteins have become denatured by heating. We assume that also in dead unheated plant tissues used as food, the protoplasm of plasmodesmata ceases to flow.

As dead unheated or heated plant material cannot be compared with living plants we thought it would be worth while to study the possible entry of di-gesting enzymes into plant cells (unheated or heated, both of them dead) through plasmodesmata. Since the protoplasmatic threads did not flow and the solid material in them consisted in the main of proteins it would be inte-resting to use a proteolytic enzyme for digestion and to learn whether this would aid the penetration of another type of enzyme. If this did occur, it would mean that the proteolytic enzyme opened a way by digesting the protoplasmatic threads and facilitating the entry of the other enzyme.

1.1. SCOPE OF THE PRESENT INVESTIGATION

1. The purpose of our study is to learn whether the heating[2] of material in distilled water at 100 °C affects principally the permeability of the cell wall and cell membrane of dead plant cells, thereby permitting a better entry of digesting enzymes.

[1] For definition of plasmodesmata, pit and plasmodesmatal canal see page 47, paragraph 3.3.
[2] We use the word 'heating' in preference to 'cooking' because the latter implies a prepara-tion of materials for consumption; that is to say the organoleptic properties should also be considered. We made only a digestion in vitro.

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Heating not only alters the cell wall, but also favours the action of proteolytic enzymes by denaturing the proteins inside the cells. Denatured protein is more susceptible to hydrolysis, and proteinaceous inhibitors usually lose their activity against the enzyme by denaturation.

We see that it is difficult to separate the factors which improve the digestibility of plant cells when heated. And if we wish to confine the problem only to permeability, we have to distinguish the denaturation effect on proteins.

We overcame this difficulty by treating unheated material with a substance that would only affect permeability (see point 2).

2. To study the first point we had to find a substance that could affect the cell wall and cell membrane permeability without denaturing the enclosed proteins. As will be seen in the present investigation Ethylenediaminetetraacetate (EDTA) fulfills these conditions.

If unheated material treated with EDTA were digested to the same degree as heated material it would be evident that heating affects principally the barrier of the substrates.

3. We wanted to ascertain whether big molecules (enzymes) would penetrate the plant material through plasmodesmata (pit).

With a view to this we studied: a. The penetration of chymotrypsin into soybean material, which possesses plasmodesmata, and into yeast and algae. These last two have no plasmodesmata. b. The penetration of lipase alone or with chymotrypsin. If chymotrypsin were to enhance the penetration of lipase in the case of soybean, but not in the case of yeast the importance of plasmodesmata would be stressed.

4. The penetration of enzymes into dead plant cells is a passive process on the part of the cell, but the enzyme itself may be active. Therefore we also studied the penetration of a compound of large molecular weight without enzyme activity, namely amylopectin. At the same time we studied the possible leakage of β-amylase from soybean material.

1.2. GENERAL CONSIDERATIONS

The use of digesting enzymes to study the entry of big molecules is convenient because we can detect their penetration with the products of hydrolysis produced inside the cells and diffusing from them. Inconvenience might arise if inhibitors of the enzymes employed occur within the cells.

We knew that in soybean material there is a trypsin inhibitor (KUNITZ, 1947). In view of this we decided to use α-chymotrypsin as the proteolytic enzyme. The activity of this enzyme is slightly reduced by the said inhibitor (KUNITZ, loc. cit.).

In addition, other proteolytic inhibitors from soybean were considered. They

were the saponins (page 39, paragraph 2.3.5.3.) and the AA inhibitor (page 77, in General Discussion).

As an enzyme other than proteolytic we used pancreatic lipase because the digestion of fats is important in the field of nutrition. Moreover α-chymotrypsin was ideal to use with pancreatic lipase, because both enzymes require similar conditions for optimal activity, i.e. pH, temperature and calcium ions.

Soybean, yeast and also algae contained sufficient proteins to be used as substrates for α-chymotrypsin, and the first two had enough fat for pancreatic lipase.

We used dead plant material as substrates for these enzymes because: a. as we have already mentioned our object was to learn whether the site of protoplasmatic threads (plasmodesmata) would be the place of entry for these enzymes; b. we wished to prevent an active uptake (pinocytosis) of big molecules by the plant cells (JENSEN and MCLAREN, 1960); c. the dead plant cells would be more representative for the vegetable material used as food.

For the digestion experiments with the two enzymes mentioned, isolated soybean cells and sections were used instead of whole seeds or cotyledons, not only because the former enabled us to work at a cellular level, but also because we assumed that with these types of substrates it would be easy to see pits under the light microscope and eventually plasmodesmata by means of electron microscopy.

When the cells of soybean, yeast and algae – heated or EDTA-treated – were observed under the light microscope they manifested no differences whatsoever from the respective unheated cells. For this reason we thought that the biochemical method would be convenient for us to learn, whether or not, these substrates facilitated the passage of chymotrypsin and/or lipase. In the case of chymotrypsin we also determined immunologically whether this enzyme penetrated soybean cells and sections. At the same time we tried to detect if the entry was through the pits (plasmodesmata).

As mentioned on page 3. paragraph 1.1., point 3 of the scope, the substrates soybean, yeast and algae were used because the first of these has plasmodesmata, whereas the last two have not.

In addition, these materials were chosen as they are, or are becoming, very valuable from a nutritional point of view.

For soybean this can be found in the book edited by MARKLEY (1950) and in specialized publications on soybean as well as in many other articles in periodicals related to food technology and nutrition.

With regard to yeast we would only mention that in addition to its being used for fermentation processes it is becoming regarded more and more as a source of proteins. This will be realized when we state that such prominent companies

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as Shell, Esso, Nestlé and B.P. are trying to produce single cell proteins, which means proteins from unicellulars (MATELES and TANNENBAUM, loc. cit.).

As for algae we merely state that they are utilized in water reclamation for a dual purpose: as an element in the oxidization process and as food for animals (ERCHUL and ISENBERG, 1968). This becomes more significant when we consider that there are plans to use algae on interplanetary trips as a source of oxygen and food for human beings. On our own planet algae might be of use in those countries which have no large areas of grassland devoted exclusively to the raising of animals.

Before ending the General Considerations, we should like to remark that BISALPUTRA et al. (1967) demonstrated that there is no cytoplasmic connection between the neighbouring cells in the red alga *Laurencia spectabilis*. Thus, these authors state that the classical term 'pit connection' is a misnomer. This is an important feature as we used algae because they have no plasmodesmata (pit).

2. EXPERIMENTS MADE WITH α-CHYMOTRYPSIN

2.1. MATERIALS AND METHODS

2.1.1 *Chemicals*

2.1.1.1. Buffer solutions

Buffer solutions used were 0.1M borate pH 8 for soybean cells, soybean sections and algae; and 0.1M phosphate pH 7.6 for yeast cells. When cells were stored Preventol[1] 115 (obtained from Bayer, Leverkusen, West Germany) (0.1 % w/v) was added to prevent microbial contamination.

2.1.1.2. The choice of an antimicrobial compound (Preventol 115)

It was necessary to find a suitable antimicrobial compound for use during storage of substrate suspensions. Toluene and chloroform interfere with the determination of chymotrypsin digestion since they have a high extinction at 280 mμ.

Half percent and 0.1 % solutions of the following compounds were prepared and their extinction read at 280 mμ against distilled water (Table 1).

TABLE 1.

Antimicrobial compound	$E^{1\ cm}_{280\ m\mu}$
0.5 % Dowicide (o-phenylphenol sodium salt tetrahydrate) obtained from N.V. Stockvis en Zonen, Rotterdam	2.64
0.5 % Preventol 115 obtained from Bayer	0.071
0.1 % Preventol 115	0.045
0.1 % Nipasept (propyl or methyl ester of p-hydroxybenzoate) obtained from Nipa Labs. Ltd. London	2.64
0.1 % Shirlan Na obtained from I.C.I.	3.00
0.5 % Cetyl pyridinium bromide (CPB) obtained from B.D.H.	0.192
0.1 % CPB	0.039
0.5 % Cetyl trimethyl ammonium bromide (CTAB) obtained from Merck	0.085
0.1 % CTAB	0.026

Since Nipasept and Shirlan Na dissolve incompletely, even at concentrations of 0.1 %, the solutions were filtered prior to the determination of the extinction.

The most promising antimicrobial substances were Preventol 115 and CTAB because of their low extinction at 280 mμ. The former did not affect the activity of chymotrypsin, the latter however inhibited chymotrypsin considerably. In table 2 are recorded the extinctions ($E^{1\ cm}_{280\ m\mu}$) determined after digestion of 1 % casein solutions (1ml) by a chymotrypsin solution (0.05 ml) at 35°C for 20 minutes as described in the Determination of chymotrypsin activity (page 8, paragraph 2.1.2.3.) in the presence and in the absence of the antimicrobial substances.

[1] The mention of trade names in this publication is for purpose of identification and does not imply endorsement by our Department.

TABLE 2.

Antimicrobial compound	$E^{1\ cm}_{280\ m\mu}$
none	0.81
0.1% Preventol 115	0.80
0.1% CTAB	0.55

The concentration of the antimicrobial substance was based on the final volume of the reaction mixture.

The effectivity of Preventol 115 was corroborated with the light microscope: no growth of microorganisms could be detected in media containing 0.1% Preventol 115, even after storing for 4 months at 5°C. It was decided to use Preventol 115 as an antimicrobial compound during storage of substrates. Preventol 115 is toxic and consists of inorganic salts of sodium. The exact formula is kept secret by Bayer.

2.1.1.3. Ethylenediaminetetraacetate (EDTA) solution

EDTA solution was prepared by dissolving EDTA-disodium salt (38 g) in distilled water (600–800 ml) and adjusting the pH to 10 with NaOH; then the final volume was made up to one litre. Preventol 115 (1 g) was dissolved in the EDTA solution (1 litre). In this way we obtained 3.8% (0.113M) EDTA solution pH 10 with 0.1% Preventol 115.

2.1.2. Enzymes

2.1.2.1. Chymotrypsinogen (CTG) and Trypsinogen (TG)

CTG and TG were prepared in the Laboratory for General and Technical Biology by crystallization from fresh bovine pancreas according to KUNITZ and NORTHROP as described by LASKOWSKI (1955).

A 0.25% CTG solution in 0.001N hydrochloric acid (100 ml) containing 1% calcium chloride[1] and a 0.025% TG solution in the same solvent (100 ml) were dialysed in different beakers against 0.001N hydrocholoric acid containing 1% calcium chloride for one night at 5°C. The solutions obtained are stable at 5°C and therefore they were used as CTG and TG stock solutions.

2.1.2.2. α-Chymotrypsin (CT)

After a number of preliminary experiments CT was prepared immediately prior to use in the following way. TG solution (1 ml) was diluted with 0.1M borate buffer pH 8 (5 ml) and CTG solution (2 ml) was added. Final volume 8 ml. The mixture was incubated at 35°C for 40 minutes; TG was converted autocatalytically into trypsin which in turn converted CTG into CT. Under these conditons CT activity remained constant for at least 180 minutes (fig. 1).

A mixture of TG solution (1 ml) and 0.1M borate buffer pH 8 (7 ml) without CTG was used as a blank for the conversion of CTG into CT by trypsin (fig. 1).

[1] Calcium chloride was added because calcium ions enhance the activity of chymotrypsin (LASKOWSKI, loc. cit.).

2.1.2.3. Determination of α-chymotrypsin activity

Casein solution in 0.1M borate buffer pH 8 (1%) was made according to RICK (1962) .The solution contained 0.005M of calcium chloride, and was stored at 5 °C. Fresh solutions were prepared weekly.

Enzyme activity was determined by the method of KUNITZ as modified by WU and LASKOWSKI and described by LASKOWSKI (loc. cit.). Casein solution (1.0 ml) was added to 0.1M borate buffer pH 8 (0.95 ml) and enzyme solution (0.05 ml) and incubated at 35 °C for 20 minutes. Then 5% trichloroacetic acid (TCA) (3.0 ml) was added and the mixture shaken. Blanks were prepared by first adding TCA and then casein solution. The mixtures were centrifuged after standing for one hour and the supernatant was filtered through fritted glass (G3). The extinction at 280 mμ was read in a one cm cuvette against distilled water. The value of the corresponding blank was substracted from the value obtained.

This assay was used to control either the activity of chymotrypsin on the other substrates (soybean, yeast and algae) or the conversion of CTG into CT by trypsin (fig. 1).

2.1.3. *Determination of the concentration of substrate suspensions*

The concentration of substrates in the various suspensions (with the exception of the soybean sections) was determined with the aid of the hematocrit (fig. 2) in the following way. After the suspension had been stirred for 30 minutes one ml was transferred with a pipette with a wide orifice into the hematocrit and centrifuged at 500 g for 15 minutes at room temperature. The volume of the sediment (cells or powder) multiplied by 100 gave the concentration (v/v) of the suspension. For example, if after centrifugation the sediment had a volume

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of 0.3 ml the concentration of the suspension was 30% (v/v). In all experiments 20% suspensions were used with the exception of the experiments with algae (Chlorella 10%, Scenedesmus 2%). The percentages of dry weight of the suspensions were determined by drying at 110°C overnight and weighing.
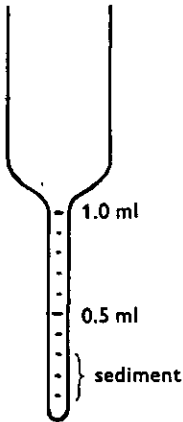
FIG. 2. Diagram of the hematocrit used for determining the concentration of the various suspensions. The calibrated part has a capacity of 1 ml.

2.1.4. *Nitrogen determinations and calculations based on nitrogen contents of the substrates*

Nitrogen contents were determined by Kjeldahl destruction followed by assay of ammonia with the microdiffusion method (CONWAY, 1962).

Model experiments with ammonium sulphate and hippuric acid performed as indicated below (2.1.4.1.) demonstrated that on the average 95% of the original nitrogen was recovered with this method.

2.1.4.1. Nitrogen contained in the sample

The nitrogen determination was carried out in duplicate, on filtrates from samples drawn during experiments (substrate plus chymotrypsin solution) and on the corresponding blanks. The filtrates were obtained as described later in paragraphs 2.1.6.1., 2.1.6.2., 2.1.6.4. and 2.1.6.5.

The filtrate (5 ml) in a digestion flask was concentrated in vacuo to a volume of about 2 ml, to remove most of the water. Then the digest mixture (5 ml) was added and the mixture heated until digestion was complete. After cooling the liquid was diluted to 25 ml in a volumetric flask and 1 ml samples were taken for determination of ammonia in Conway dishes. Diffusion was allowed to proceed at room temperature for 3 hours. Then the boric acid solution was titrated using a microburette (2 ml) with 0.02N hydrochloric acid. The results are expressed as g N/100 ml.

Five ml of filtrate was used in order to have at least 100 μg of total nitrogen, as recommended by CONWAY (loc. cit.). However it was our experience that even with less than 100 μg nitrogen, the results were satisfactory. This was verified by using the method of JOHNSON as described by UMBREIT and BURRIS

(1964) which is sufficiently sensitive for quantities of 10 to 40 µg of nitrogen.

2.1.4.2. Nitrogen (%) on dry weight of the substrate

A part of the substrate suspension, when ready for the experiment as described later under paragraph 2.1.6., was dried for one night at 110 °C. With a portion of this material, wrapped in a cigarette-paper, we made nitrogen determinations in duplicate. As a blank, paper only, without material, was used. The data of the different materials corrected for the blank are shown in table 3, column 1.

2.1.4.3. Calculation of quantity of nitrogen in the reaction mixture (A)

First of all we shall define the term reaction mixture. It is the substrate suspension in buffer plus the same buffer plus chymotrypsin solution. The quantity of nitrogen in the reaction mixture is the amount of nitrogen of the substrate plus the amount of nitrogen of the enzyme. This last value was so insignificant when compared with that of the substrate that it was ignored.

To show how this was calculated we shall give an example with unheated soybean cells. With the other materials, the same reasoning was applied.

In our experiment to determine the digestion of unheated soybean cells by chymotrypsin, we had in a final volume of 80 ml (reaction mixture) 2.45 g of dry material (see page 16, paragraph 2.1.6.1.). We found that the nitrogen (%) in the dry weight of unheated soybean cells is 6.0%; so that in 2.45 g there is 0.146 g nitrogen and in order to refer to 100 ml of reaction mixture we multiplied by factor 1.25 (100/80). Therefore $0.146 \times 1.25 = 0.183$ gN/100 ml. This value is the quantity of nitrogen in 100 ml of reaction mixture of unheated soybean cells, which is presented with the values for the other materials in table 3, column 2.

2.1.4.4. Calculation of the nitrogen released from the substrate by α-chymotrypsin

This was defined as the amount of nitrogen contained in the filtered sample as a percentage of quantity of nitrogen in the reaction mixture. It was calculated as follows:
$$\frac{\text{nitrogen contained in the sample (g/100 ml)}}{\text{quantity of nitrogen in the reaction mixture (g/100 ml)}} \times 100$$

2.1.4.5. Calculation of the fraction of cell wall nitrogen in relation to total cell nitrogen (B)

We may assume that the percentage of nitrogen in isolated cell wall is of the order of magnitude of 2.5%. This value is the mean of the following ones: 1.9%, 2.8% (for the primary cell wall of full-grown cotton hairs quoted by ROELOFSEN, loc. cit.); 1.0%, 2.0% (for cell wall of baker's yeast as quoted by PHAFF, 1963) and 4.6% (for *Chlorella pyrenoidosa*, determined by NORTHCOTE et al. 1958). Furthermore we assume that the cell wall represents approximately 16% of the dry weight of the whole cell. This value is an average of: 16% for

Phaseolus vulgaris, which belongs to the same tribe as soybean (LAMPORT, 1965), 19% for baker's yeast (GRYLLS, 1961) and 14% for Chlorella (NORTHCOTE et al. loc. cit.). Thus the amount of cell wall nitrogen is roughly 0.4% (2.5% of 16%) of the whole cell weight. Consequently a rough approximation of the fraction of cell wall nitrogen in relation to total cell nitrogen was calculated as follows: $\dfrac{0,4\%}{\text{nitrogen } (\%) \text{ on dry weight of the substrate}}$. These data are recorded in table 3, column 3.

2.1.4.6. Calculation of the amount of cell wall nitrogen in the reaction mixture

This was effected by multiplying (A) × (B). The data recorded in table 3, column 4, are expressed in per cent.

TABLE 3.

Column No. Substrate	1 N (%) on dry weight of the substrate	2 Quantity of N in the reaction mixture (A) (gN/100 ml)	3 Fraction cell wall N in relation to total cell N (B)	4 Cell wall N in the reaction mixture (A) × (B) (%)
Soybean				
Unheated cells	6.0	0.183	0.07	0.013
Heated cells	7.6	0.232	0.05	0.012
Unheated sections	5.5	0.175	0.07	0.012
Heated sections	5.9	0.184	0.07	0.013
Unheated sections-EDTA	4.8	0.150	0.08	0.012
Heated sections-EDTA	6.9	0.213	0.06	0.013
Yeast				
Unheated yeast (STS or LTS)	6.3	0.301	0.06	0.018
Heated yeast (STS or LTS)	7.5	0.379	0.05	0.019
Unheated yeast (STS or LTS)-EDTA	6.6	0.306	0.06	0.018
Heated yeast (STS)-EDTA	7.2	0.425	0.06	0.026
Unheated yeast-EDTA-heated	5.2	0.300	0.08	0.024
Scenedesmus spec.				
Unheated	7.2	–	0.06	–
Heated	7.5	0.063	0.05	0.003
Unheated-EDTA	6.8	0.066	0.06	0.004
Heated-EDTA	7.4	0.065	0.05	0.003
Unheated-EDTA-heated	7.5	0.059	0.05	0.003
Chlorella spec.				
Unheated	6.2	0.153	0.06	0.009
Heated	6.9	0.170	0.06	0.010
Unheated-EDTA	8.4	0.180	0.05	0.009
Heated-EDTA	8.5	0.185	0.05	0.009
Unheated-EDTA-heated	7.9	0.140	0.05	0.007

2 1.5. *Substrates*

The substrates chosen for chymotrypsin: soybean *(Glycine max.* L.), baker's yeast *(Saccharomyces cerevisiae)* and the green algae *(Scenedesmus spec.* and *Chlorella spec.).*

2.1.5.1. Soybean

Soybean seeds, imported from China were purchased from N.V. Speelman's Oliefabrieken, Overschie. The variety 'Acme' was obtained from the Department of Agronomy and Plant Genetics, Institute of Agriculture, Minnesota, USA. Results obtained with these varieties were identical.

Cotyledons. Prior to use, soybean seeds were soaked for 20 hours in 20 parts of distilled water (w/v) and dehulled.

Unheated cells. EDTA at pH 6 or 8, sodium hexametaphosphate at pH 4 and glycine-NaOH buffer (0.1 M pH 10) failed to macerate the cotyledons. Maceration was effected by EDTA at pH 10 in the following way. Cotyledons (100 g wet weight) were kept suspended in 3.8 % EDTA solution containing 0.1 % Preventol 115, pH 10 (1 litre) at 37 °C for two days. After that period the pH was 9.7. After decanting the liquid the softened cotyledons were suspended in 0.1 M borate buffer pH 8 and desintegrated with the aid of a 'Vibromischer' (Chemie-Apparatebau A.G., Zürich) to obtain a suspension of single cells.

The cell suspension obtained was centrifuged and the cells washed with 0.1 M borate buffer pH 8 several times and stored in 0.1 M borate buffer pH 8 containing 0.1 % Preventol 115.

Heated cells. Cells obtained by EDTA maceration were heated in distilled water at 100 °C for 5, 10, 15 or 20 minutes.

Cells of heated seeds. The maceration procedure was applied to seeds which had been heated in distilled water for 5, 10, 15 or 20 minutes.

Unheated sections. Sections of 100 µ were cut as shown in fig. 3. The sections were washed several times with 0.1 M borate buffer pH 8 to remove the cell contents from disrupted cells. They were stored in 0.1 M borate buffer pH 8 containing 0.1 % Preventol 115 at 5 °C for 16 weeks. Each week this buffer was renewed to remove the proteins which were gradually extracted by the buffer, probably from broken cells.

Heated sections. These sections were cut from cotyledons which just before sectioning had been heated in distilled water at 100 °C for 20 minutes. They were washed and stored in the same way as unheated sections.

Sections-EDTA (unheated and heated). After 16 weeks of storage the sections (1 part) were submitted to the action of 3.8 % EDTA with 0.1 % Preventol 115, pH 10, (10 parts) for two days. EDTA was removed by rinsing carefully with 0.1 M borate buffer pH 8. Then the experiments were carried out immediately without storing for a long time or shaking the sections (to avoid damaging them).

Soybean powder (subcellular). Soybean powder was obtained by ball-milling vacuum-dried cotyledons in the presence of solid carbon dioxide at –30 °C for 30 minutes. The powder was sieved through a 23 mesh sieve. Microscopical inspection showed that the material passing through the sieve did not contain

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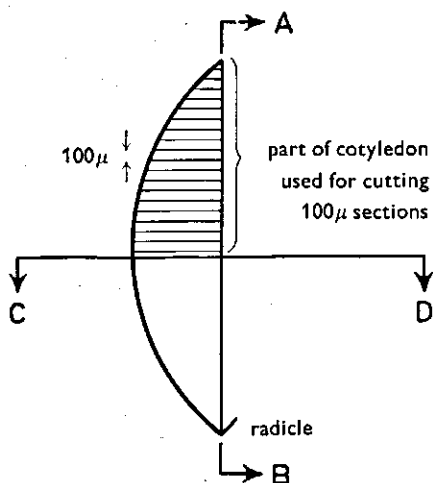
FIG. 3. *Diagram showing how soybean was sectioned.* Soybean sections with a thickness of 100μ were obtained with the aid of the microtome by cutting the upper part of the soybean cotyledons parallel with the plane C-D. The lower part of the cotyledons containing the radicles was discarded.

From microphotographs of sections cut parallel with the plane A-B it was deduced that the 100μ sections have a proportion of 1 part of damaged cells to 3 parts of undamaged cells.

whole cells. The powder was stored at 5°C in a desiccator over silicagel. Portions of this powder (P) were treated in the following way, giving several types of powder:

Powder A. Treatment with 10 parts (w/v) of 3.8% EDTA solution pH 10 with 0.1% Preventol 115, at 37°C for two days *(unheated-EDTA)*. A portion treated with 10 parts (w/v) of 0.1 M borate buffer pH 10 with 0.1% Preventol 115, at 37°C for two days served as a blank *(unheated-control)*.

After centrifuging, the powders were stirred with 5 parts of 0.1 M borate buffer pH 8 for 30 minutes and again centrifuged. Washing was repeated three times after which all the EDTA had been removed; this could be shown by the cobalt method (SEN SARMA, 1960). The centrifuged powder was stored at 5°C in the same buffer to which calcium chloride was added (final concentration 0.005 M).

Portions of unheated-EDTA and unheated-control were heated in 5 parts of 0.1 M borate buffer pH 8 at 100°C for 20 minutes under reflux; in this way *heated-EDTA* and *heated-control* were prepared.

Before the digestion experiments with chymotrypsin the powder suspensions were diluted to obtain a concentration of 20%, checked with the aid of the hematocrit.

Powder B. Powder P was heated at 120°C for 120 minutes in an autoclave and the same steps as for powder A were made resulting in the preparations *heated-EDTA* and *heated-control*.

Powder C. Powder P was extracted twice with acetone at room temperature to remove the lipids and the same steps as for powder A were followed.

Powder D. The powder P was extracted twice with 10 parts (w/v) of 80% ethanol at 55°C for 24 hours. After each extraction the suspension was centrifuged (11,000 g) at 5°C for one hour. The sediment was suspended in distilled water and stirred for one hour, then part of the suspension was dialysed against

water at 5°C for one night, perevaporated and lyophilised *(unheated ethanol extracted powder)*. The remaining part of the suspension was heated at 100°C for 20 minutes, then dialysed, perevaporated and lyophilised *(heated ethanol extracted powder)*. Perevaporation was effected by hanging the dialysis tube containing the suspension in a thermostat at 30°C with forced ventilation. The constant air current caused rapid evaporation.

Both lyophilised powders were suspended in 0.1 M borate buffer pH 8 to a concentration of 20% determined with the hematocrit, prior to the experiment.

Powder E. Powder B was extracted twice with 80% ethanol at 55°C, surpended in distilled water, perevaporated and lyophilised, then suspended in 0.1 M borate buffer pH 8, to a concentration of 20% controlled with the aid of the hematocrit.

Powder F. Powder P was extracted twice with 80% ethanol at 5°C instead of 55°C and the same steps as for powder D were followed.

Powder G. Powder P was treated with 10 parts of 3.8% EDTA solution pH 10 containing 0.1% Preventol 115, at 37°C for two days. A blank was prepared by treating a portion of powder P with 0.1 M borate buffer pH 10 containing 0.1% Preventol 115, at 37°C for two days. Subsequently both suspensions were submitted to dialysis for two days against distilled water containing 0.1% Preventol 115 and two days more against 0.1M borate buffer pH 8 with 0.1% Preventol 115. After 4 days of dialysis at 5°C, the powder suspensions had a pH of 8. A part of each suspension (EDTA and control) was heated under reflux at 100°C for 20 minutes in the same buffer which remained at pH 8. Thus the respective heated powders were obtained.

Powder H. The powder P was suspended directly in 0.1 M borate buffer pH 8 without dialysis and without centrifuging. Part of the suspension was heated under reflux in the same buffer at 100°C for 20 minutes. In this way the unheated-control and the heated-control were obtained.

2.1.5.2. Yeast

Baker's yeast was obtained from the 'Koninklijke Nederlandse Gist en Spiritusfabriek', Delft.

The yeast was stored in 0.1 M phosphate buffer pH 7.6 containing 0.1% Preventol 115, instead of in borate to avoid flocculation of the yeast cells (JANSSEN, 1958). Unless otherwise stated the yeast cells used in the experiments were dead. The unheated cells died during storage in 0.1 M phosphate buffer pH 7.6 containing 0.1% Preventol 115. That the cells were dead was demonstrated with the phosphate methylene blue reaction (JøRGENSEN-HANSEN, 1948). Furthermore it was found that after inoculation of the cells in petri-dishes with malt-agar no growth occurred at 30°C.

During the experiments it was found that the period of storage of the heated yeast cells was important. Therefore experiments were performed with cells which had been stored at 5°C for a short time (6–8 days, STS) and with cells which had been stored for a rather long time (25–30 days, LTS).

The following preparations were made:

Unheated yeast-STS. The yeast was stored in 0.1 M phosphate buffer pH 7.6 with 0.1% Preventol 115 for one week and when stored for 25–30 days *unheated yeast-LTS* was obtained.

Heated yeast-STS. The yeast was heated in distilled water at 100°C for 20 minutes and stored in 0.1 M phosphate buffer pH 7.6 with 0.1% Preventol 115 for one week. When stored for 25–30 days *heated yeast-LTS* was obtained.

Unheated yeast (STS or LTS)-EDTA. The same procedure was used as for isolated soybean cells. EDTA was removed by washing with 0.1 M phosphate buffer pH 7.6. To obtain *heated yeast-STS-EDTA* the same procedure as for unheated yeast-EDTA was followed using heated yeast-STS.

Unheated yeast-EDTA-heated. The cells were treated as unheated yeast-EDTA then EDTA was removed by washing with distilled water. The cells were heated in distilled water at 100°C for 20 minutes, after which they were washed 3 times with 0.1 M phosphate buffer pH 7.6.

Live yeast. Yeast received freshly from the factory was suspended in 0.1 M phosphate buffer pH 7.6 and immediately used for the digestion by chymotrypsin.

2.1.5.3. Algae

Scenedesmus spec. and *Chlorella spec.* were cultivated at room temperature in a solution with macro and micro nutrients according to SANWAL (1963) without sterilization, provided with light by TL fluorescent tubes, Philips 20 W/33, and with a mixture of air $+5\%$ CO_2. Each week the algae were harvested. For the preparation of EDTA-Fe solution the prescription of WARRIS (1953) was followed.

For the preparation of the medium twice distilled rain water was used, since it was found that once distilled tap water caused poor growth. The latter may be due to the high chlorophenol content of tap water.

The harvested algae were stored in 0.1 M borate buffer pH 8 with 0.2% Preventol 115, in which they soon died *(unheated algae)*. This was verified by rinsing the algae with twice distilled water to remove Preventol 115, and incubating them again in the same conditions as previously mentioned. No growth occurred.

Heated algae were obtained by heating a suspension of algae in distilled water at 100°C for 20 minutes.

Algae-EDTA were obtained by treatment with EDTA as described for soybean and yeast cells.

In this way the following preparations were obtained:
unheated algae, heated algae, unheated algae-EDTA, unheated algae-EDTA-heated and heated algae-EDTA.

2.1.6. *Digestion of the proteins in the substrates by chymotrypsin*

The degree of digestion of the proteins in a substrate effected by chymotrypsin was estimated by determining the increase in extinction at 280 mμ of the solution obtained after removal of the substrate. The value of the extinction of this

solution is proportional to the amount of aromatic compounds present in the solution. The aromatic compounds are released from the substrate by the enzyme and are mainly the amino acids, tryptophan, tyrosine and phenylalanine and peptides containing these amino acids. Since in each substrate the amino acid composition of the proteins is constant the amount of aromatic amino acids and peptides is proportional to the total amount of the products of hydrolysis of the proteins of the substrates under consideration. Therefore the extinction at 280 mμ of the solution is proportional to the amount of the products of hydrolysis of the proteins liberated into the solution.

In addition the nitrogen content of the solutions was determined.

In order to avoid the substrate being a limiting factor the optimum substrate concentrations were determined for soybean cells and for algae-EDTA by plotting the digestion during the first 20 minutes against the substrate concentration. For algae-EDTA the digestion during the first 60 minutes was used. From the results obtained with soybean cells, satisfactory concentrations for soybean sections and for yeast cells could be deduced.

Prior to the digestion experiments the substrate suspensions were washed until the extinction of the supernatant was below 0.100 to be sure that only negligible amounts of proteins were outside the cells. Soybean cells and sections and algae were washed with 0.1 M borate buffer pH 8, while yeast cells were washed with 0.1 M phosphate buffer pH 7.6. The antimicrobial compound Preventol 115 was not used during washing because it had been found capable of extracting substances (probably proteins) from the cell material that absorbed at 280 mμ. Soybean cells were washed in batches until the supernatant of a suspension of 20% concentration controlled with the hematocrit (v/v) had a satisfactorily low extinction at 280 mμ. Soybean sections were washed for 16 hours in a Soxhlet-type extractor described schematically in fig. 4. The yeast cells were kept in buffer overnight at 35°C and were then washed by stirring the centrifuged cells for 30 minutes followed by centrifuging (800 g for 15 minutes). This process was repeated 3 times. The algae were treated similarly until the extinction of the supernatant was satisfactorily low.

2.1.6.1. Digestion of soybean cells

The washed soybean cell suspension was diluted to a concentration of 20% in 0.1 M borate buffer pH 8. To 70 ml of this suspension (containing 2.45 g dry material) 9 ml borate buffer was added and the mixture stirred in a water-bath at 35°C. After 30 minutes chymotrypsin solution (1 ml) was added. A blank was prepared by adding borate buffer (1 ml) instead of enzyme solution to the cell suspension. All suspensions were stirred at the same rate.

After various intervals approximately 5 ml samples were taken from the suspension with a pipette with a wide orifice. The samples were directly and rapidly filtered through a fritted glass-filter G3. The extinction of the filtrate was determined directly in the spectrophotometer at 280 mμ.

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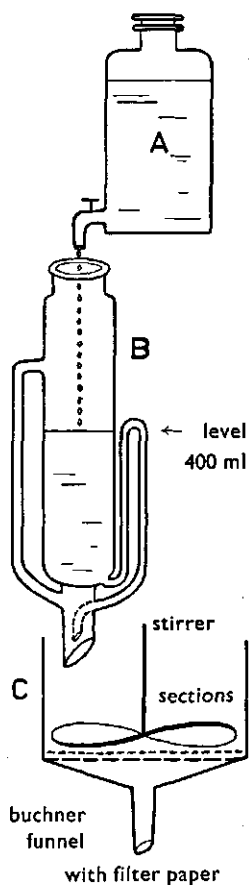
FIG. 4. *Diagram of the apparatus used for washing the sections.*
The 0.1M borate buffer pH 8 dripped from the container A into
the Soxhlet accessory B until it reached the upper level on the
siphon. Then the buffer solution (400 ml) was released into the
buchner funnel with filter paper containing the sections. The
suspension of sections in the buffer solution was filtered while
being stirred. In the meantime a new volume of buffer accumu-
lated in the Soxhlet.

For determination of nitrogen the filtrates were acidified by adding a drop of
concentrated sulphuric acid to avoid bacterial growth and stored at 5°C over-
night.

An experiment was also made in which the sample solutions, instead of being
read directly at 280 mμ were first treated with 5% TCA (2 ml of filtrate + 3 ml
5% TCA) to remove proteins; after one hour the mixtures were centrifuged for
30 minutes at 700 g and the supernatants read in the spectrophotometer. This
experiment was done for comparison with digestion of powder suspensions.

2.1.6.2. Digestion of soybean sections

After washing the sections (wet weight 14.7 g, dry weight 2.5 g) borate buffer
was added until the volume was 75 ml. Then chymotrypsin solution (5 ml) was
added and hydrolysis allowed to proceed under the conditions described above
for cells. The five-fold amount of chymotrypsin as compared with the amount
used in the experiments described under 2.1.6.1. was applied to compensate for
the difference in the ratio surface/enzyme.

For sampling it was found convenient to stop stirring for 15 seconds in order to allow the sections to sink to the bottom. Then the sample was taken from the supernatant and stirring was resumed. The sample was filtered through a fritted glass-filter followed by direct reading of the extinction. Then the sample was immediately returned to the reaction mixture.

For sections-EDTA the same procedure was followed except for the proportions which were reduced by a factor 10 to save material. Readings at 280 mμ were done in cuvettes of 1 mm, therefore the extinction was multiplied by 10 to be comparable with the results of a 1 cm cuvette.

2.1.6.3. Digestion of soybean powder (subcellular)

With the substrates mentioned in the preceding paragraphs the extinction of the filtered sample was directly determined. This could be done because the appearance of the products in the medium was attributable to the action of chymotrypsin. With powder, in which the cells were completely broken up, the filtered digest contained low-molecular material resulting from the hydrolysis of proteins by chymotrypsin in addition to intact and partly hydrolysed protein. Since all of these contribute to the extinction of the solution it was necessary to remove the intact proteins in order to reveal the effect of the enzyme on the substrate. This was done by precipitating the proteins with TCA. This modification permitted us to avoid washing the powder suspension to reach a low extinction at 280 mμ before digestion.

The suspension for an experiment was prepared and adjusted to 20% concentration with the aid of the hematocrit. The technique used was similar to the determination of chymotrypsin activity (page 8, paragraph 2.1.2.3.). Instead of casein solution, soybean-powder suspension was used.

2.1.6.4. Digestion of yeast

The proportions chosen for the experiments with yeast were the same as for soybean cells because the nitrogen content of yeast is very similar to that of soybean cells (table 3, column 1, on page 11). 70 ml yeast suspension contained 3.7 g dry material. The ratio cell surface/enzyme for yeast cells is larger than that for soybean cells because the former are considerably smaller than the latter.

The procedure and precautions were the same as for soybean cells except for the following slight modification. The samples taken for reading in the spectrophotometer were centrifuged before filtration to avoid the formation of a cake on the fritted glass-filter G4. The samples were stored at 5°C, in order to reduce the hydrolysis by chymotrypsin. Next day the samples were centrifuged for 30 minutes at 700 g and the supernatants filtered through a fritted glass-filter G4 and the filtrate read at $E_{280 \, m\mu}^{1 \, cm}$.

2.1.6.5. Digestion of algae

The procedure was the same as with yeast, except for Chlorella-EDTA; before filtering the sample it was necessary to centrifuge at 11,000 g instead of

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700 *g* in order to obtain a clear filtrate. The following proportions were used:

Scenedesmus spec.

The 2% cell suspension (70 ml) contained 0.70 g dry material and was mixed with borate buffer (9 ml) and chymotrypsin solution (1 ml). The blank was the same solution without enzyme. The final volume was 80 ml in all experiments.

Chlorella spec.

The 10% cell suspension (70 ml) contained 1.75 g dry material and was mixed with borate buffer (9 ml) and chymotrypsin solution (1 ml). The blank was the same solution without enzyme. The final volume was 80 ml in all experiments.

The optimum substrate concentration was determined as described for soybean cells, using an incubation period of 60 minutes, except for the quantities of suspension and solutions all of which were reduced fourfold (final volume 20 ml). Since this final volume had to be sufficient for taking 13 samples (from zero to 120 minutes, with intervals of 10 minutes) each sample was about 1 ml. We were able to take samples of such small volume, because a. for the extinction determinations we employed cuvettes of 1 mm light path instead of 1 cm. With the former cuvettes 0.25 ml of filtrate was sufficient for use in the spectrophotometer, whereas for the latter it was necessary to have at least 3 ml of filtrate; b. the filtration of the samples to obtain the respective filtrates was effected through a G4 fritted glass-filter of small diameter, specially made to prevent a considerable part of the filtrate from remaining within the filter plate.

In cases where additional 5 ml portions of filtrate were needed for the nitrogen determinations (after 0, 50 or 120 minutes of digestion) the experiments were made with final volumes of 40 ml.

2.2. RESULTS OF THE EXPERIMENTS ON THE DIGESTION OF THE PROTEINS IN THE SUBSTRATES BY CHYMOTRYPSIN

The results of the chymotrypsin digestion experiments are presented in graphs, where the extinction at 280 mμ of the filtrate of the digestion mixture is recorded as a function of digestion time.

In addition to some of these experiments others were carried out to determine the nitrogen content, and the results of these are also presented graphically. In the latter case the quantity of nitrogen (mg) per 100 ml of the filtrate is recorded as a function of digestion time. This was done as a control on the spectrophotometric method.

2.2.1. *Determination of the optimal ratio of enzyme quantity to amount of soybean suspension*

Unheated cells. First the amount of 20% cell suspension was kept constant (20 ml) while the amount of enzyme solution was varied (1, 2 or 4 ml); the mixtures were made up to a final volume of 80 ml with 0.1 M borate buffer pH 8. The same mixture with 0.1 M borate pH 8 instead of chymotrypsin solution

served as a blank. From the results corrected for the blank (fig. 5a) it could be concluded that under the conditions chosen a quantity of 1 ml of chymotrypsin solution was satisfactory. With this quantity of enzyme solution a series of determinations were carried out in which the amount of 20% cell suspension varied (20, 50, 60 or 70 ml). The mixtures were made up to a final volume of 80 ml with 0.1 M borate buffer pH 8; the incubation time was 20 minutes. Apart from the blanks consisting of cell suspensions without enzyme solution, the chymotrypsin solution (1 ml) was incubated with the supernatants of the cell suspension. As the extinctions were very low they were ignored. In fig. 6 the extinctions of digests of unheated cells are presented, corrected for the blanks.
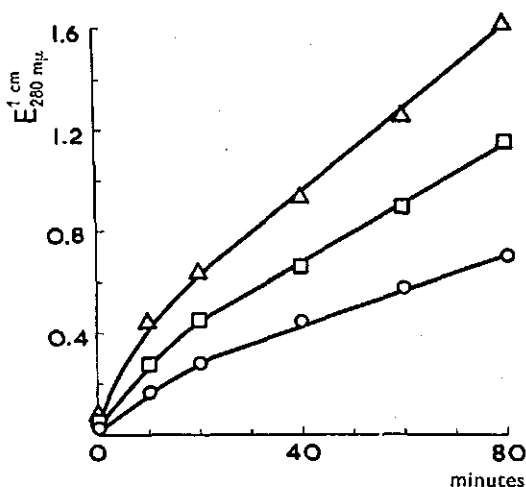
FIG. 5a. Twenty per cent unheated soybean cell suspension (20 ml) incubated with different quantities of chymotrypsin solution. The data are corrected for appropriate blanks. 1 ml (O——O), 2 ml (□——□) and 4 ml (△——△) chymotrypsin solution.
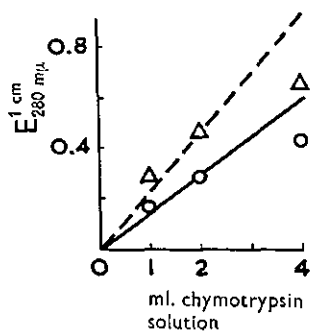
FIG. 5b. The extinction values of fig. 5a, obtained after 10 (O——O) or 20 minutes (△----△) of digestion are recorded as a function of the amount of chymotrypsin solution.

Heated cells. The amount of enzyme solution was kept constant (1 ml) and the amount of 20% heated cell suspension was varied (20, 40, 60, 70 or 75 ml); and the mixtures were made up to a final volume of 80 ml with 0.1 M borate buffer pH 8; the incubation time was 20 minutes. The respective blanks were similar except for the enzyme solution for which was substituted 1 ml 0.1 M

borate buffer pH 8. In fig. 6 the extinctions corrected for the appropriate blanks are recorded.

As a result of these preliminary determinations it was decided to use 70 ml cell suspension of 20% concentration as the optimum substrate for heated and unheated cells in all subsequent experiments.
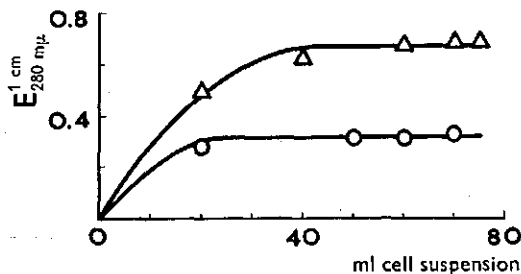
FIG. 6. *Determination of the optimal ratio of enzyme to soybean cell suspension.* Chymotrypsin solution (1 ml) was incubated for 20 minutes at 35°C pH 8, with different amounts of 20% cell suspension. Unheated cells (O——O), heated cells (△——△). The data are corrected for appropriate blanks.

2.2.2. *Digestion of soybean cells by chymotrypsin*

Unheated cells. In fig. 7 we see that right from the start of the experiments the substrate present within the cells is digested by chymotrypsin. The blank is a horizontal line. Fig. 8 shows the data of the nitrogen determinations. Here the curve is similar to the curve of fig. 7. Again the blank is a horizontal line.

Heated cells and cells of heated seeds. Fig. 7 shows the digestion of cells heated for 20 minutes; again the blank is a horizontal line. Identical results are obtained when cells are heated for 5, 10, 15, 20 or 25 minutes. Likewise there is no difference in the results obtained with cells from seeds which had been heated for 5, 10, 15 or 20 minutes. The nitrogen determinations (fig. 8) show a close correlation with the spectrophotometric data.

In the period of digestion between 20 and 80 minutes the graph presents a rather straight line. During this time the increment of the extinction for each 10 minute interval is between 0.07 and 0.09 for unheated cells, while for heated cells and for cells of heated seeds it is between 0.18 and 0.20.

In table 4 we give the amount of nitrogen released from unheated and heated soybean cells (in per cent). With an 80-minute period of digestion this is 9.8% in the case of unheated cells and 22.2% for heated cells.

Digestion of soybean cells by chymotrypsin and determination of the extinction at 280 mμ in TCA treated samples

The extinctions of the TCA-treated liquid phases of the digests of unheated cells are recorded in fig. 9. Again the blanks form a horizontal line. The results of a similar experiment with heated cells are also presented in fig. 9. Here too the blank is a horizontal line.

The increment of the extinction per 10 minute interval between 20 and 80 minutes is 0.02 for unheated cells and 0.04 for heated cells.
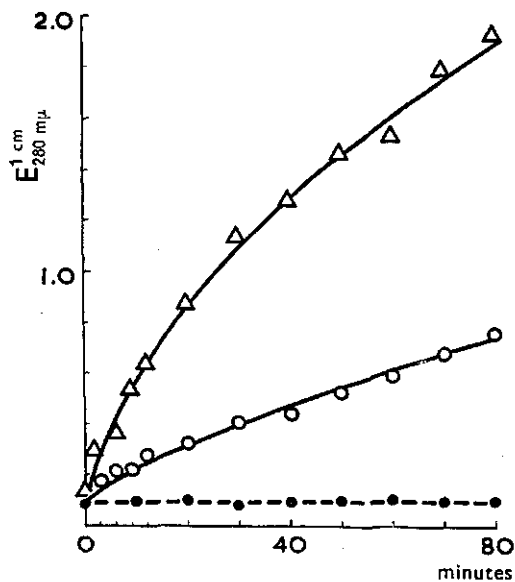
FIG. 7. *Digestion of soybean cells by chymotrypsin.* 20% soybean cell suspension in 0.1M borate buffer pH 8 (70 ml) plus 9 ml of the same buffer plus 1 ml enzyme solution. Unheated cells (O——O), heated cells (△——△). The same mixtures with 1 ml 0.1M borate buffer pH 8 instead of chymotrypsin solution served as blanks. Blanks of unheated and heated cells have practically identical values (●- - -●)
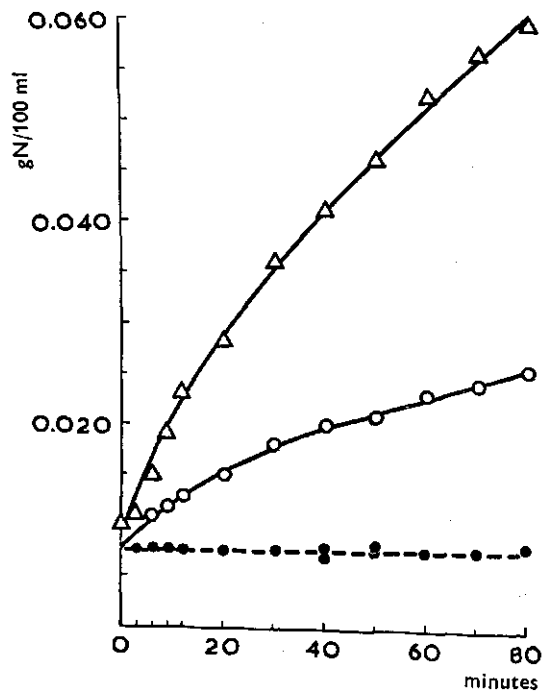
FIG. 8. *Digestion of soybean cells by chymotrypsin.* Nitrogen determination of filtrates from the samples obtained from the mixtures described in fig. 7. Unheated cells (O——O), heated cells (△——△). The blanks of unheated cells are similar to those of heated cells (●- - - -●).

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TABLE 4. Amount of nitrogen released from unheated and heated soybean cells by α-chymotrypsin.

Sample minutes	Unheated soybean cells (%)	Heated soybean cells (%)
0	0	1.1
3	–	1.6
6	1.6	3.5
9	2.2	4.9
12	2.7	6.9
20	3.8	9.1
30	5.5	12.6
40	6.6	14.7
50	7.1	16.8
60	8.2	19.1
70	8.7	21.0
80	9.8	22.2

2.2.3. *Digestion of soybean sections by chymotrypsin*

This experiment was made to exclude any influence of EDTA on the permeability of the cell walls.

Fig. 10 shows the results obtained with unheated sections and with heated sections, both digested by chymotrypsin. The increment per 10 minute interval was for the unheated sections 0.09 and for the heated sections 0.40. This means that the increment for the heated sections is 4.4 times greater than the increment for the unheated sections.
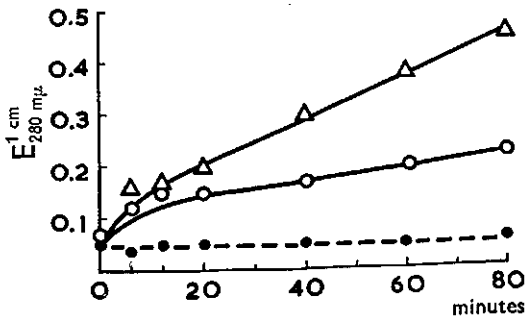
FIG. 9. *Digestion of soybean cells by chymotrypsin and determination of the extinction at 280 mμ in TCA treated sample.* Unheated cells plus chymotrypsin (O——O), heated cells plus chymotrypsin (△——△). Blanks of unheated cells are similar to blanks of heated cells (●- - -●).

The nitrogen content of samples after a period of digestion of 120 minutes was 3 mg per 100 ml for unheated sections and 44 mg per 100 ml for heated sections.

The nitrogen released from the substrate by α-chymotrypsin is 1.7% for unheated sections and 23.9% for heated sections. In reality the value 1.7% for unheated sections should be divided by a factor 5 to compare roughly with the unheated cells, since in the experiments with the former the quantity of enzyme solution used was 5 times that of the latter. The fact that after 120 minutes 0.4%

of the nitrogen of unheated sections was present in the filtrate while after 80 minutes this figure was 9.8 % in the case of unheated cells indicates that chymotrypsin penetrates the sections only very slightly.
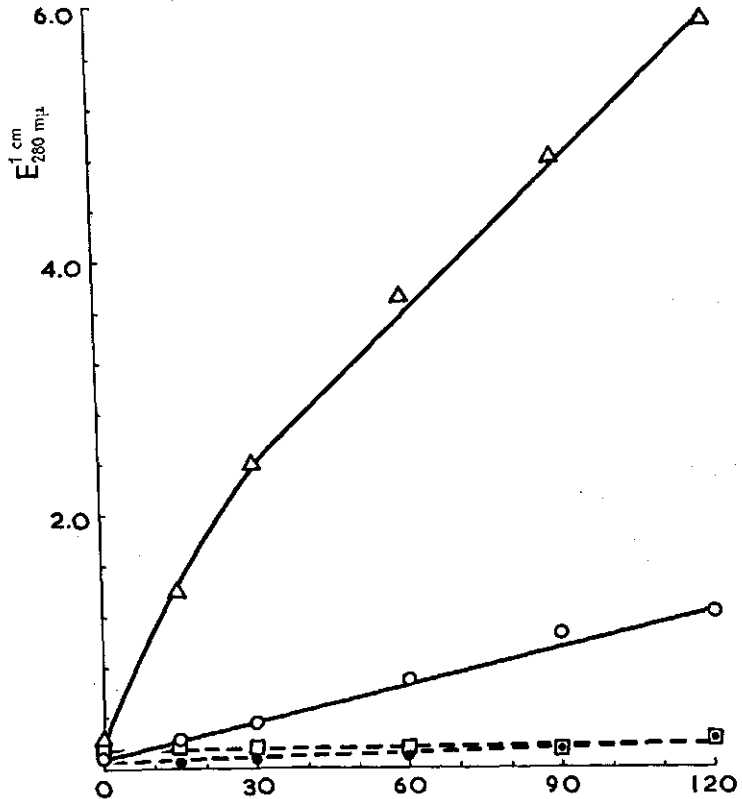
FIG. 10. *Digestion of soybean sections by chymotrypsin.* Unheated sections plus chymotrypsin (O——O), blank (●- - -●). Heated sections plus chymotrypsin (△——△), blank (□- - -□).

Digestion of sections-EDTA by chymotrypsin

In order to be able to compare the results of the digestion of (EDTA-treated) cells with (not-EDTA-treated) sections it was necessary to perform a digestion experiment with EDTA-treated sections.

Fig. 11 records the results of digestion of unheated sections-EDTA and heated sections-EDTA. The increment per 10 minutes for unheated sections-EDTA is 0.17 while in the case of heated sections-EDTA it is 0.29; so the increment per 10 minutes for the heated -EDTA is 1.7 times as high as unheated-EDTA.

The nitrogen content per 100 ml of the sample is 27 mg for the unheated sections-EDTA and 49 mg for heated sections-EDTA. In the former case the

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amount of nitrogen released from the substrate by α-chymotrypsin is 18%, while in the latter it is 23%, both after 120 minutes of digestion.

In the unheated condition the effect of EDTA treatment is indeed large, the amount of nitrogen released from the substrate by α-chymotrypsin being 18% in EDTA treated sections and 1.7% in untreated sections. Comparison of heated sections (23.9%) and heated sections-EDTA (23%), shows that once the material has been heated EDTA no longer has an effect. It is concluded that EDTA has a marked influence only on unheated material. That the result of the digestion of unheated-EDTA (18%) is rather similar to that of heated (23.9%), allows the conclusion that heating in distilled water affects the permeability of the cell wall and membranes in rather the same way as EDTA does.
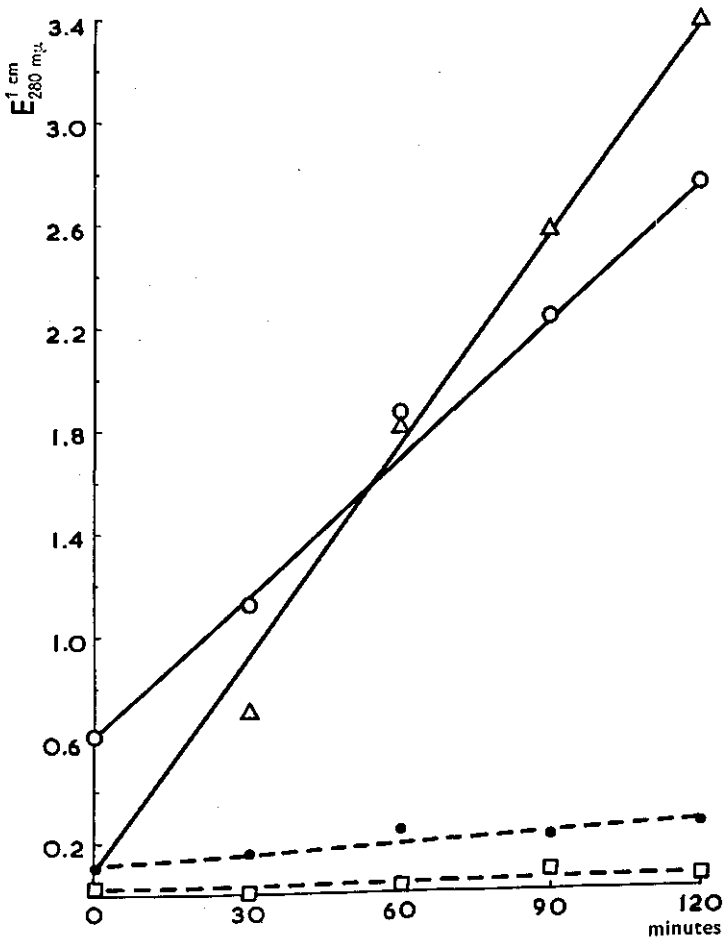
FIG. 11. *Digestion of soybean sections-EDTA by chymotrypsin.* Unheated sections-EDTA plus chymotrypsin (○——○), blank (●- - -●). Heated sections-EDTA plus chymotrypsin (△——△), blank (□- - -□).

2.2.4. *Digestion of soybean powder (subcellular) by chymotrypsin*

This experiment had the following objectives: a. to learn whether a soybean substrate which is not enclosed by the cell wall would be more susceptible to chymotrypsin after treatment with EDTA; b. to determine the increments per 10 minutes during digestion of unheated and heated powders and to see if the differences are comparable to those found in the experiments with other soybean preparations (cells, sections-EDTA)

Powder A: unheated-EDTA, heated-EDTA, unheated-control, heated-control. A 20% suspension was not digested when chymotrypsin was used. Since an excess of substrate can sometimes inhibit the enzyme, the concentration of the suspension was decreased to 1%; however, no digestion occurred.

Since neither unheated powder (EDTA and control) nor heated powder (EDTA and control) of the type A was digested by chymotrypsin it was considered possible that contamination of the powder with metal originating from the ball-mill was the cause of this inhibition. Therefore casein was submitted to ball-milling and a casein solution was prepared and used as a substrate for chymotrypsin as described on page 8, paragraph 2.1.2.3. Results of the experiments are shown in table 5.

TABLE 5.

Tube no	NORMAL CASEIN $E_{280\,m\mu}^{1\,cm}$				BALL-MILLED CASEIN $E_{280\,m\mu}^{1\,cm}$			
		Blank	Net value	Average		Blank	Net value	Average
1	0.82	0.081	0.74		0.86	0.088	0.77	
				0.76				0.77
duplo	0.85	0.069	0.78		0.84	0.077	0.76	

It is obvious that there is no difference between non-treated casein and casein submitted to ball-milling. Consequently ball-milling is not responsible for the inhibition of digestion by chymotrypsin.

The *powders B* and *C* were likewise not digested by chymotrypsin.

Powders D (unheated and heated ethanol-extracted powder) were both digested by chymotrypsin. The inhibitor was not inactivated by the heat treatment (compare powder B) but it was extracted by 80% ethanol at 55°C. This extraction was made in accordance with the method devised by BIRK et al. (1963) to remove the thermostable proteinase inhibitor of soybean.

There was practically no difference between the unheated and heated powder of this type. This can be explained by the denaturing action of ethanol at 55°C resulting in denatured protein in the unheated preparation, while the heated powder contained protein which had been denatured by heat.

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Powder E: heated-EDTA and heated-control were digested by chymotrypsin. It should be noted that the powder heated at 120 °C (powder B) was not digested, whereas when the same heated powder was extracted twice with 80 % ethanol at 55 °C (powder E) it could be digested.

Powder F: was not digested by chymotrypsin.

Powder G: unheated powder (EDTA and control) heated powder (EDTA and control) were digested by chymotrypsin (fig. 12). For the calculation of the increment per 10 minutes we considered the digestion period between 20 and 60 minutes appropriate.

The increment per 10 minutes from 20 to 60 minutes is for:

Unheated powder-EDTA   0.03
Heated powder-EDTA   0.06
Unheated powder-control   0.03
Heated powder-control   0.06

This shows that once the cell wall is not a barrier to the cell contents (subcellular powder) EDTA no longer has a function. Further it is concluded that when the barrier is removed the increment per 10 minutes for heated material is twice as large as in the case of unheated. Besides, this experiment demonstrates that EDTA does not make the substrate more susceptible to chymotrypsin (unheated EDTA and control present the same increment per 10 minutes and the same occurs with heated EDTA and control).

These experiments (powder G) are of importance for comparisons with unheated and heated cells and sections.
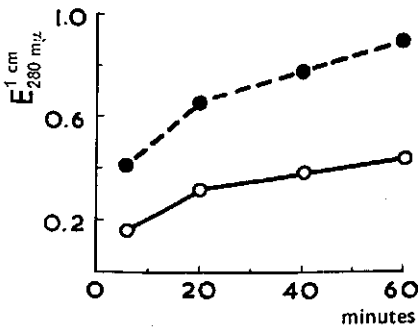
FIG. 12. *Digestion of powder G by chymotrypsin.* Unheated-EDTA and unheated-control have similar values (O——O). Heated-EDTA and heated-control have also similar values (●- - -●). The data are corrected for appropriate blanks.

Powder H: unheated-control and heated-control were digested by chymotrypsin. The increments per 10 minutes of these powders from 20 to 60 minutes were 0.06 for unheated-control and 0.13 for heated-control; so the increment per 10 minutes of heated powder is twice as large as that of unheated powder. On comparing the unheated-control of powder H with the unheated-control of powder G (0.06 and 0.03 respectively), and the heated-control of powder H with the heated-control of powder G (0.13 and 0.06 respectively) we find that the increment of powder H is larger than that of powder G. This implies that through

dialysis we lost low molecular compounds which can be attacked by chymo-trypsin. It is not possible to use the results of our experiments with powder H for comparison with other results, because when the powder is treated with EDTA the latter must be removed and therefore dialysis must be applied. This inevitably results in removal of part of the substrate.

2.2.5. *Digestion of yeast by chymotrypsin*

All the data for yeast as well as for yeast-EDTA are corrected for appropriate blanks.

The results of the digestion experiments with unheated yeast (STS and LTS), live yeast, and heated yeast (STS and LTS) are recorded in fig. 13 (extinction values at 280 mμ). Nitrogen determinations were made after 0, 50 and 120 minutes (fig. 14). We observe that there is a correlation between the spectropho-tometric and nitrogen determinations. The amounts of nitrogen released from yeast as well as from yeast-EDTA by α-chymotrypsin are shown in table 6.

Observing the spectrophotometric curves of fig. 13 we see that neither unheat-ed yeast STS and LTS, nor live yeast is digested by chymotrypsin. They all present a horizontal line. Heated STS presents a sigmoid line while LTS is digested at a fairly constant rate which is definitely higher than that of STS. The sigmoid shape of the line of STS indicates that this material still possesses a barrier after being heated. Observing fig. 14 (nitrogen determinations) we see that there is a very slight difference between unheated yeast-STS and LTS whereas the difference between heated-STS and LTS is much larger. This means that storing the yeast has an influence on its permeability; it is very marked with heated yeast while with unheated yeast it is very insignificant.

From our previous experiments with soybean sections we knew that the most impermeable substrate is affected the most intensively by EDTA treatment; we wanted to ascertain whether the same would occur with yeast material which, in spite of the fact that it had been heated, still possessed a barrier. Therefore the following experiments were carried out with yeast STS and not with LTS.

The results of digestion experiments with unheated yeast-EDTA (STS-EDTA and LTS-EDTA had the same results), unheated yeast-EDTA-heated and heated (STS)-EDTA are shown in fig. 15 (extinctions at 280 mμ) and in fig. 16 (nitrogen determinations). We observe that there is a correlation between the spectrophotometric and nitrogen determinations. Unheated yeast-EDTA, heated (STS)-EDTA and unheated yeast-EDTA-heated are all digested by chymotrypsin.

Comparison of the amount of nitrogen released after 120 minutes from un-heated yeast STS (0%) and LTS (0.3%) with that of the same material treated with EDTA (10.1%), and from heated yeast-STS (3.2%) with that of the same material treated with EDTA (8.9%) demonstrates that the most noticeable effect of EDTA was on unheated substrate and to some extent on heated-STS.

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When the amount of nitrogen released after 120 minutes of digestion from un-heated yeast-EDTA (10.1 %) is compared with that of unheated yeast-EDTA-heated (14.7 %) it can be concluded that once the material has been treated with EDTA, the effect of heating is far less important than in material that has not been treated with EDTA.

Furthermore we see that the amount of nitrogen released after 120 minutes of digestion from unheated yeast-EDTA (10.1 %) is similar to that of heated yeast-LTS (10.6 %). This permits the conclusion that heating in distilled water affects the permeability of the cell wall and membranes in rather the same way as EDTA.

TABLE 6. Amount of nitrogen released from yeast and yeast-EDTA by α-chymotrypsin

| Sample | Unheated yeast | | Heated yeast | | Unheated- | Heated (STS)- | Unheated |
minutes	STS	LTS	STS	LTS	EDTA	EDTA	EDTA-heat-ed
0	0	0	0	0.4	1.0	0.9	2.0
50	0	0.3	0.8	5.0	6.2	4.9	9.7
120	0	0.3	3.2	10.6	10.1	8.9	14.7

2.2.6. *Determination of the optimal ratio of enzyme quantity to amount of algae suspension*

The amount of chymotrypsin solution was kept constant (0.25 ml) and the concentration of the unheated algae-EDTA suspension (17.5 ml) was varied (for *Scenedesmus spec.*: 0.5 %, 1 %, 5 %, 10 % or 20 %; for *Chlorella spec.*: 1 %, 5 %, 10 % or 20 %) the mixtures were made up to a final volume of 20 ml with 0.1 M borate buffer pH 8; the incubation time was 60 minutes. The respective blanks were similar except for the enzyme solution, which was replaced by borate buffer pH 8 (0.25 ml).

In fig. 17 the extinctions corrected for the appropriate blanks are recorded as a function of the concentration of the substrate. The readings on the spectrophotometer were made with cuvettes of 1 cm light path.

We observe in fig. 17 that our graphs are similar to those found normally when the optimum ratio of substrate to enzyme is determined. The horizontal part of the line in the case of *Scenedesmus spec.* is from 0.5 % to 10 % concentration, thereafter turning downwards to reach zero at 20 % concentration. It would appear as if above 10 % concentration there is inhibition of the enzyme due to excess of substrate. The same occurs in the case of *Chlorella spec.*, with the difference that the horizontal part of the line is situated between 5 % and 10 %. Therefore for *Scenedesmus spec.* it was decided to use a concentration of 2 % and for *Chlorella spec.* 10 %. The rather low percentage (2 %) of *Scenedesmus spec.* was chosen, because the quantities of available material were limited.
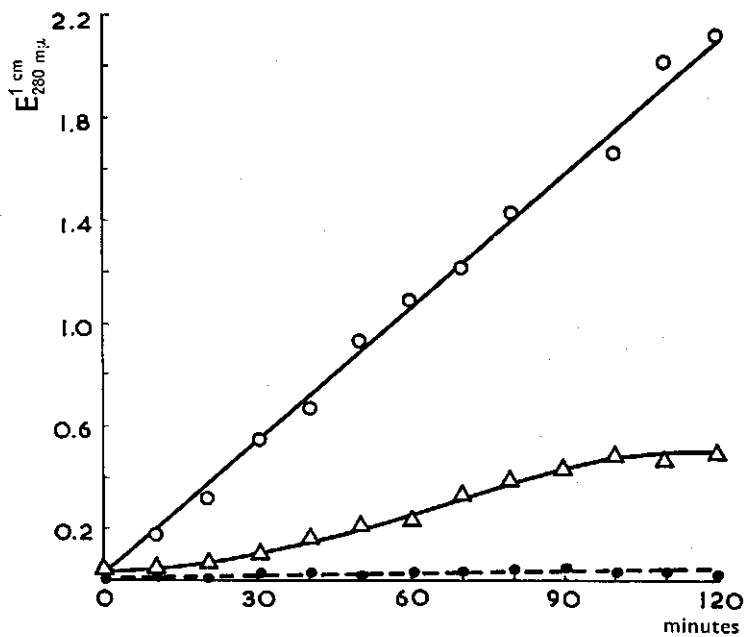
FIG. 13. *Digestion of yeast by chymotrypsin.* Unheated yeast (STS or LTS) and live yeast (●- - -●), heated yeast-STS (△——△), heated yeast-LTS (○——○).

FIG. 14. *Digestion of yeast by chymotrypsin.* Nitrogen determination of filtrates from samples after 0, 50 and 120 minutes of digestion. Unheated yeast-STS (●- - -●), unheated yeast-LTS (□- - -□), heated yeast-STS (△——△) and heated yeast-LTS (○——○).
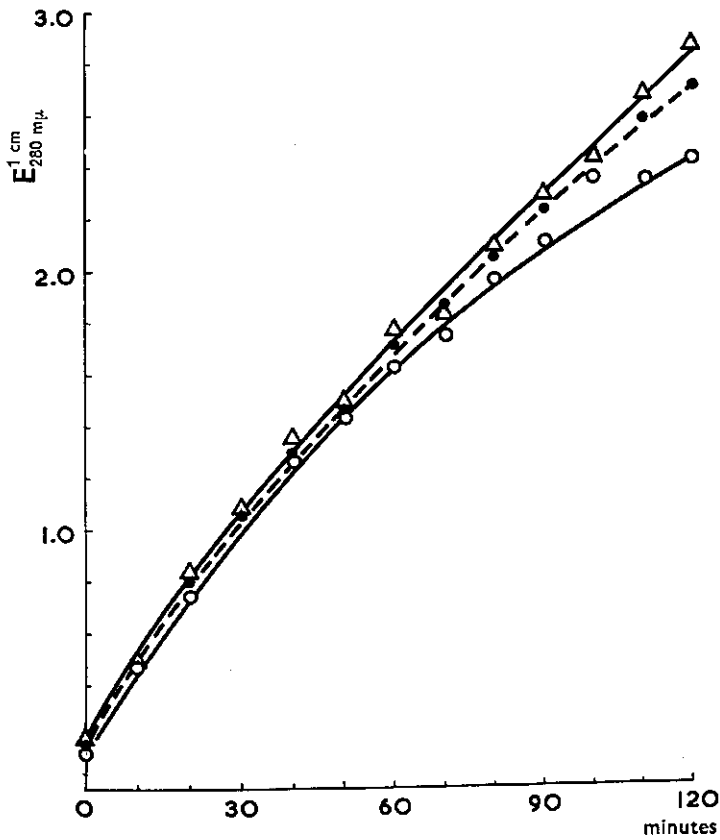
FIG. 15. *Digestion of yeast-EDTA by chymotrypsin.* Unheated yeast (STS or LTS)-EDTA (O——O), unheated yeast-EDTA-heated (△——△), heated yeast (STS)-EDTA (●- - -●).

FIG. 16. *Digestion of yeast-EDTA by chymotrypsin.* Nitrogen determination of filtrates from samples after 0, 50 and 120 minutes of digestion. Unheated yeast (STS or LTS)-EDTA (●——●), heated yeast (STS)-EDTA (O- - -O) and unheated yeast-EDTA-heated (△——△).
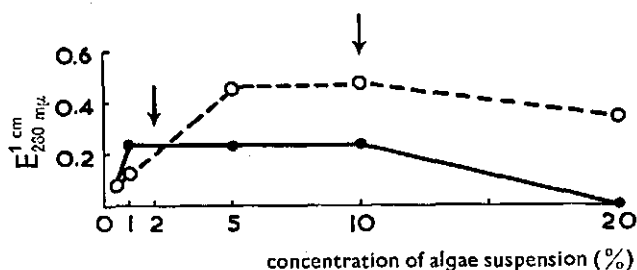
FIG. 17. *Determination of the optimal ratio enzyme quantity to amount of algae.* Chymotrypsin solution (0.25 ml) was incubated for 60 minutes at 35 °C pH 8, with different concentrations of unheated algae-EDTA suspension (17.5 ml). *Scenedesmus spec.* (●——●), *Chlorella spec.* (○- - -○). The arrows (↓) indicate the concentrations chosen for subsequent experiments.

2.2.7. *Digestion of algae by chymotrypsin*

The graphs representing the results of the experiments in which algae were digested by chymotrypsin (extinction at 280 mμ) are based on averages of six values (three duplicate experiments). The maximum deviation of these six values from the mean was ± 10%. In the case of soybean and yeast only one representative result was mentioned, and averages were not made because reproducibility was such that the graphs were very similar. The reproducibility of the results of the experiments with algae is lower than that of soybean or yeast because of the smaller scale of the experiments.

In the case of algae we did not study the effect of storage on the cell wall permeability as was done with yeast, because of scarcity of material. The algae harvested at different times were mixed in order to obtain sufficient substrate for the experiments.

All the data for algae as well as for algae-EDTA are corrected for appropriate blanks.

The results of the digestion experiments with *Scenedesmus spec.*– unheated, heated, unheated-EDTA, heated-EDTA and unheated-EDTA-heated– are recorded in fig. 18 ($E^{1\,mm}_{280\,m\mu}$ was multiplied by 10 to obtain $E^{1\,cm}_{280\,m\mu}$). Nitrogen determinations of the samples were made after 0, 50 and 120 minutes of digestion (fig. 19).

The results of the digestion experiments with *Chlorella spec.* (unheated, heated, unheated-EDTA, heated-EDTA and unheated-EDTA-heated) are recorded in fig. 20 ($E^{1\,mm}_{280\,m\mu}$ was multiplied by 10 to obtain $E^{1\,cm}_{280\,m\mu}$). Nitrogen determinations of the samples were made after 0, 50 and 120 minutes of digestion (fig. 21).

We observe that for *Scenedesmus spec.* as well as for *Chlorella spec.* there is a correlation between the spectrophotometric and nitrogen determinations.

From the amounts of nitrogen released from Scenedesmus and from Chlorella by α-chymotrypsin (table 7 and 8 respectively) the following conclusions can be drawn: 1. EDTA treatment does not enhance the digestibility of heated material; 2. EDTA treatment of unheated algae, though giving rise to a rather

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slight increase in digestibility as compared to untreated material, has far less effect on digestibility than the heating procedure; 3. heat treatment after EDTA-treatment of the algae causes considerably better digestibility of the protein of Scenedesmus cells than heating alone or than EDTA treatment of heated cells. For Chlorella heat treatment after EDTA-treatment does not cause better digestibility than heating alone.
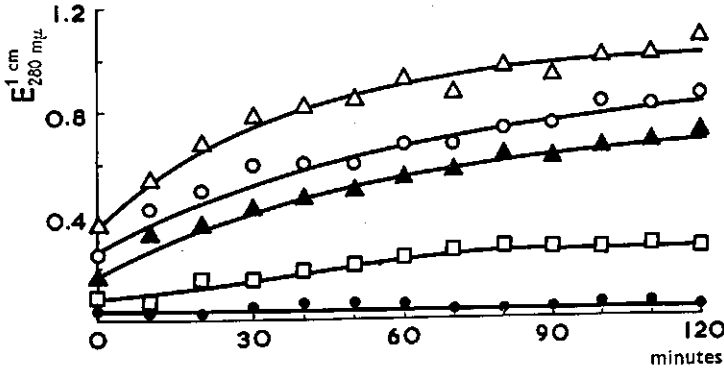
FIG. 18. *Digestion of Scenedesmus spec. by chymotrypsin.* Unheated (●——●), unheated-EDTA (□——□), heated (▲——▲), heated-EDTA (○——○) and unheated-EDTA-heated (△——△).
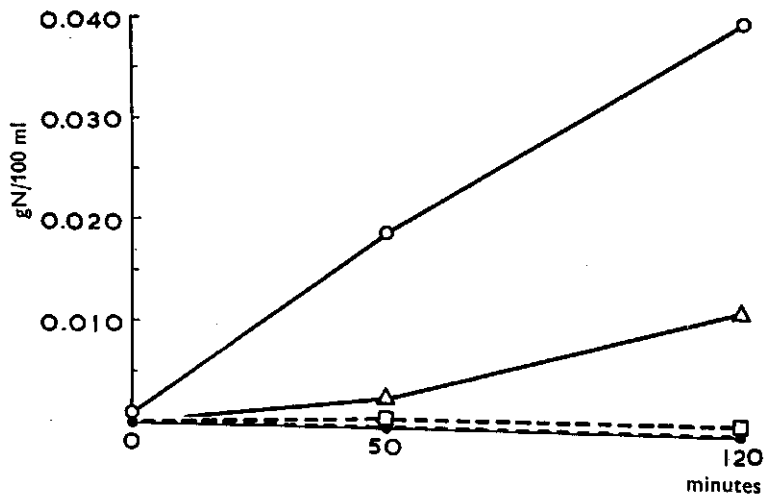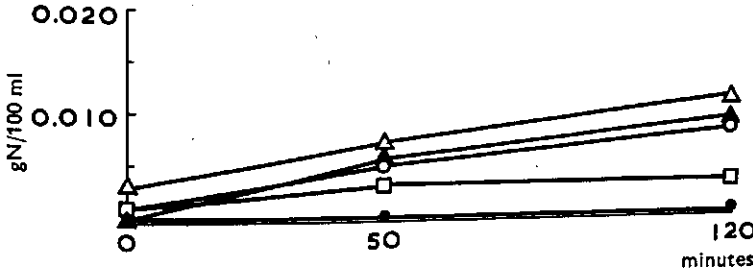
FIG. 19. *Digestion of Scenedesmus spec. by chymotrypsin.* Nitrogen determination of filtrates from samples after 0, 50 and 120 minutes of digestion. Unheated (●——●), unheated-EDTA (○——○), heated (▲——▲), heated-EDTA (□——□) and unheated-EDTA-heated (△——△).

TABLE 7 Amount of nitrogen released from *Scenedesmus spec.* by α-chymotrypsin.

Sample minutes	Unheated	Heated	Unheated-EDTA	Heated-EDTA	Unheated-EDTA-heated
0	0	0	1.5	1.5	5.1
50	0	9.5	4.6	7.7	11.9
120	0	14.3	4.6	12.3	18.6
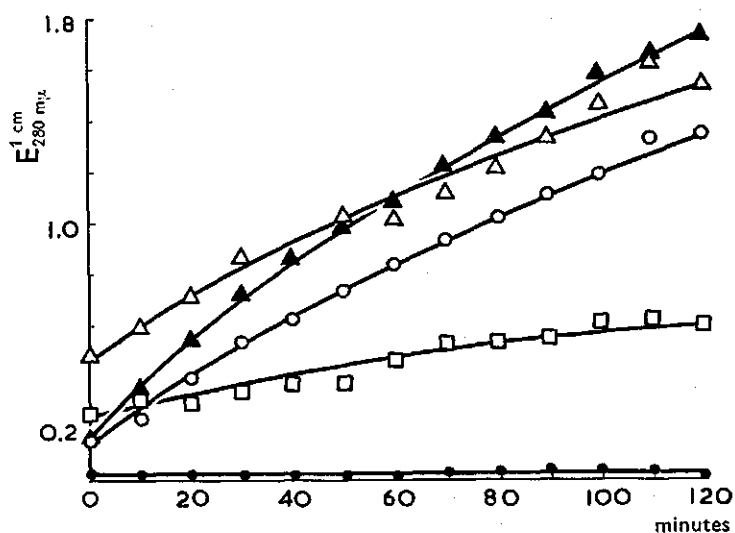
Fig. 20. *Digestion of Chlorella spec. by chymotrypsin.* Unheated (●——●), unheated-EDTA (□——□), heated (▲——▲), heated-EDTA (○——○) and unheated-EDTA-heated (△—— △).
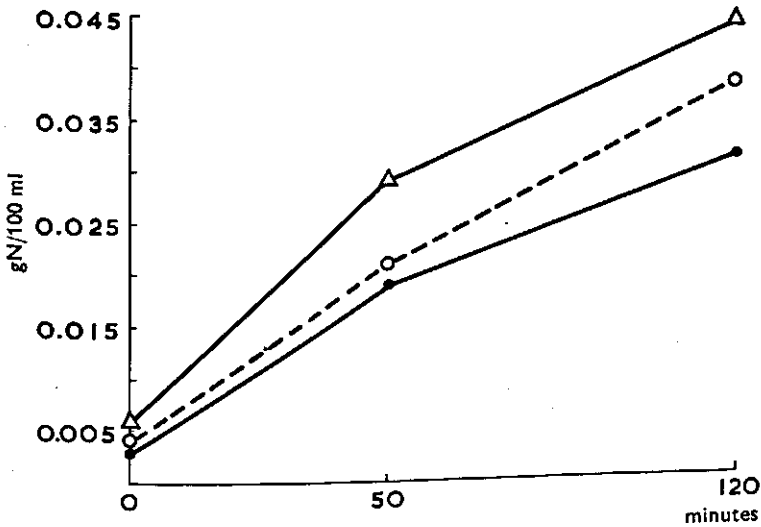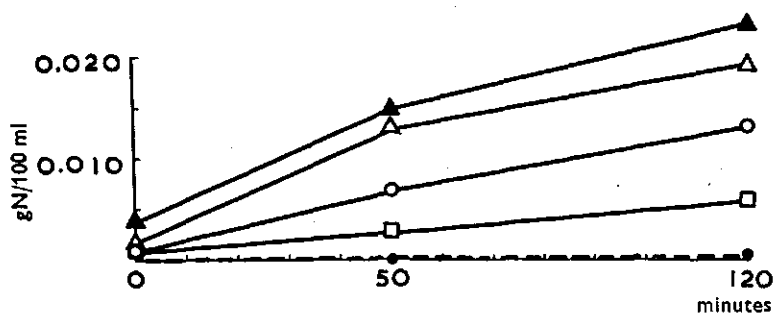

Fig. 21. *Digestion of Chlorella spec. by chymotrypsin.* Nitrogen determination of filtrates from samples after 0, 50 and 120 minutes of digestion. Unheated (●——●), unheated-EDTA (□----□), heated (▲——▲), heated-EDTA (○——○) and unheated-EDTA-heated (△—— △).

TABLE 8. Amount of nitrogen released from *Chlorella spec.* by α-chymotrypsin

Sample minutes	Unheated	Heated	Unheated-EDTA	Heated-EDTA	Unheated-EDTA-heated
0	0	2.4	0.6	0.5	1.4
50	0	8.8	1.7	3.8	9.3
120	0	13.5	3.3	7.0	13.6

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2.3. DISCUSSION ON DIGESTION BY CHYMOTRYPSIN

Before we start with the discussion of the results themselves we should like to comment on the antimicrobial compound (Preventol 115), EDTA, soybean trypsin inhibitor, also called Kunitz inhibitor and on the process of maceration.

2.3.1. *Antimicrobial compound (Preventol 115)*

The choice of an antimicrobial compound for the storage of the suspensions of the various substrates was made according to the following criteria: a. it should not interfere with the digestion of proteins by chymotrypsin; b. it should not affect the permeability of the substrates during storage, especially of unheated substrates. This excludes the substances with biocidal properties dependant on surface activity (PETHICA, 1958).

We considered Preventol 115 suitable because it fulfilled both requirements.

Requirement a. was important because for purposes of comparison we wanted to use a part of the substrate suspension directly from storage without first washing it. Later we found that it was better to avoid the use of Preventol 115 for the incubation with chymotrypsin at 35°C and for the washings at room temperature, since Preventol 115 apparently extracted proteins. However, when some experiments had been made with soybean cells (heated and unheated) digested with chymotrypsin we found there were no differences between the corrected values of experiments made in the presence and absence of Preventol 115. The uncorrected values of the former produced higher results than in the latter case, both for the experiment itself and for the corresponding blank.

When washing the substrates until a low extinction at 280 mμ of the filtrate was attained, before the incubation with chymotrypsin, we did not use Preventol 115 during rinsing in order to minimize dissolution of proteins. Moreover, we did not employ it during the incubation with enzyme. Therefore requirement a. became less important.

During the storage of the suspensions at 5°C Preventol 115 did not cause much leakage of proteins. The extinction value ($E^{1\,cm}_{280\,m\mu}$) of the liquid phase of soybean cell suspension attained, after two weeks of storage, a maximum of approximately 0.200–0.250, which remained constant. Some experiments demonstrated that this was a leakage of material for soybean cells which absorbed at 280 mμ. It was not due to a bacteria or mould, or to a proteinase within the soybean cells.

However, this leakage of material absorbant at 280 mμ did not represent a significant amount of proteins, because the nitrogen contents of soybean cells in table 3, column 1, which were obtained after they had been stored and washed to prepare them for incubation with chymotrypsin coincided with those of soybean seeds (MORSE, 1950) which were not macerated, not stored in the liquid medium or washed. The same reasoning can be applied to yeast (table

3, column 1; compared with data provided by GRYLLS, loc. cit.) and to algae (values of table 3, column 1 coincide with those given by FOWDEN, 1961 and DAM et al., 1965).

That Preventol 115 did not affect the permeability of membranes, was further supported by the experience that unheated yeast, unheated Scenedesmus and unheated Chlorella were not penetrated by chymotrypsin.

Requirement b. was very important, because if the barrier of unheated material were affected after storage it would not be possible to distinguish the effect of EDTA or the effect of heating on unheated substrate.

We assumed that Preventol 115, 0.1 % (w/v) in phosphate buffer pH 7.6 or borate buffer pH 8 killed yeast and algae after 2–3 days at 5°C which was confirmed by their lack of viability. We assumed that also soybean cells and sections were dead despite the fact that we were unable to test their viability, as we had done with yeast and algae.

Although the substrates – soybean, yeast and algae – were dead, it does not mean that they were altogether chemically inactive. For example, β-amylase, urease and lipoxygenase were still active in soybean (page 55. paragraph 4.3.). This means that also the enzymes of the lysosomes might remain active to produce autolysis of the cells. The fact that unheated yeast, unheated Scenedesmus and unheated Chlorella were not penetrated at all by chymotrypsin, demonstrated that if autolysis did occur, it did not affect the barrier of the cell wall and cell membrane, thereby assisting the entry of chymotrypsin.

2.3.2. Ethylenediaminetetraacetate (EDTA)

As our principal purpose is to determine how far heating affects permeability of the cells to permit a better passage of digesting enzymes, we had to find a substance that would decrease the cell wall barrier of unheated material without denaturing proteins.

We found that EDTA fulfils this condition. It is known that this substance affects permeability (LEIVE, 1965) by sequestering cations that are important in the structure of biological membranes (KAVANAU 1965). According to our findings protein from EDTA-treated subcellular powder is not more susceptible to chymotrypsin, and furthermore EDTA does not denature proteins (page 55, paragraph 4.3.).

EDTA decreases the barrier of the cell wall, but it is very difficult to establish that this effect was confined only to the cell wall. As EDTA is a low molecular compound it can also diffuse into the cells and affect the permeability of the various membranes present within the cells. Therefore many times in this publication we shall use the expression: EDTA affected cell wall and cell membrane permeability.

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It is worth while noting that when once the substrate had been treated with EDTA, this chemical was removed by successive washings. Therefore EDTA was absent when the materials were digested by chymotrypsin and also – as will be seen later – when hydrolyzed by lipase.

2.3.3. *Maceration*

Isolated soybean cells were obtained by the maceration of cotyledons (un-heated or heated) with EDTA (ROELOFSEN, loc. cit.). After being heated in dis-tilled water at 100 °C for 20 minutes the cotyledons were not softened, that is to say, no maceration had occurred. Only after EDTA-treatment were they mace-rated. This means: a. that enzymes of the soybean were not involved in this process; b. rupture of hydrogen bonds is not sufficient for the maceration of soybean cotyledons, as hot water is known to be an H-bond breaking agent.

Furthermore, 0.1 M glycine-NaOH buffer pH 10 did not macerate the cotyle-dons, thereby demonstrating that the alkaline medium by itself cannot do it. Therefore EDTA is specific in this action. This can be explained because EDTA sequesters cations, solubilizing pectate and perhaps hemicellulose. Considering that there are only traces of pectins present in the carbohydrates of soybean seed (DAUBERT, 1950) we assume that hemicellulose complexed with cations is the main component in the cementing material of soybean cotyledons. Hemi-cellulose, once deprived of its complexed cation, is more soluble in an alka-line medium. This could explain why hexametaphosphate (at pH 4), which is also a compound sequestering cation, does not macerate soybean cotyledons.

The sequestering powers of EDTA at pH 8 and 10 are similar (MARTELL and CALVIN, 1952). However, EDTA at pH 8 did not apparently macerate soybean cotyledons under the conditions used, although it was possible to macerate them when the cotyledons were suspended in EDTA pH 8 for 3–4 days instead of two (VAN RIEL unpublished data).

Before closing the discussion on maceration we should like to point out that this process is more complicated than just the solubilization of hemicellulose and pectate. H-bonds and proteins are also involved in the cementing material (GINZBURG, 1961). Another difficulty in the study of cementing material arises from the probability that also substances from within the cells are extracted together with the cementing material (VAN RIEL, unpublished data).

2.3.4. *Soybean trypsin inhibitor (Kunitz inhibitor)*

The penetration of enzyme into substrate may be easily followed by scanning the products of hydrolysis formed within the cells and leaking to the outside medium. If, however, the enzyme were inhibited by some agent within the cell, its entry would not be detected.

Soybean contains the Kunitz trypsin inhibitor, which, to a less marked extent, also inhibits α-chymotrypsin (KUNITZ, loc. cit). Moreover, the reaction of this

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inhibitor with α-chymotrypsin is *reversible*, as they form a highly dissociable complex (KUNITZ, loc. cit; WU and LASKOWSKI, 1955). Therefore we may state that not all molecules of chymotrypsin are inactivated simultaneously, even if they do react with the inhibitor.

On observing fig. 5b, which was deduced from fig. 5a by fixing the time of incubation of soybean cells with chymotrypsin at 10 or 20 minutes and by recording the extinction values as a function of the amount of chymotrypsin solution, we see that that point which relates to 4 ml enzyme solution is below the line determined by the first two points (1 and 2 ml enzyme solution). This is because with 4 ml of chymotrypsin solution the amount of substrate is the limiting factor.

Furthermore, when we observe the determination of the optimum ratio enzyme/ substrate (fig. 6) we see that this graph is similar to the classical one obtained with a homogeneous substrate without inhibitor. This means that we did not have the effect of the inhibitor even if it were still present.

It is probable that we removed the Kunitz inhibitor, which is a globulin, by suspending the soybean material in 0.1 M borate buffer pH 8, 0.1 % Preventol 115 (w/v) and washing several times with borate buffer before the experiment was carried out.

In view of the foregoing considerations, we may conclude that the soybean trypsin inhibitor does not constitute a problem for the digestion of substrate by chymotrypsin.

2.3.5. *Digestion of soybean substrate by chymotrypsin*
2.3.5.1. Cells
The fact that unheated and heated soybean cells were penetrated by chymotrypsin right from the start of the experiment can be explained by the solubilization of pectins (traces) and maybe hemicellulose by EDTA. Consequently a part of the matrix of the cell wall was removed, leaving pores delimited partly by the cellulose (skeleton) of the cell wall. Moreover it may be that EDTA or heat treatment effected contraction of the plasmodesmata, thereby leaving the plasmodesmatal canals open for chymotrypsin; alternatively it is possible that the removal of the cementing material left the plasmodesmata unprotected and that chymotrypsin was able to contact the protoplasmatic threads from the outset of the experiment.

That no sigmoid line was obtained during our experiment with unheated cells seems to denote that the latter no longer had a barrier. This shows that the unheated cells were not intact.

It is possible to account for the fact that the degree of penetration of chymotrypsin is the same for soybean cells heated for 5, 10, 15 and 20 minutes and for

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cells from cotyledons heated 5, 10, 15 and 20 minutes, because the heating was with 100% relative humidity at 100°C. Under these conditions we very quickly obtained a maximum denaturation of the proteins.

2 3.5.2. Sections

In unheated sections which had not been EDTA-treated we observed a very slight penetration of chymotrypsin. This slight penetration might be caused by chymotrypsin digesting the cytoplasmatic threads (plasmodesmata). This supposition is supported by the experience that unheated yeast and unheated algae (both without plasmodesmata) were not penetrated at all by chymotrypsin.

The fact that unheated sections-EDTA undergo a similar penetration as heated sections demonstrates that heating of material affects principally permeability. As expected, heated sections were digested to a somewhat greater extent than unheated sections-EDTA, because heating affected both the cell wall barrier and the proteins, the latter becoming more susceptible to chymotrypsin.

2.3.5.3. Subcellular powder

After removal of EDTA and buffer from the suspensions of powder A (unheated-EDTA, unheated-control, heated-EDTA and heated-control), powder B (autoclaved: heated-EDTA and heated-control), powder C (defatted with acetone) and powder F, chymotrypsin did not digest them.

As: 1. when the stock powder P and autoclaved powder B were extracted with 80% ethanol at 55°C, the extracted powders (D and E) could be digested by chymotrypsin; 2. powder F, which is in effect powder P extracted with 80% ethanol at 5°C instead of at 55°C, was not digested by chymotrypsin; thus we were induced to think that we had been confronted with a thermoresistant inhibitor, which is insoluble in water and acetone and which only 80% ethanol at 55°C can extract. These characteristics correspond to the thermoresistant inhibitor discovered by BIRK et al (1963), which is a saponin.

This saponin acts as an inhibitor when there are not enough proteins in the reaction mixture. At this stage it tends to combine with the enzyme-protein (ISHAAYA and BIRK, 1965). This is what happened when we removed EDTA or buffer from the powder suspension by centrifuging and discarding the supernatant. Most albumins of the powder were eliminated.

For this reason we decided to remove EDTA or buffer by dialysis. The dialysis tube would retain the albumins and in fact proteins in general, thereby preventing the creation of the conditions which could allow saponin to act as an inhibitor. Powder G that had been washed by dialysis was digested by chymotrypsin. Powder H, which was merely powder P directly suspended in borate buffer pH 8 for the experiment, without being centrifuged was likewise digested by chymotrypsin. This corroborates the assumption that when the proteins are not removed the saponin does not act as an inhibitor. This explains the

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phenomenon that in cells and sections whose cell wall and cell membrane were still retaining the proteins, saponin did not inhibit chymotrypsin.

As powder G was the one that permitted EDTA-treatment and control, we use the results of tests with this powder for comparison with unheated or heated cells.

On observing the values of powder G we concluded 1. that EDTA does not render the proteins of soybean more susceptible to chymotrypsin; 2. that once the material has no barrier (the cell wall is broken because it consists of subcellular powder) EDTA does not enhance digestion by chymotrypsin. This is yet another proof that EDTA specifically affects permeability.

We have seen that: 1. all heated EDTA-treated soybean substrates (cells and sections) have increments per 10 minutes double those of unheated EDTA-treated substrates; 2. the same occurs with heated subcellular powder in comparison with unheated subcellular powder, both preparations no longer having whole cell walls.
Consequently when relating points 1. and 2. we are induced to think that in the case of soybean cells and sections-EDTA, heating caused only denaturation of proteins as the permeability of the cell wall was already strongly affected by the EDTA-treatment.
In experiments with sections which were not treated with EDTA the heated sections were found to have an increment per 10 minutes 4.4 times greater than unheated. This means that unheated sections still possessed a strong barrier. We deduce that heating altered the barrier formed by the cell wall; in addition to this effect the denaturation of the proteins of heated material contributed to a considerable degree to the increased digestibility.

2.3.6. *Yeast digested by chymotrypsin*
First of all we should like to state that all explanations relating to yeast cell walls were deduced from the review of PHAFF (loc. cit.), the scheme proposed by LAMPEN (1968) and the book by ROGERS and PERKINS (1968).

We may say in a few words that the major components of yeast cell walls are glucan (skeleton), mannan and protein.

Live yeast and unheated yeast-LTS were not penetrated by chymotrypsin during our experiments. This shows that the barrier of the latter is similar to that of the former, despite its being dead.

Unheated yeast-STS was very slightly penetrated by chymotrypsin. This could be detected by means of the nitrogen released from the substrate and not by means of the spectrophotometric values.

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It is possible that the storage of yeast in 0.1 M phosphate buffer pH 7.6, 0.1 % Preventol 115 for approximately one month at 5 °C (unheated yeast-LTS), solubilized a fraction of mannan from the cell wall, whereas no solubilization occurred in unheated yeast-STS.

It is interesting to note that after EDTA-treatment unheated yeast (STS or LTS) is penetrated by chymotrypsin to a spectacular extent. This would suggest that cations play an important role in the structure of yeast cell wall. As far as we know, literature about yeast cell wall does not mention the importance of metal bridges. It could be that once the cations are removed mannan, possibly phosphorylated mannan, mannan-protein and even a part of the glucan are solubilized. This would decrease the barrier of the yeast cell wall.

We assume that in the case of heated yeast-STS mannan-protein from the cell wall is solubilized. After heated yeast had been stored for one month at 5 °C in 0.1 M phosphate buffer pH 7.6, 0.1 % Preventol 115 it is possible that the same degradation of the cell wall occurred as in the case of unheated-EDTA. This could explain why the values of digestion for unheated-EDTA and heated-LTS are similar.

The foregoing assumptions could also explain why the difference in digestibility between heated-STS and LTS is greater than the difference between unheated-STS and LTS.
The experience that heated-STS still possesses some barrier which is removed by EDTA-treatment would seem to indicate that in heated yeast-STS metal elements still fulfil a function, and they are not removable by heating alone.

Moreover, it may be that by EDTA or heat treatment compounds of the cell wall were removed, thus permitting hydrolysis of proteins from the wall by chymotrypsin, which would contribute to its degradation. Thus the entry of enzyme would be facilitated by its own catalytic activity. However, the fact that heated-LTS was penetrated to a greater extent than STS indicates that during the storage some solubilization of compounds from the cell wall occurred. These compounds were not removed by the action of chymotrypsin and/or heating.
A comparison of unheated-EDTA-heated with unheated-EDTA, where the values of the former are slightly higher than the latter, demonstrates that EDTA-treatment affected cell wall permeability most. The difference in digestibility is due to the denaturation of proteins by heating, which renders them more susceptible to chymotrypsin.

2.3.7. *Digestion of algae by chymotrypsin*
For the determination of the optimal ratio of enzyme quantity to the amount of algae suspension we used 1. unheated-EDTA, since we expected that EDTA-treatment would allow the penetration of chymotrypsin; 2. an incubation time

of 60 minutes, as the entry of enzyme into algae is slower than into soybean cells.

In the case of algae, just as with soybean sections and yeast, EDTA did not enhance the entry of chymotrypsin into heated material. Again we see that EDTA only affects material that still possesses a barrier. However, the increase of digestibility by EDTA-treatment on unheated algae was not so high as with soybean sections and yeast. This indicates that the compounds of the cell wall of algae are associated in some way with metals, but when cations are removed the degradation of the cell wall is not so great as it is with soybean and yeast.

The experience that a heat treatment after EDTA-treatment causes considerably better penetration into Scenedesmus cells than heating alone or than EDTA-treatment of heated cells can be accounted for, because the cell wall of Scenedesmus consists of an inner cellulosic and an outer pectic layer, with a thin layer in between (BISALPUTRA and WEIER, 1963). It is assumed that heating would have more effect on the middle and inner layers if the outer pectin layer has been removed by EDTA-treatment. When the Scenedesmus cells were heated in the presence of all the layers of the cell wall the outer layer and perhaps also the middle one protected the inner layer in some way or other.

As for Chlorella cells a heat treatment after EDTA-treatment does not improve the digestibility compared with heating alone, because the walls of these cells have microfibrils of α-cellulose enclosed in a continuous matrix. This matrix consists in the main of a combination of glycoprotein and hemicellulose (NORTHCOTE et al., loc. cit.). Consequently, heating immediately affects the entire cell wall.

Before ending this discussion we should like to remark that in all types of substrates, the amount of nitrogen released by α-chymotrypsin was larger than the quantity of cell wall nitrogen in the respective reaction mixtures (table 3, column 4). Consequently the nitrogen of the samples did indeed arise from proteins within the cells and not from proteins from the cell wall. This means that chymotrypsin entered the substrates.
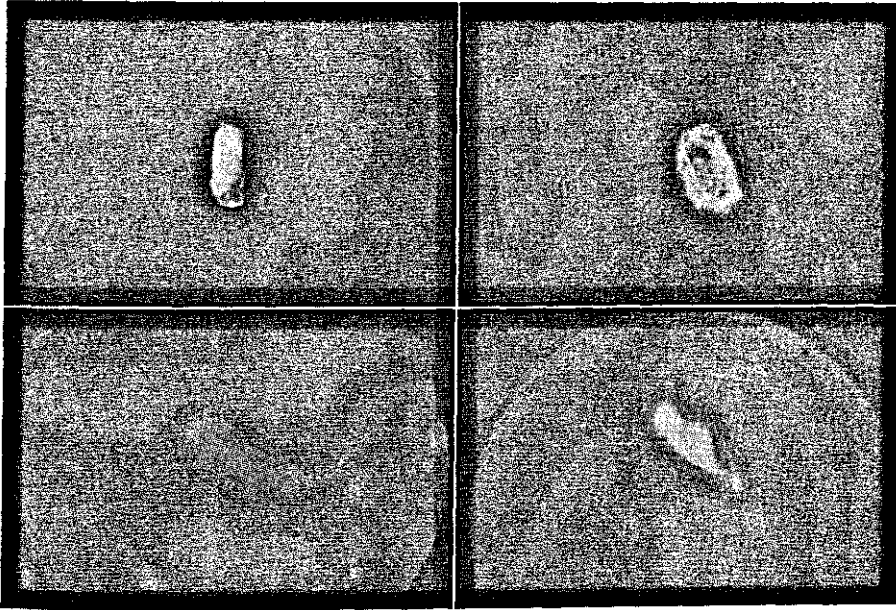
FIG. 22. *Localization of α-chymotrypsin within unheated or heated soybean cells with the fluorescent antibody technique.* Upper: Enzyme + antiserum + FA. Lower: Control (1, 2, 3 or 4). Magnification × 240.

3. FLUORESCENT ANTIBODY TECHNIQUE

The fluorescent antibody technique was used with the following purposes: a. to confirm immunologically the evidence obtained biochemically that chymotrypsin penetrated into soybean cells and sections. If this confirmation were obtained, then the biochemical method alone would be sufficient to demonstrate whether the enzyme entered yeast and algae; b. to study the way chymotrypsin penetrated into the soybean substrate. We specially wanted to ascertain whether chymotrypsin would penetrate through plasmodesmata (pits).

3.1. MATERIALS AND METHODS

The fluorescent antibody technique we applied was the 'sandwich' method of WELLER AND COONS (1954) as given by VAN DER VEKEN et al. (1962). It is so called (sandwich) because it brings about reaction of the antigen (chymotrypsin), the appropriate antibody (antiserum) and the fluorescent antibody. Therefore the soybean cells incubated with chymotrypsin were fixed and treated with unlabelled antiserum (from rabbit) and then with an antibody (from goat) coupled with a fluorescent dye directed against the globulins of the rabbit's antiserum.

The unlabelled antiserum and fluorescent antibody mentioned above, were previously absorbed with normal substrate (without chymotrypsin) to avoid unspecific reactions between the antiserum or fluorescent antibody with the substrate.

Antiserum against chymotrypsin (antigen) was obtained by inoculation of a rabbit with chymotrypsin solution, according to URIEL and AVRAMEAS (1964) with slight modifications. The specificity of antiserum against chymotrypsin was checked with the micro-precipitin test of VAN SLOGTEREN as described by VAN DER VEKEN et al. (loc. cit.) and with a gel diffusion precipitin reaction (VAN DER VEKEN et al. loc. cit.) In both cases simultaneous determinations were done against chymotrypsin and against extract of malt containing other proteolytic enzymes (TEN HOOPEN, 1968). This extract had antigenic properties (TEN HOOPEN, unpublished data).

The reaction of the antiserum obtained was positive with chymotrypsin whereas it was negative with the extract containing other proteolytic enzymes.

The antigen dilution end-point (1:23) and the titre[1] of the antiserum (1:8) were determined with the previously mentioned microprecipitin test of VAN

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[1] 'In every analytical method, there is a minimum limit to the quantities of the two reacting substances required for a positive reaction. For antisera this minimum quantity is termed the titre and indicates the highest dilution at which the antiserum produces a visible reaction with the antigen. In the case of the antigen it is called the dilution end-point' (VAN DER VEKEN, loc. cit.).

SLOGTEREN. The dilutions of antiserum and antigen for the micro-precipitin test and gel diffusion precipitin reaction were made with 0.9% NaCl solution.

Fluorescent Antibody (FA) from goat against γ-globulin of rabbit was purchased from Difco (code 2351).

Phosphate saline buffer (PSB) pH 7.2 was purchased from Difco.

The lyophilized FA and PSB were dissolved in rain-water that had been twice distilled.

Mounting fluid (MF) was purchased from Difco (code 2329).

Soybean cells (unheated and heated) were the same as those used in the previous experiments.

Unheated soybean sections were the same as those used in the previous experiments but 10μ instead of 100μ thick. This was to make possible the observation of the sections under the microscope.

Absorption of antiserum and FA

In the literature it is recommended that the antiserum and FA be absorbed with normal tissue which had been powdered, in order to obviate unspecific reactions (CREMER AND VAN DER VEKEN, 1964; HERS 1963; DIFCO, 1964).

We absorbed these substances with isolated soybean cells (unheated and heated as appropriate) but not powdered.

Soybean-cell suspensions were filtered through filter paper and lyophilized. This material was washed by stirring with portions of twice distilled rain-water for 15 minutes; then the suspension was centrifuged for 30 minutes at 22,000 g at 5°C. The residue was suspended in PSB for 15 minutes and again centrifuged. We immediately added antiserum (1.5 ml) to about 30 mg of the wet cells. The resultant mixture was stirred cautiously for one hour at room temperature. After centrifugation for 30 minutes at 22,000 g at 5°C the supernatant was submitted to the same procedure and the residue discarded.

The same treatment was applied to the FA, but with the exclusion of light.

The antiserum and FA were also twice absorbed with unheated soybean sections that had not been powdered, in a way similar to that described for soybean cells.

'Sandwich' method

As we mentioned already this method is so called because it brings about reaction of the antigen (chymotrypsin), the appropriate antibody (antiserum) and the FA. The first step is to bring about a reaction between the antigen and the antibody. After this, the excess of antiserum that did not react with the chymotrypsin is removed by washing and FA is added. Then the excess of FA that did not react with the γ-globulin fraction of the antiserum is removed.

Soybean cells

Twenty per cent soybean-cell suspension (7 ml) was mixed with chymotrypsin solution (1 ml), or with borate buffer (1 ml) for the blank. The enzyme concentration was therefore ten times higher than in the previous experiments with

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chymotrypsin (page 16, paragraph 2.1.6.1.) and was well within the antigen's dilution end-point. After the desired period of digestion an aliquot of the cell suspension was taken and filtered through a fritted glass-filter G3. A small quantity of cell material (ca. 30 mg) was taken from the fritted glass-filter into a centrifuge tube. The contents of the tube were fixed with 5 drops of acetone pro-analar. The acetone was removed from the samples by evaporation in a thermostat at 37°C for 20–30 minutes. After the addition of 0.4 ml twice absorbed undiluted antiserum the tube was shaken for one hour at room temperature. We did not dilute the antiserum because it had a low titre (1:8) and some dilution had already occurred during the absorption process. Then 3 ml of PSB were added and the suspension was stirred gently for 15 minutes and subsequently centrifuged for 30 minutes at 22,000 g at 5°C. The washing process with PSB was repeated 3 times. Then one drop of twice absorbed FA was added and the mixture was shaken in darkness at room temperature for one hour. The excess of FA that did not react was removed by washing three times with PSB.

One drop of the sediment was mounted on a large coverglass, which served as a slide, and one drop of MF was added. Another coverglass was placed over the mount and the preparation was sealed with beeswax.

Soybean sections

Unheated sections (0.100–0.147 g) in borate buffer (1 ml) were mixed with chymotrypsin solution (0.5 ml). The blank consisted of the same mixture, but instead of chymotrypsin solution borate buffer was used. As the antigen dilution end-point was 1:32 the dilution of 1:2 was considered appropriate. All other details were similar to those described for the soybean cells.

As a control on the validity of the 'sandwich' method the following combinations were made:

Experiment enzyme + antiserum + FA
Control 1 :   –   antiserum + FA
Control 2 : enzyme   –   + FA
Control 3 : enzyme + antiserum   –
Control 4 : cells or sections only

Observations of the preparations

Observations were made with a Zeiss fluorescence microscope with a dark field condensor, a mercury vapor arc lamp (Philips 150) and a filter combination for inducing fluorescence in the range 5500–6000 Å. For this purpose we employed one heat absorbing filter (BG 22, 2 mm thick) and two exitor filters (BG 12 and UG 1, both 2 mm thick) to filter the incident light beam from the lamp before it irradiated the specimen. Residual UV light was removed by placing a barrier filter (OG 4, 2 mm thick) between the ocular lens and the object.

Preparations were photographed by means of a Zeiss camera, on an Anscochrome colour film, T 100–135–12 PNI. The exposure time was one minute. When photographing, only one exitor filter (BG 12) was used.

3.2. RESULTS

A photograph of soybean cells treated as described (enzyme + antiserum + FA) is presented in fig. 22 (p. 42a), where a green colour can be observed. The control is entirely blue, without a fluorescent greenish colour.

The picture of unheated cells is the same as that of heated cells. Furthermore when heated or unheated cells were processed after 3, 6, 9, 12 or 60 minutes of incubation with chymotrypsin, no differences were found on inspection of the preparations under the microscope. There were no differences between any of the controls (1, 2, 3 or 4) or between the controls belonging to heated and unheated cells; all of them being entirely blue.

The results for unheated sections were similar to those for cells. No differences were seen between sections digested by chymotrypsin after 10 or 120 minutes. The controls likewise showed great similarity to one another.

The fluorescent green colour which characterised the positive test indicates where the antigen-antibody reaction occurred, and it is therefore concluded that chymotrypsin is localized inside the cells (isolated cells or cells in sections). The absence of this green colour in the various controls demonstrates that the test is specific for chymotrypsin (comparing positive test and control 1) and that there was no aspecific fluorescing material (control 2, 3 or 4).

3.3. DISCUSSION

For the purpose with which we are concerned we should like to mention one advantage (1) and to comment on one disadvantage (2) of this immunological technique.

1. The immunological method has the advantage that localization of chymotrypsin inside the cells can be effected without destroying them.

The use of the 'sandwich' method was preferred to using a labelled protein other than chymotrypsin, or to the use of the same chymotrypsin conjugated with a fluorescent dye, because: a. the use of another protein would not be representative for our own experiments; and b. conjugating the chymotrypsin with a fluorescent dye gives rise to the possibility that the activity of the enzyme would be affected. Therefore preference was given to the 'sandwich' method because the enzyme could then be used in the same form as it had been employed in previous experiments. Furthermore the possibility exists that when the antiserum was added after different periods of digestion of the soybean substrate the reaction of the antibody to chymotrypsin would stop the activity of the enzyme. However according to URIEL (1963) the catalytic activity of the enzyme is not always inhibited by the antigen-antibody reaction. This could explain why we did not find differences between the preparations after different periods of digestion.

2. One of the main problems in the use of the 'sandwich' method in virology (plant or medical) arises from the fact that it is sometimes impossible to detect viruses which are present in a very low concentration (CREMER and VAN DER VEKEN, loc. cit.). In our case this was not a problem because we were able to

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vary the amount of enzyme solution and, if need be, also its concentration. This moreover rendered it possible for us to work with an antiserum of low titre.

On the basis of the results obtained, we conclude that the method applied is indeed specific for the localization of chymotrypsin. We do not, however, find differences after different times of digestion. This allows the conclusion that this method is useful to verify that the enzyme can diffuse into the cells, and that it does so within 3 minutes. A comparable phenomenon occurs in the localization of influenza virus antigen in host cells. The synthesis of the viral subunits within the host cells can be detected but the eclipse phase cannot be followed as a function of time (HERS, personal communication).

Before proceeding with the discussion we consider it appropriate to define the words: plasmodesmata, plasmodesmatal canals, plasmodesmatal pores and pits. These are deduced from the publication of VOELLER (1964). *Plasmodesmata* are cytoplasmatic threads penetrating the cell wall and forming intercellular bridges. The channels through which the plasmodesmata pass are the *plasmodesmatal canals*. When we observe the transversal section of the plasmodesmatal canals we view the *plasmodesmatal pores*. The plasmodesmata may be distributed randomly in the cell wall or they may be grouped together within depressions or narrow regions of the wall, known as *pits*.

To define the difference between plasmodesmata and plasmodesmatal canals or plasmodesmatal pores is most important, because research workers sometimes confuse them. For example, when an isolated cell wall has been treated in such a way that proteins, pectins and fats are removed (purification of the cell wall) it is logical that the electron microscopic photographs of such a preparation will show the plasmodesmatal pores, but not the cytoplasmatic threads (plasmodesmata). According to VOELLER (loc. cit.) STUGGER, using ultra thin sections of plant cells (*Allium cepa*), was able to distinguish between the diameter of the pores (1100–1900 Å) and the thickness of the plasmodesmata thread (300–400 Å). He attributed this difference to shrinkage during the preparation of the tissues for the electron microscope and believed that the cytoplasmatic connections of living or intact cells fill the pores completely.

Reverting to the discussion we would say that the impossibility of detecting the mode of penetration with the fluorescent antibody technique does not preclude the possibility of chymotrypsin having entered the soybean substrate through plasmodesmata. When the soybean cells were stained with zinc chloride iodine (Behrens reagent[1]) as described by ROELOFSEN (loc. cit.), pits could be observed under the light microscope. Furthermore we made electron microscopic photographs of isolated soybean cell wall taken from unheated cells. We prepared isolated cell wall as described by ROELOFSEN (loc. cit.), with slight modifications. After shadowing it with platina, electron microscopic photographs were made. With the aid of these photos we determined the mini-

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[1] Behrens reagent consists of: 20 g $ZnCl_2$, 6.5 g KI, 1.3 g iodine in 10.5 ml water.

mum diameter of plasmodesmatal pores and found values of 150–200 Å. According to MATTHEWS et al. (1967) the α-chymotrypsin molecule is ellipsoid with axes of 45Å, 35Å and 38Å. Therefore it is very probable that chymotrypsin can penetrate the plasmodesmatal pores.

4. PENETRATION OF AMYLOPECTIN INTO SOYBEAN MATERIAL

The penetration of amylopectin into soybean cells was studied with the following purposes.

1. So far we studied the penetration of chymotrypsin into soybean material.

It seemed desirable to study the diffusion of a non-protein substrate of large molecular weight (amylopectin) into the soybean material. It could be expected that amylopectin after penetration would be hydrolysed by the β-amylase present within the cell.

2. It remained to be demonstrated that the increased permeability after EDTA tieatment was specifically brought about by EDTA and was not an aspecific effect of the alkaline medium (pH 10). Because of this borate buffer pH 10 was used. As borate ions might form complexes with neutral polysaccharides of the cell wall and increase its barrier, we treated soybean material also with glycine buffer pH 10.

3. If β-amylase, would still be active after treatment of unheated soybean material with EDTA, this would indicate that EDTA did not denature the proteins.

Similar experiments carried out with urea as substrate were intended to test for the presence of soybean urease in situ after EDTA-treatment.

4.1. MATERIALS AND METHODS

The β-amylase activity of soybean material (or extract) was determined with the dinitrosalicylic acid method as described by BERNFELD (1955). Amylopectin was used as a substrate. The determinations were made with a qualitative perspective. No optimal ratio enzyme-substrate was determined. The activity of the enzyme was determined under the conditions of pH and temperature as given by BERNFELD (loc. cit.) for β-amylase of sweet potato.

Acetate buffer, 0.016 M, pH 4.8.

Amylopectin, was purchased from Avebe, Veendam, Amylopectin P, No. 1684 (Potato Starch).

Amylopectin solution, was prepared by dissolving amylopectin (1 g) in acetate buffer (100 ml).

3,5-dinitrosalicylic acid reagent, was prepared as indicated by BERNFELD (loc. cit.).

Borate-NaOH buffer, 0.1 M, pH 10, 0.1 % Preventol 115 (w/v).

Glycine-NaOH buffer, 0.1 M, pH 10, 0.1 % Preventol 115 (w/v).

EDTA solution, 3.8 % (0.113 M), pH 10, 0.1 % Preventol 115 (w/v), was prepared as indicated on page 7, paragraph 2.1.1.3.

Unheated cotyledons were obtained from soybean seeds as described on page 12, paragraph 2.1.5.1. *Heated cotyledons* were obtained from seeds which had been heated in distilled water at 100°C for 20 minutes. One part of the wet

cotyledons (unheated or heated) was kept suspended respectively in 10 parts of: EDTA solution, borate buffer or glycine buffer (w/v), at 37 °C for two days. After that period the pH-values of the three suspensions were ca. 9.7. After decanting the respective liquids the cotyledons were rinsed by suspending them in 10 parts of acetate buffer for 15 minutes at room temperature. Then the acetate buffer was decanted and a new portion of it was added (1:10 w/v). After draining the second portion of acetate buffer through cheese-cloth, the cotyledons were ready for incubation with amylopectin solution for β-amylase determinations. Then we had the following preparations of unheated as well as of heated cotyledons: *cotyledons-EDTA, cotyledons-borate* and *cotyledons-glycine*. Only cotyledons-EDTA were softened, which is an indication of maceration.

Another experiment was done to see whether β-amylase leaked or not from the cotyledons. Consequently we tried to detect β-amylase in the filtrate. After rinsing the cotyledons (EDTA, borate or glycine) 3 times with acetate buffer, the third portion of acetate, which was kept suspended for 30 minutes instead of 15 minutes, was not discarded, but filtered through fritted glass-filter G3. In this way we obtained *extracts from cotyledons-EDTA, cotyledons-borate* and *cotyledons-glycine.*

Cotyledons-borate and cotyledons-glycine, after being rinsed three times with acetate buffer for obtaining the respective extracts, were suspended separately in EDTA solution for two days at 37 °C. Another portion of cotyledons-borate was again suspended in borate buffer for two days at 37 °C. Afterwards the liquids (EDTA or borate) were removed by decanting them, and rinsing the cotyledons 3 times with acetate buffer. These cotyledons were named as follows: *cotyledons-borate-EDTA, cotyledons-glycine-EDTA* and *cotyledons-borate-borate*. The portions of acetate buffer belonging to the first and third rinsing operation of cotyledons-glycine-EDTA were filtered through fritted glass-filter G3 and tested for the presence of β-amylase. They constitute the *first and third extract of cotyledons-glycine-EDTA.*

Soybean sections (unheated or heated) were obtained as described previously (page 12, paragraph 2.1.5.1.) with the difference that they were used directly without storing them for 16 weeks. The sections were treated with EDTA or borate buffer as described for cotyledons. The same procedure was applied for rinsing these materials. Therefore we had *sections-EDTA* and *sections-borate.*

Soybean cells (unheated or heated) were obtained as described in page 12, paragraph 2.1.5.1. They were stored for one month in 0.1 M borate buffer pH 8, 0.1 % Preventol 115, at 5 °C. Prior to use for the β-amylase determinations, the cell suspensions were filtered through fritted glass-filter G3 to remove 0.1 M borate buffer pH 8. The soybean cells accumulated on the plate of the glass-filter. One part of this wet material was twice rinsed with 10 parts of acetate buffer (w/v) in a similar way as for cotyledons and sections.

For the determination of β-amylase activity the dinitrosalicylic acid method as described by BERNFELD (loc. cit.) was slightly modified. This method is based on the increase of reducing power of a solution of amylopectin during

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digestion by α or β-amylase and the formation of a brown colour by dinitro-salicylate with reducing sugars. The extinction of the solution containing the brown reaction products was determined at 540 mμ in a Beckmann (G 2400) or Unicam (Cambridge SP 600) spectrophotometer, with glass cuvettes of 1 cm light path. In the soybean material α-amylase is virtually absent (NEWTON and NAYLOR, 1939; GERTLER and BIRK, 1965); therefore the extinction at 540 mμ is practically wholly due to maltose produced by β-amylase. We present only the extinction values, without converting them into maltose units.

Cotyledons, sections or cells incubated with amylopectin solution.

One part of wet weight of the different types of cotyledons was incubated at 20°C with 10 parts of amylopectin solution (w/v). The suspension was not stirred. As blanks the corresponding heated cotyledons were used. Another control consisted of incubating the cotyledons (EDTA or borate) with acetate buffer only (no amylopectin). After different periods of incubation (10, 60 or 120 minutes) a part of the supernatant (2 ml) was pipetted and filtered through fritted glass-filter G3. This filtrate (1 ml) was reacted with dinitrosalicylic acid reagent (1 ml). The tube containing this mixture was heated for 5 minutes in boiling water. After cooling, distilled water (10 ml) was added and the ex-tinction read at 540 mμ. With sections and cells the same procedure was applied, with the exception that for cells the filtrate that reacted with dinitrosalicylic acid reagent was obtained from a sample where the whole suspension was pipetted.

Extracts incubated with amylopectin

Extracts from the different types of cotyledons (1 ml) were incubated with amylopectin solution (1 ml), at 20°C. After different times of incubation a part of the mixture (0.5 ml) was reacted with dinitrosalicylic acid reagent (0.5 ml). The tube containing this mixture was heated for 5 minutes in boiling water. After cooling, distilled water (5 ml) was added and the extinction read at 540 mμ.

4.1.1. *Urease test*

The urease test was made according to CASKEY and KNAPP as described by CIRCLE (1950) slightly modified. It consisted of incubation for 30 minutes at 30°C of soybean material (ca. 0.2 g) with 10 ml 0.05 M phosphate buffer solution pH 6.3 containing 0.3 g urea and 2 drops of 1% phenol red solution. It was found that when urease is active, the suspension will change from yellow to red.

The following types of soybean material were tested. Cells (unheated or heated), sections (unheated or heated), sections-EDTA (unheated or heated), unheated powder P, unheated powder-EDTA and steamed powder.

Cells and *sections* were the same as those employed for penetration of amy-lopectin into soybean material (paragraph 4.1.). Unheated powder P was ob-tained as previously described (page 12, paragraph 2.1.5.1.).

Unheated-powder-EDTA was obtained by grinding cotyledons-EDTA, which were prepared as described under paragraph 4.1. *Steamed powder* is the unheated powder P steamed at 105 °C for 20 minutes.

4.2. RESULTS

The results of the β-amylase determinations are presented in graphs, where the extinction at 540 mμ is recorded as a function of time. The graphical representations were made with values corrected for the appropriate blanks.

The data relating to the different types of cotyledons are shown in fig. 23. Cotyledons-borate and cotyledons-glycine present a horizontal line. This means no reaction of amylopectin with β-amylase. Cotyledons-EDTA, cotyledons-borate-EDTA and cotyledons-glycine-EDTA present a gradient which implies that reaction does occur between β-amylase of these cotyledons and amylopectin. The fact that cotyledons (borate or glycine), after being treated with EDTA, have β-amylase activity, demonstrates that these buffers did not denature the β-amylase inside the cotyledons. Furthermore, this demonstrates that apparently the effect of EDTA, and not the alkaline medium, is specific for altering the permeability of the cell wall and cell membranes. The experiment with cotyledons-borate-borate (horizontal line) indicates that the β-amylase activity of cotyledons-borate-EDTA is not due to the fact that cotyledons-borate were merely kept two days more at 37 °C.

The control experiment with cotyledons (EDTA or borate) and acetate buffer only (no amylopectin) results in a horizontal line. This means that the reduction of dinitrosalicylic acid reagent was produced by maltose and not by other compounds of soybean material.
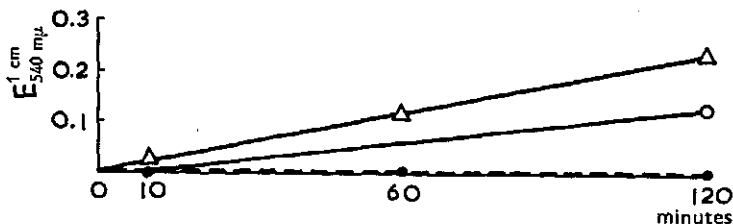
FIG. 23. *β-amylase determinations of cotyledons incubated with amylopectin solution.* Cotyledons-borate, cotyledons-glycine of cotyledons-borate-borate (●- - -●). Cotyledons-borate-EDTA or cotyledons-glycine-EDTA (○——○). Cotyledons-EDTA (△—△).

The results of the experiments made with extracts are recorded in fig. 24. Extracts from cotyledons (borate and glycine) present a horizontal line, whereas extracts from cotyledons-EDTA and cotyledons-glycine-EDTA (first and third) have a gradient line. This means that β-amylase leaked from cotyledons only after treatment with EDTA. The results of the first extract from cotyle-
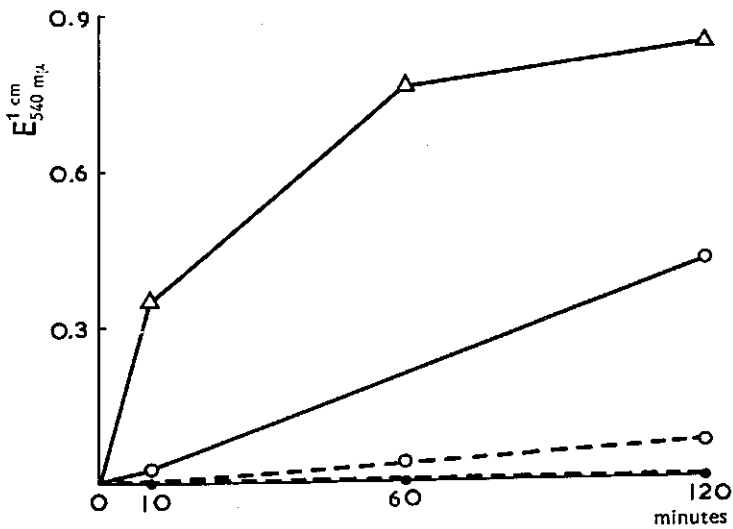
FIG. 24. β-*amylase determinations of extracts from cotyledons incubated with amylopectin solution.* Extracts from cotyledons-borate of cotyledons-glycine (●- - -●). Extracts from cotyledons-EDTA (△——△). First extract from cotyledons-glycine-EDTA (○——○) Third extract from cotyledons-glycine-EDTA (○- - -○).
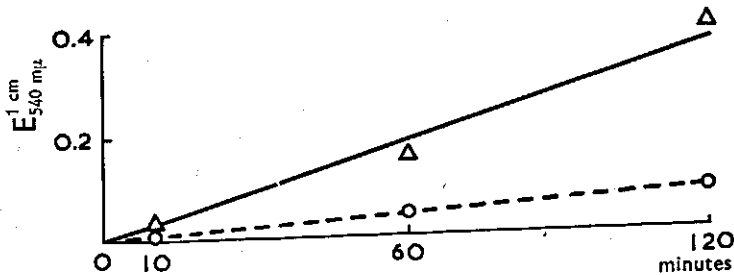
FIG. 25. β-*amylase determinations of sections and cells incubated with amylopectin.* Sections-EDTA (△——△). Sections-borate or cells (○- - -○).

dons-glycine-EDTA show a steeper upward slope than in the case of the third extract. This indicates that in the first extraction more β-amylase was present.

The data of the experiments made with sections are recorded in fig. 25. Sections-EDTA present a steep line, whereas sections-borate present a line with a very slight slope. This could be explained by assuming the presence of β-amylase adsorbed on the broken cells of the sections. It should be recalled that the sections were used directly, without their having been stored for 16 weeks and rinsed each week. Moreover, as stated in the text of fig. 3 (page 13) the sections had one part of damaged cells to 3 parts of undamaged cells; therefore it would not be surprising if part of the β-amylase of the damaged cells was still present.

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The graphical representation of the results obtained with cells (fig. 25) present a slight slope. This means that even after being stored for one month at 5 °C in 0.1 M borate buffer pH 8, 0.1 % Preventol 115, the cells still retained β-amylase activity. This is not surprising when one considers that NEWTON et al. (1943) found that at room temperature concentrates from soybean extracts containing β-amylase were still active after 30–34 days.

The extinctions of the different blanks did not increase during incubation, which proved that the reactions in EDTA-treated unheated material were indeed due to enzymic activity.

4.2.1. *Results of the urease test*

Each of the following types of material gave a positive result: unheated cells, unheated soybean sections, unheated soybean sections-EDTA, unheated powder P, unheated powder-EDTA. As controls, that gave a negative result, we used: heated cells, heated sections, heated sections-EDTA, steamed powder, unheated powder P in phosphate buffer (no urea) and 3 % urea solution only (no soybean material).

4.3. DISCUSSION

The experiments which led to the β-amylase determinations were carried out for qualitative purposes only. Hence, some conclusions that were arrived at from experiments made with cotyledons were assumed to apply qualitatively to all the soybean material (cotyledons, sections and cells). We employed cotyledons because it was necessary to have a non-EDTA-treated material. It would have been possible to use sections, but this would have involved tedious work to obtain sufficient quantities of them. Cells could not be employed because they had themselves been obtained from EDTA-treated cotyledons.

The choice of β-amylase as a naturally present enzyme was made because this permitted the use of a substrate of large molecular weight (amylopectin: 50,000–1,000,000 as quoted by FOSTER, 1965). There is however no categorical indication that amylopectin penetrated into the cells of soybean material treated with EDTA, because extracts of cotyledons-EDTA contained β-amylase. Therefore the probability exists that the reaction between β-amylase and amylopectin occurred within and/or outside the cells.

The fact that it is impossible to demonstrate that only a penetration of amylopectin takes place does not preclude the possibility that EDTA is specific for affecting permeability. If we consider that β-amylase is a large molecular compound (molecular weight 61,700 GERTLER and BIRK, 1965) which is extracted only when cotyledons are treated with EDTA, leads us to conclude that EDTA is indeed specific for affecting the permeability of the cell wall and cell membrane.

LAUFER et al. (1944) extracted β-amylase to a maximum degree, from whole defatted soybean seeds with distilled water only, at 20 °C for $2\frac{1}{2}$ hours. This could

induce one to think that there was a contradiction with our findings where two aqueous buffers failed to bring about the extraction of the aforesaid enzyme. The explanation of this difference could be that the extraction by LAUFER et al. (loc. cit.) was performed with defatted beans. The solvent employed presumably affected the permeability of the soybean membranes, without denaturing β-amylase, which is resistent to the action of organic solvents (NEWTON et al., loc. cit.).

After expressing the previous considerations we would suggest that there are three possible reasons to account for the positive reaction of β-amylase when soybean was treated with EDTA. The first two are related to the leakage of β-amylase and the third one to its activity. 1. EDTA, as we had already assumed, affects only the permeability of membranes. Proteins bodies (and consequently β-amylase) in soybeans are surrounded by membranes (TOMBS, 1967). 2. EDTA affects the cationic bridges. Perhaps β-amylase is attached to soybean material by cationic bridges, or cationic elements help to make for a stronger attachment than would exist without them. This could be something similar to the attachment of ATPase in *Streptococcus fecalis*. ATPase is attached to the membrane by hydrophobic bonds, but when Mg is present the attachment is stronger (ABRAMS and BARON, 1968). 3. β-amylase is blocked by some metal which EDTA removes. β-amylase molecule of soybean possesses SH groups involved in its active center (GERTLER and BIRK, 1966). It may be that in our case the positive reaction between β-amylase and amylopectin is due not to one of the possibilities above mentioned, but to a combination of them.

In addition to the β-amylase determinations, the urease test was carried out to ascertain whether this enzyme was active or not. We did not study the possible penetration of substrate into soybean material, because urea is a low molecular compound. Therefore we confined ourselves to the result of this qualitative test, merely to see whether EDTA-treated material still had urease activity. Considering that apart from β-amylase and urease – and as it will be seen later (page 73, paragraph 5.2.4.) – also lipoxidase is still active after EDTA treatment, we conclude that EDTA does not denature these enzymes. Therefore, using the activity of enzymes as criteria for denaturation of proteins, the conclusion is allowed that EDTA is not a protein denaturing agent.

5. EXPERIMENTS MADE WITH CHYMOTRYPSIN
AND/OR LIPASE

The purpose of these experiments was to learn whether chymotrypsin would enhance the penetration of lipase or not. The effect was studied in the case of soybean (with plasmodesmata) and yeast (without plasmodesmata). Algae (without plasmodesmata) were not used, because we considered it sufficient to work only with yeast for detecting the effect of the presence or absence of plasmodesmata.

5.1. PRELIMINARIES OF THE EXPERIMENTS MADE WITH CHYMOTRYPSIN AND/OR LIPASE

5.1.1. *Materials, Methods and Results*

Olive oil. The acid value of this oil (2.7) was determined in accordance with the method applied in the British Pharmacopoeia, as described by PEARSON (1962). According to the Merck Index (1960) the acid value of olive oil is between 0.2–2.8. Therefore we considered it all right to employ this olive oil.

Gum arabic solution, olive oil emulsion and sodium taurocholate were prepared according to DESNUELLE et al. (1955).

Gum arabic solution. Gum arabic (10 g) that had been previously pulverized in a mechanical mortar was suspended in distilled water (100 ml). After it had been vigourously stirred the turbid solution was filtered.

Olive oil emulsion. Gum arabic solution (165 ml) and crushed ice (15 g) were mixed with olive oil (20 ml). This mixture was shaken violently in the Griffin flask-shaker for 15 minutes at room temperature. The resultant olive-oil emulsion can be kept at 5 °C in closed flasks for a long time.

Sodium taurocholate (NaT). NaT (20 g) was dissolved in distilled water (100 ml).

Commercial lipase powder. The lipase powder used (pork pancreas) was purchased from Mann Research Laboratories Inc., New York, Cat. 4633, G 1895 and stored at 5 °C in a dessiccator containing silica gel.

Purified lipase powder. This term refers to the lipase prepared from pork pancreas according to the method by MELIUS and SIMMONS (1965).

Diisopropylfluorophosphate (DFP). This chemical was purchased from Calbiochem, Los Angeles, D.27,858, Lot 54,046. A 1 M stock solution was prepared by dissolving DFP (1 g) in isopropanol (5.4 ml). The DFP was dissolved in isopropanol as a safety measure (BALLS and JANSEN, 1952). The solution was stored at 5 °C.

DFP solution (10^{-4}M, 10^{-3}M or 10^{-2}M) were prepared by appropriate dilutions of the 1 M DFP stock solution with distilled water. The glassware used for DFP was cleaned after rinsing first with NaOH as recommended by SAUNDERS (1957).

Lipase solutions. Portions of commercial lipase powder (10 mg) were suspend-

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ed at 5°C in 1 ml of the various DFP solutions (10^{-4}M, 10^{-3}M or 10^{-2}M). After being stirred for 2 or 20 hours at 5°C the suspensions were centrifuged at 5°C for 15 minutes at 700 g. The resultant supernatant was the lipase solution. Therefore we had the following types of lipase solutions: in DFP 10^{-4}M-(2 or 20 hours), 10^{-3}M-(2 or 20 hours) and 10^{-2}M-(2 or 20 hours).

Commercial lipase powder (10 mg) was likewise suspended at 5°C in distilled water (1 ml) without DFP. After being stirred for 15 minutes the suspension was centrifuged at 700 g for 15 minutes at 5°C. The supernatant obtained was the *lipase solution in water*.

Purified lipase solution. This was obtained by suspending purified lipase powder (10 mg) in distilled water (1 ml). After the suspension had been stirred at 5°C for 15 minutes it was centrifuged at the same temperature, at 700 g for 15 minutes. The supernatant obtained was the purified lipase solution.

Chymotrypsin solutions. We prepared chymotrypsin solution 5 times more concentrated than the solution used in previous experiments (page 7, paragraph 2.1.2.2.). This enzyme is designated as CT 5st.

We ascertained whether CT 5st had really five times more activity than the previous solution, by diluting the enzyme 5 times with 0.1 M borate buffer pH 8 and incubating a portion of it (0.05 ml) in casein solution as previously described (page 8, paragraph 2.1.2.3.). We diluted the enzyme CT 5st to prevent the casein from becoming a limiting factor in the reaction process. The extinction at 280 mμ was the same as in the case of chymotrypsin. This shows that the CT 5st really had an activity five times that of the chymotrypsin.

Chymotrypsin solution in NaCl. Hitherto we had prepared chymotrypsin by incubation of CTG (2 ml) and TG (1 ml) with 0.1 M borate buffer pH 8 (5 ml) as described on page 7, paragraph 2.1.2.2. In the present instance we followed the same procedure, but instead of using borate buffer, the pH was adjusted to 8 and the final volume was made up to 8 ml. The activity of these enzyme solutions (chymotrypsin and CT 5st) in NaCl pH 8, was checked as described on page 8, paragraph 2.1.2.3. Both had the same activity as they had when borate buffer was used. This activity was also constant for 180 minutes at 37°C.

5.1.1.1. Determination of proteolytic activity of the different lipase solutions and the percentage of proteolytic inhibition by DFP

Quantities (0.05 ml) of lipase solution in distilled water and in DFP, and purified lipase solution were incubated with casein solution as for the determination of chymotrypsin activity (page 8, paragraph 2.1.2.3.) with the slight difference that the incubation was at 37°C instead of at 35°C (table 9).

Purified lipase solution did not show any proteolytic activity, whereas lipase solution in distilled water did ($E_{280\,m\mu}^{1\,cm} = 0.520$). This extinction was considered 100% activity. The extinctions of the lipase solutions in DFP were converted to percentages assuming a linear relation between extinction and percent remaining activity. The percentage of inhibition by DFP is calculated by substracting the remaining activity (%) from 100%.

TABLE 9. Determination of proteolytic activity of the different lipase solutions and the percentage of proteolytic inhibition effected by DFP.

Lipase solution in:	$E_{280 m\mu}^{1 cm}$	Remaining proteolytic activity (%)	Percentage of proteolytic inhibition
Distilled water (no DFP)	0.520	100	0
10^{-4}M DFP 20 hours	0.222	43	57
10^{-4}M DFP 2 hours	0.350	67	33
10^{-3}M DFP 20 hours	0.147	28	72
10^{-3}M DFP 2 hours	0.189	36	64
10^{-2}M DFP 20 hours	0.140	27	73
10^{-2}M DFP 2 hours	0.127	24	76
Purified lipase solution	0	0	

5.1.1.2. Influence of inhibition of the proteolytic activity of lipase solution in 10^{-3}M DFP-20 hours at 37 °C

Lipase solution in 10^{-3}M DFP-20 hours, was placed in a water bath at 37 °C. After it had reached this temperature aliquot parts (0.05 ml) were submitted at different times (0, 40, 80 or 120 minutes) to the casein digestion test as described on page 8, paragraph 2.1.2.3. with the slight difference that the mixture was incubated at 37 °C. The inhibition percentage was calculated as previously described and also on the basis that an extinction of 0.520 is 100% activity. Results corrected for appropriate blanks are recorded in table 10, in which it is shown that after 120 minutes at 37 °C inhibition remained constant between 70 and 77%.

TABLE 10. Influence of inhibition of the proteolytic acitivity of lipase solution in 10^{-3}M DFP–20 hours at 37 °C.

Time (minutes)	$E_{280 m\mu}^{1 cm}$	Remaining activity(%)	Percentage of proteolytic inhibition
0	0.158	30	70
40	0.153	29	71
80	0.142	27	73
120	0.117	23	77

5.1.1.3. Modified Casein Digestion Method (MCD)

This method as described by MOOIJMAN (1964) was adopted to see whether all of the DFP in the lipase solution (10^{-3}M DFP-20 hours) reacted with seryl or histidyl groups of the commercial lipase powder or if there was still some DFP free to inhibit any chymotrypsin that might by added later when working with both enzymes simultaneously (lipase and chymotrypsin).

The MCD method consisted of incubation at 37 °C of lipase solution (10^{-3}M DFP-20 hours) (1.0 ml) plus 0.1 M borate buffer pH 8 (0.1 ml) plus chymotrypsin solution (1.0 ml) plus 2 % casein solution containing 0.005 M Ca ions (1.0 ml). Thirty minutes after the addition of the casein solution 5 % trichloroacetic acid (TCA) (3.0 ml) was added. The mixture was shaken and allowed to stand at room temperature for one hour and then centrifuged at 700 g for 30 minutes. The supernatant was filtered through a fritter glass-filter G3 and the extinction of the filtrate was measured in the spectrophotometer at 280 mμ. The corresponding blank was the same, but in this case TCA had been added before the chymotrypsin. As a reference the same reaction mixture was used, but instead of lipase solution (containing the inhibitor DFP) distilled water was employed. The corresponding blank consisted of the same mixture except that TCA had been added prior to chymotrypsin. The extinctions at 280 mμ corrected for the appropriate blanks were for the experiment (with DFP) 0.35 and for the reference (with H_2O) 1.89. The activity remaining was one fifth of the original activity. This allowed us to assume that if CT 5st was used the non-inhibited portion would have approximately the same activity as the reference (no inhibitor). To corroborate this assumption, the same MCD method using 4 % casein solution instead of 2 %, was adopted for CT 5 st. As a reference we used chymotrypsin and distilled water (no DFP of the lipase solution). The results corrected for the appropriate blanks are for CT 5st 3.8 and for the reference 3.7. This demonstrates that CT 5st combined with DFP of lipase solution is similar to the reference. Therefore DFP of lipase solution (1 ml) combined with CT 5 st (1 ml), i.e. 1:1 (v/v) inhibits a part of the chymotrypsin activity, leaving an active fraction. This situation is similar to that when chymotrypsin is alone without inhibitor.

5.1.1.4. Determination of lipase activity (fig. 26)

This test was made according to the method of DESNUELLE et al. (loc. cit.) and MARCHIS-MOUREN et al. (1959), slightly modified. Olive oil emulsion (10 ml) plus sodium taurocholate (NaT) (0.3 ml) plus distilled water (19.7 ml) was poured into a titration flask, which was then placed in a water bath at 37 °C. Then the pH of the mixture was adjusted to 9.1–9.2 with 1N NaOH (several drops) and the lipase solution to be tested (0.5 ml) was added. The pH dropped due to the fatty acids liberated. Small amounts of 0.1N NaOH were added to bring the pH to 9.0 when the original adjustment was 9.1 and to 9.1 when the original adjustment was 9.2. The results are recorded in graphs, where the amount of 0.1 N NaOH used for the titration (accumulative values of the partial titrations) are recorded as a function of time (fig. 27). The slope of the line, i.e. the number of μequivalents of acid liberated per 10 minutes, was used for expressing the enzyme acivity.

In order to obtain more points for plotting the line we did not employ buffer solution. The line obtained had the same slope as the one where buffer was used.

Another experiment consisted in determining the lipolytic activity of 1.5 ml

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or 2.5 ml lipase solutions in 10^{-3}M DFP-20 hours (instead of 0.5 ml). In these cases the amount of distilled water was decreased to obtain a final volume of 30 ml. The appropriate blanks had 10^{-3}M DFP (1.5 ml or 2.5 ml) instead of lipase solution. The quantity of acid liberated per 10 minutes is proportional to the amount of lipase solution employed: 5, 17 and 24 μequivalents acid per 10 minutes respectively produced by 0.5, 1.5 and 2.5 ml lipase solution (fig. 27).
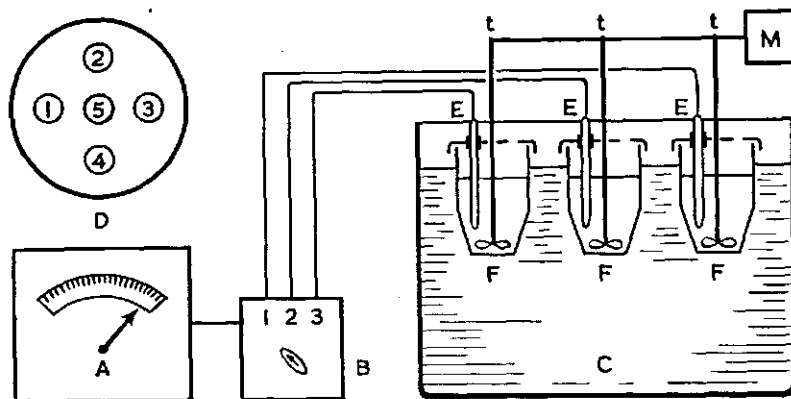
FIG. 26. *Diagram of the installation used for the determination of lipase activity.* (A) pH-meter, Metrohm type E196 connected to the 3 electrodes (E) through a switch box (B), so that each of the electrodes is selected individually by moving the switch. This allows readings to be taken at 2 minute intervals. The three identical stirrers (t) had a common motor (M) to ensure that the velocity of stirring was equal for the three titration flasks (F) (Metrohm), which were placed in a water-bath at 37°C (C). The three flasks in identical conditions allowed us to make an experiment in duplicate and a blank simultaneously. (D) Top of a titration flask. The cover has five holes (1, 2, 3, 4 and 5). The electrode passed through hole 1, fitting perfectly. The stirrer passed through hole 5. Each time that it was necessary to add 0.1N NaOH hole 4 was uncovered and a pipette of one ml capacity was introduced without touching the contents. Holes 2 and 3 were closed with plastic plugs.

5.1.1.5. Determination of lipase activity when lipase and chymotrypsin are present simultaneously.

The lipase activity was determined by the method previously mentioned, but also in this case we added chymotrypsin dissolved in NaCl solution (0.5 ml) and as a corresponding blank NaCl solution (0.5 ml) without chymotrypsin. Another blank was prepared by adding chymotrypsin in NaCl (0.5 ml) without lipase solution and without DFP solution.

The results are presented in fig. 27. It will be seen that after 40–50 minutes the line representing the lipase activity in the presence of chymotrypsin decreases in comparison with lipase alone. This is because chymotrypsin digests lipase. Chymotrypsin alone yields a practically horizontal line, therefore chymotrypsin is not responsible for the titration values when incubated with olive oil emulsion and does not contain lipase as an impurity.

We should like to point out that when the NaCl solution (with or without chymotrypsin) was used the reaction mixture contained Ca ions. In the final

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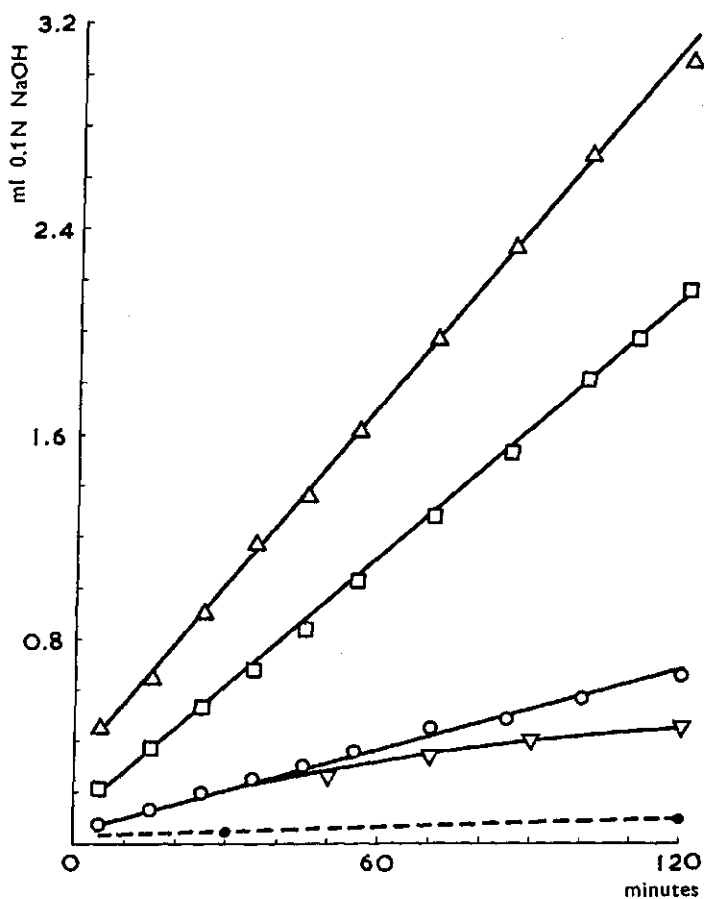
FIG. 27. *Determination of lipase activity.* 0.5 ml lipase solution or 0.5 ml lipase solution in presence of NaCl (0.5) ml without chymotrypsin (O——O), 1.5 ml lipase solution (□—–—□), 2.5 ml lipase solution (△—— △). Lipase solution (0.5 ml) in presence of 0.5 ml chymotrypsin solution in NaCl (▽——▽). Corresponding blanks made with 10^{-3}M DFP (0.5 ml, 1.5 ml or 2.5 ml) or chymotrypsin without lipase and without DFP (●- - -●).

volume (30 ml) these ions were present in a concentration of approximately 0.6 mM.

5.1.2. *Discussion*

The use of the purified lipase solution would had been the best for our experiments because it has no proteolytic activity. This was important as we had to study the effect of lipase on its own and in combination with chymotrypsin, the latter being a proteolytic enzyme. However we could not employ the purified lipase solution because the amount of pure lipase powder obtained was too small for digestion experiments with soybean and yeast. Consequently we used the commercial lipase powder for preparing lipase solution. The drawback is

that this lipase solution in distilled water possesses proteolytic activity. Therefore we added DFP which is an irreversible inhibitor of proteolytic enzymes. Moreover, even when we used lipase solution alone it was still better to inhibit the proteolytic enzymes in order to prevent the lipase from being digested (SARDA et al., 1957, 1964; BENZONANA et al., 1964).

The inhibition of the proteolytic activity presented in the lipase solution in DFP reached a maximum 72–76% in 10^{-2}M DFP((2 or 20 hours) and in 10^{-3}M DFP-20 hours. When the lipase activity of the different lipase solutions in DFP was determined it was found that DFP was inhibiting the lipase activity as a function of its concentration. It is known that DFP is a specific inhibitor of esterases because it combines with histidyl and seryl groups which are the active sites of these enzymes. Furthermore, it is known that DFP inhibits the activity of milk lipase (ROBERTSON et al. 1966).

The 10^{-4}M DFP solution had little or no inhibitory effect on the lipase solution; however the proteolytic inhibition was low (33–57%). On the other hand 10^{-2}M DFP inhibited the lipase too strongly. A compromise was found with 10^{-3}M DFP-20 hours, which effected a proteolytic inhibition similar to 10^{-2}M but afforded a good lipase activity (5 μequivalents acid per 10 minutes).

Once we had decided to use lipase solution in 10^{-3}M DFP-20 hours for further experiments we ascertained whether the proteolytic inhibition of this solution would remain constant for 2 hours at 37°C, as these conditions will be used for further experiments with soybean and yeast. We observed in table 10 that the inhibition of proteolytic activity remained constant (74% ± 4%). Furthermore, it is worth while to notice that the lipase activity is constant from zero to 120 minutes (fig. 27). However, when chymotrypsin is present in the lipase solution, the lipase activity decreases after 40–50 minutes (fig. 27), the line tending to become horizontal. This shows that chymotrypsin digests lipase, and this fact will be interesting for the interpretation of later results of experiments with sections digested by both enzymes.

When one uses up to 2.5 ml of lipase solution the activity is proportional to the amount used. This is important because in the experiments with soybean sections we shall use five times more lipase solution than will be the case with isolated soybean cells.

The MCD method was used to determine whether all of the DFP had combined with the proteolytic enzymes of the commercial lipase powder. We found, however, that part of the chymotrypsin was inhibited when added simultaneously with lipase solution in DFP. When CT 5st was used this enzyme was inhibited by the DFP of the lipase solution to such a degree that there remained an activity similar to that which occurs in chymotrypsin in the absence of DFP. Therefore in the experiments with soybean and yeast, when the two enzymes are used simultaneously, lipase solution and chymotrypsin 5st are mixed in a proportion of 1:1 (v/v) prior to addition to the substrate.

Chymotrypsin or CT 5st will be prepared in NaCl solution and not in buffer.

5.2. Digestion by lipase of substrates (soybean and yeast) treated or not treated with chymotrypsin

5.2.1. Materials and methods

Soybean cells (unheated or heated). These were obtained as described on page 12, paragraph 2.1.5.1. The heated cells used had been heated for 20 minutes.

Soybean cell suspension. The cells, which had been stored in 0.1 M borate buffer pH 8, 0.1 % Preventol 115, were washed twice with a large excess of distilled water. They were suspended in distilled water to a concentration of 20 % with the aid of the hematocrit (page 8, paragraph 2.1.3.).

Filtrate of unheated cells. When the 20 % unheated cell suspension in distilled water was prepared it was filtered through a Buchner funnel containing a filter paper. The resultant filtrate was used for the experiment.

Soybean sections (unheated or heated). These were obtained and treated as described on page 12, paragraph 2.1.5.1. The sections, which had been stored in 0.1 M borate buffer pH 8, 0.1 % Preventol 115, were washed twice with a large excess of distilled water.

Yeast cells. This was the same preparation as was previously described on page 14, paragraph 2.1.5.2. All the experiments were carried out with 'short-time storage' (STS). Accordingly we had the following types: unheated, heated, unheated-EDTA, heated-EDTA and unheated-EDTA-heated. These yeast preparations which had been stored in 0.1 M phosphate buffer pH 7.6, 0.1 % Preventol 115, were washed as described on page 16, paragraph 2.1.6., but distilled water was used instead of 0.1 M phosphate buffer pH 7.6. The 20 % suspensions were prepared with the aid of the hematocrit.

Helix Pomatia (HP). 'Suc d'helix pomatia' was purchased from 'Industrie Biologique Française'.

Unheated yeast-HP. This was unheated yeast treated with HP in the manner to be described later on page 66, paragraph 5.2.1.4.

Olive oil emulsion. This was prepared in the manner previously described on page 56, paragraph 5.1.1.

Heated olive oil emulsion. This was prepared by heating the whole of the olive oil emulsion in a bath with boiling water (100 °C) for 20 minutes.

Heated olive oil. This was obtained by heating olive oil in a bath with boiling water for 20 minutes.

Emulsion of heated olive oil. This was prepared in the same manner as the olive oil emulsion, except that heated olive oil was used.

Subcellular soybean powder. An unheated preparation was obtained as described for powder P, on page 12, paragraph 2.1.5.1. Heated powder was obtained in the same way as powder P, but with the use of heated cotyledons in distilled water at 100 °C for 20 minutes.

Emulsion of subcellular soybean powder (unheated or heated). This was prepared in the same manner as the olive oil emulsion, but subcellular powder (20 g) was used instead of olive oil (20 ml).

Ether. Ether was purified as described by VOGEL (1957). Later we used 'Aether

puriss. absol. pro narcosi' purchased from the 'Koninklijke Nederlandse Gist- en Spiritusfabriek N.V.', Delft.

Chloroform. This was purified in the manner described by VOGEL (loc. cit.).

Margaric acid methyl ester. Margaric acid (heptadecanoic acid), made by Koch and Light (Colnbrook, England) and obtained as a gift from Unilever Research Laboratorium in Vlaardingen, was methylated in accordance with the method of STOFFEL et. al. (1959). Later we used the heptadecanoic acid methyl ester purchased from Sigma (St. Louis, U.S.A.)

Sodium taurocholate (NaT). This was prepared as described on page 56, paragraph 5.1.1.

Palmitic acid methyl ester and stearic acid methyl ester were prepared by methylating palmitic and stearic acids separately according to STOFFEL et al. (loc. cit.).

Oleic acid methyl ester, linoleic acid methyl ester and *linolenic acid methyl ester* were purchased from Sigma (St. Louis, U.S.A.) as was also a 100 mg ampoule containing 20 mg of each of palmitic, stearic, oleic, linoleic and linolenic acid methyl esters.

5.2.1.1. Penetration of lipase into substrates (soybean and yeast) treated or non-treated with chymotrypsin.

As in the case of penetration of chymotrypsin we digested the soybean and yeast substrates in vitro with lipase, in order to measure the entry of this enzyme.

If lipase were to penetrate into the substrate it would release fatty acids from the fats. Therefore when titrating the reaction mixture with 0.1 N NaOH to a constant pH we have a measure of fatty acids liberated by lipase. The NaOH data (accumulative values of the partial titrations) are recorded as a function of time. The slope of the line, i.e. μequivalents of acids per 10 minutes is a measure of the enzymic activity. This indicated the penetration of lipase.

5.2.1.2. Digestion of soybean cells (unheated or heated) by chymotrypsin and/or lipase.

Twenty per cent cell suspension (35 ml) plus distilled water (4.0 ml) was poured into a titration flask, which was then placed in a water bath at 37 °C. The pH of the mixture was adjusted to between 8 and 9 with 1N NaOH, and chymotrypsin solution (0.5 ml) was added. During the incubation with chymotrypsin the pH was maintained between 8 and 9 by adding 0.1N NaOH. After 80 minutes the reaction mixture was adjusted to pH 9.1–9.2 and lipase solution (0.5 ml) was added, the moment at which this was done marks our zero time. When the pH dropped 0.2 units 0.1N NaOH were added drop by drop to bring the pH to 9.0–9.1. This experiment is designated (CT 80' + L) which means soybean cells incubated with chymotrypsin for 80 minutes and then with lipase. The corresponding blank for the experiment comprised the same procedure, but DFP 10^{-3}M (0.5 ml) was used instead of lipase solution (CT 80'–).

Another experiment consisted of using NaCl solution without chymotrypsin

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(0.5 ml) and adding lipase solution (0.5 ml) after about 80 minutes (–80' L). The corresponding blank comprised the same procedure, but with the use of DFP 10^{-3}M (0.5 ml) instead of lipase solution (–80'–).

So far we have described the experiments in which chymotrypsin had been previously incubated to lipase. We shall now describe the experiment in which both enzymes were used simultaneously.

Twenty per cent cell suspension (35 ml) plus distilled water (4.0 ml) was poured into a titration flask, which was then placed in a water bath at 37 °C. Then the pH was adjusted to 9.1–9.2 with 1N NaOH. After this 1.0 ml of mixture of CT 5 st and lipase solution (1:1 v/v) were added (page 62, paragraph 5.1.2.). This was the zero time. When the pH dropped by 0.2 units it was adjusted to 9.0–9.1 as previously mentioned. This experiment is designated (CT + L) which means that soybean cells were incubated with both enzymes simultaneously. The blank was prepared by adding 1.0 ml of a mixture of CT 5st plus DFP 10^{-3}M (1:1 v/v) to the cell suspension and was designated (CT–). Another experiment carried out by adding 1.0 ml of a mixture of NaCl solution without chymotrypsin but with lipase solution (1:1 v/v) to the cell suspension and incubating the mixture as mentioned; this was designated (–L). The blank consisted in adding 1.0 ml of a mixture of NaCl solution and DFP 10^{-3}M (1:1 v/v) and this was designated (– –).

It should be noticed that when NaCl solution (with or without chymotrypsin) was used the reaction mixture contained $CaCl_2$. In the final volume (40 ml) this salt was present in a concentration of approximately 0.4 mM.

Another blank experiment consisted in the digestion of the filtrate from unheated soybean cells. The experiment was the same as was previously described, but instead of using cell suspension we employed filtrate (35 ml). This experiment was likewise made in the presence of NaT (0.2 ml) or the absence of NaT. We carried out the following experiments: (CT + L), corresponding blank (CT –) and (– L), the corresponding blank being (– –).

The proportions of materials used for the digestion of soybean cells were determined after some preliminary experiments. We knew that 1 ml of chymotrypsin solution was optimal with 70 ml of 20 % soybean cell suspension in a final volume of 80 ml (page 21, paragraph 2.2.1.). Therefore 0.5 ml of chymotrypsin solution in a final volume of 40 ml was all right. Once we had fixed the amount of 20 % unheated soybean cell suspension (35 ml) treated for 80 minutes with chymotrypsin we varied the amount of lipase solution (0.25 ml, 0.5 ml or 1.0 ml) changing the amount of distilled water as necessary: 4.25 ml, 4 ml or 1.0 ml. The incubation time with lipase was 60 minutes. The appropriate blanks consisted of the same, but we used DFP 10^{-3}M (p.25 ml, 0.5 ml or 1.0 ml) instead of the lipase solution. We decided to use 9.5 ml lipase solution because by doing so we were able to obtain a constant rate of hydrolysis (straight line). We used unheated cells because there was no difference in the results obtained with unheated or heated cells.

Once we had determined the amount of substrate and lipase, we varied the quantity of NaT (0.1, 0.2 or 0.3 ml) changing the amounts of distilled water needed to give a final volume of 40 ml. No significant differences were obtained by using different quantities of NaT, or by using none at all.

5.2.1.3. Digestion of soybean sections (unheated or heated) by chymotrypsin and/or lipase

These experiments were carried out in a manner similar to those with soybean cells. The main differences were: 1. the quantities were reduced proportionally to a final volume of 20 ml (instead of 40 ml) in order to permit the use of a smaller quantity of sections; 2. we used five times the amount of chymotrypsin solution and lipase solution as compared with the amount used for the cells. This was done to compensate for the difference in the ratio surface/enzyme.

5.2.1.4. Digestion of yeast by chymotrypsin and/or lipase

These experiments were made in the same manner as with soybean cells, with the exception that NaT (0.8 ml) was employed and the amount of distilled water was changed to give a final volume of 40 ml. This amount of NaT was determined with unheated yeast-EDTA treated with chymotrypsin for 80 minutes. We found that when using NaT (0.2, 0.4, 0.8 and 1.6 ml) with 0.8 ml we had a line with a significantly steeper slope than when not using NaT. For 1.6 ml NaT the initial rate of lipolysis was as for 0.8 ml, but afterwards decreased.

Another experiment was carried out with unheated yeast pretreated with the enzyme preparation from Helix pomatia (HP). The 20% unheated yeast suspension (35 ml) plus distilled water in a titration flask was placed in a water bath at 37°C, and HP (0.2 ml) was added. This incubation lasted for 6 hours. The pH of the unheated yeast suspension was 5.2–5.8 and remained so during the incubation with Helix enzyme. This pH is suitable for the lysis of yeast cell wall by this enzyme preparation (EDDY and WILLIAMSON, 1957). After the incubation of the yeast with Helix enzyme NaT (0.8 ml) was added and the pH immediately adjusted to 9.1–9.2 with 0.1N NaOH. Then 1 ml of a mixture of NaCl without chymotrypsin and lipase (1:1 v/v) was added (– L), and for the corresponding blank (– –) 1.0 ml of a mixture of NaCl and DFP 10^{-3}M (1:1 v/v).

5.2.1.5. Digestion by lipase of olive oil emulsion, heated olive oil emulsion, emulsion of heated olive oil, emulsion of unheated subcellular soybean powder and emulsion of heated subcellular soybean powder

These experiments were carried out as described for the determination of lipase activity on page 59, paragraph 5.1.1.4.

5.2.2. *Analysis of higher fatty acids liberated from soybean cells by lipase*

With the potentiometric method we titrated all the acidic groups, whether or not from fatty acids. In view of this we carried out two experiments in order to

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verify the specificity of the potentiometric method. 1. We extracted with ether the fatty acids liberated by lipase and titrated this extract again with NaOH. 2. We analysed qualitatively and quantitatively the fatty acids of the ether extract by gas-liquid chromatography (GLC).

Ether extract

After 120 minutes digestion of soybean cells (unheated or heated) by chymotrypsin and/or lipase at pH 9 we filtered the reaction mixture through a fritted glass-filter G3. Then the soybean cells remaining on the filter were rinsed twice with distilled water to remove as much as possible of the neutralized fatty acids from the cells. The filtrate containing the neutralized fatty acids was collected in an erlenmayer and acidified with 1–2 drops of concentrated sulphuric acid to pH 2 (reconstitution of the fatty acids) and either kept for one night at 5 °C or directly extracted with ether. This was performed either by three successive extractions with a separating funnel or with a continuous extractor (Kutscher-Stendel) for two days at room temperature. The digestion of soybean cells as well as the other steps were performed in diffuse light; the continuous extraction itself in the dark. It was decided to work in diffuse light or absolute darkness to prevent oxidation of unsaturated fatty acids by light.

The resultant ether extract was either titrated with alcoholic NaOH solution in the presence of phenolphthalein, or it was submitted to the methylation process for GLC. The titration value was compared with that obtained with the potentiometric method after 120 minutes of digestion of the soybean cells by chymotrypsin and/or lipase.

Methylation of fatty acids

The ether extract was dried in a stream of nitrogen at 40 °C. The dried residue was methylated according to the method of STOFFEL et al. (loc. cit.). This is a micro-method for the preparation of methyl esters for GLC by esterification with methanol and hydrochloric acid. With oleic acid as a model we determined that 95 % of the acid was methylated.

The methyl esters were stored solvent-free in sealed ampoules in an atmosphere of nitrogen or in dilute solutions in petroleum ether at 5 °C in the dark.

Preparation of the methyl esters for GLC

The methyl esters in petroleum ether were dried in a stream of nitrogen at 40 °C. The dried residue was dissolved in a solution of chloroform (0.025 ml) containing margaric acid methyl ester (0.63 mg) as internal standard. Afterwards this solution was made up to 0.2 ml with chloroform. From this solution 1 μl was injected into the gas chromatograph using a Hamilton syringe (Hamilton Co., Whittier, Calif., USA).

Gas-Liquid Chromatograph (GLC)

The apparatus used was the F and M Scientific Hewlett-Packard, 5750 Research Chromatograph. The optimal conditions for chromatography (see text of fig. 30) were determined with a standard mixture of palmitic, stearic, oleic, linoleic and linolenic acids methyl esters. These fatty acids were those expected to be present in the products of digestion of soybean by lipase. Afterwards we determined the retention time of each of them.

For the identification of the peaks obtained from the mixture of esters from the digestion of soybean by chymotrypsin and/or lipase the retention times were used. The quantitative determination was done with margaric acid methyl ester as internal standard (SCHMIT and WYNNE). Our modification consisted in calculating the peak areas with a planimeter, and we compared these areas with the area of a known quantity of margaric acid.

The quantitative determination by GLC was corrected for the appropriate blanks, likewise carried out with GLC.

The amount of fatty acid (μg) divided by the respective equivalent weight gave the amount of this fatty acid expressed as μequivalents. Summation of the μequivalents pertaining to the several fatty acids (palmitic, stearic, oleic, linoleic and linolenic) gave the total amount of μequivalents. This was compared with the value obtained potentiometrically.

5.2.3. *Results*

The data (μequivalents acid per 10 minutes) of the digestion experiments of soybean and yeast by chymotrypsin and/or lipase are recorded in table 11. Each of the data of this table represents the range of six values which were deduced from graphs obtained after three duplicate experiments. These μequivalents of acid were deduced from graphs which had been corrected for the corresponding blanks. Two typical graphs are presented in figures 28 and 29. Fig. 28 is concerned with unheated soybean cells digested by chymotrypsin and lipase

TABLE 11. Digestion of soybean and yeast by α-chymotrypsin and/or lipase. The values presented are μequivalents acid per 10 minutes.

	SOYBEAN		
	Unheated sections	Heated sections	Unheated or heated cells
CT 80' + L	5–8	5–5.2	4–4.5
CT + L	1–1.4	4.8–5.0	4–4.5
L	1–1.3	2–2.4	2–3

	YEAST		
	Unheated	Unheated Yeast-HP	Unheated-EDTA	Heated, heated-EDTA or unheated-EDTA-heated
CT + L	0		2–3	5–6
L	0	7–8	2–3	5–7

We have used the designation 'L' because there was no difference between (–80' L) and (–L). In the case of yeast we have used the designation (CT + L) merely because there was no difference between the two enzymes incubated simultaneously or α-chymotrypsin before lipase CT 80' + L).
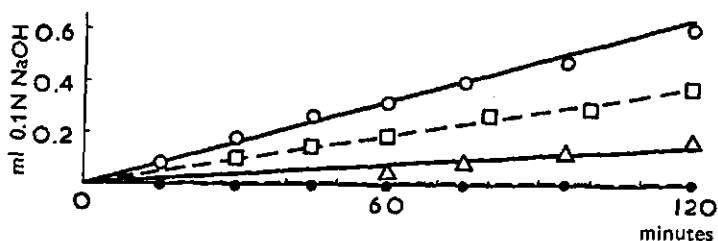
FIG. 28. *Digestion of unheated soybean cells by chymotrypsin (80 minutes) and/or lipase. Chymotrypsin and lipase (CT 80' + L) (O———O), corresponding blank (CT 80'–) (△———△). Lipase alone (–80'L) (□- - -□), corresponding blank (–80'–) (●- - -●).*
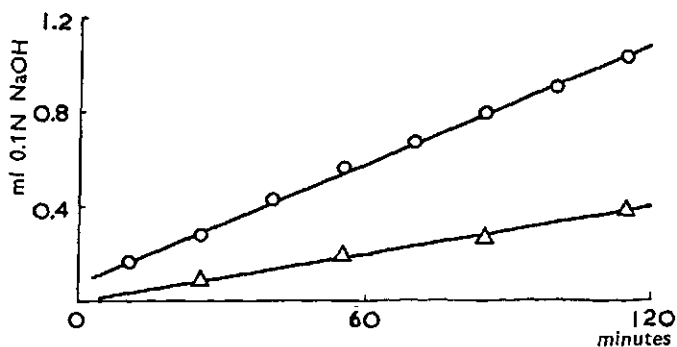
FIG. 29. *Digestion of heated yeast by lipase. Lipase alone (–L) (O———O), corresponding blank (– –) (△———△).*

(CT 80' + L) with its corresponding blank (CT 80'–); and with lipase alone (–80'L) with its corresponding blank (–80'–). Fig. 29 relates to the digestion of heated yeast by lipase (–L) and to its corresponding blank (– –).

The data in table 11 allow the following conclusions:

1. There is no difference in the digestibility of substrates when these are incubated with chymotrypsin for 80 minutes before the addition of lipase (CT 80' + L) or with the two enzymes simultaneously (CT + L). The exception arises in the case of unheated sections, where the two enzymes, when incubated simultaneously did not result in the same value as in the case of substrate incubated for 80 minutes with chymotrypsin before the addition of lipase.

The same results are obtained when instead of chymotrypsin NaCl is used for 80 minutes before the addition of lipase (–80'L) or when the NaCl solution and lipase are added simultaneously (–L).

2. In all cases of soybean material (sections or cells) chymotrypsin and lipase together gave higher values than lipase alone; except in the case of the unheated sections, where the two enzymes incubated simultaneously gave the same value as lipase alone.

Furthermore, unheated or heated soybean cells present the same values.

3. In the case of yeast, chymotrypsin and lipase gave values similar to those of lipase alone.
Heated, heated-EDTA and unheated-EDTA-heated gave similar results.

The values obtained with unheated yeast are zero. In the case of unheated yeast-HP digested by lipase alone 7–8 μequivalents acid are liberated per 10 minutes. We made the digestion of unheated-yeast-HP with lipase alone and not with chymotrypsin and lipase, because we used this as a check to ascertain whether the zero of the unheated yeast was due to cell wall permeability or to a lipase inhibitor within the cells.

The digestion of filtrate from unheated soybean cells by chymotrypsin and/ or lipase is a horizontal line. This means that the quantities of acid recorded in table 11 have originated from products of hydrolysis produced within the cells and not outside.

5.2.3.1. Results of the experiments on the digestion by lipase of the olive oil emulsions and soybean powders emulsions

The hydrolysis of fat by lipase of olive oil emulsion, heated olive oil emulsion-emulsion of heated olive oil, emulsion of unheated subcellular soybean powder and emulsion of heated subcellular soybean powder, gave the same results for unheated or heated material. This means that substrate does not become more susceptible to lipase when heated than when it is unheated.

5.2.3.2. Results of the analysis of higher fatty acids liberated from soybean cells by lipase

The titration values of the ether extracts were: 80% of the potentiometric value for the one obtained with the continuous extractor; and 50% of the potentiometric value for the one obtained with the separating funnel.

The GLC analysis of fatty acids from products of digestion of heated soybean cells gave the five fatty acids expected by us, i.e.: palmitic, stearic, oleic, linoleic and linolenic (fig. 30). The sum of the unsaturated fatty acids was 75–80% of the total fatty acids, while the sum of the saturated fatty acids was 15–20%. This proportion is characteristic for soybean oil fatty acids (MORSE, 1950; SWERN, 1964; SINGH et al., 1968).

The total amount of fatty acids was 50% of the expected value determined with the potentiometric method.

The gas-chromatogram of the products of digestion of unheated soybean cells did not clearly present the peaks pertaining to the unsaturated fatty acids (oleic, linoleic and linolenic). Therefore, this gas-chromatogram was not used for quantitative determinations.

5.2.4. *Discussion*

Once we had determined the optimum ratio soybean cells/lipase we no longer found a favourable action of sodium taurocholate. This may be because the
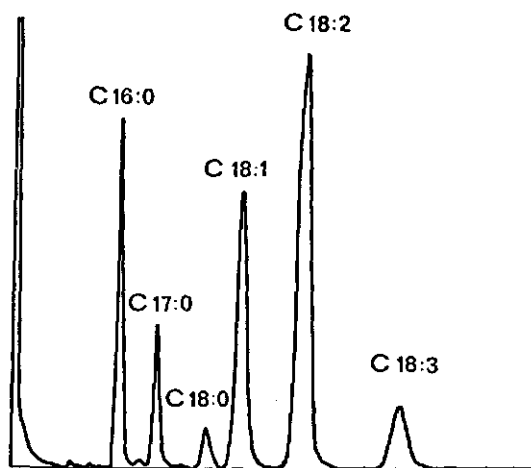
FIG. 30.

Gas-chromatogram of the fatty acids originated after 120 minutes digestion of heated soybean cells by chymotrypsin and lipase. The conditions were: column 6 ft, stainless steel, diameter 1/8", packed with 6% diethylene glycol succinate on 80–100 mesh Diatoport S (Hewlet-Packard). Carrier gas was nitrogen (15 ml per minute). Detector, flame ionisation (hydrogen 30 ml per minute, air 390 ml per minute). The column oven operated isothermically at 175°C. The temperature of the injection port was 200°C and of the detector 232°C. Range 10^2. Attenuation 16. Recorder speed 1/4 inch per minute.

Methyl ester of: palmitic acid (C 16:0), margaric acid (C 17:0), stearic acid (C 18:0), oleic acid (C 18:1), linoleic acid (C 18:2) and linolenic acid (C 18:3).

fat in soybean cells is already emulsified by its own lecithin and albumins, both of which are present in soybean and are good emulsifiers (WILLS, 1965).

As neither sodum taurocholate nor sodium desoxycholate gave better results we decided not to use them.

For the experiments with yeast we used sodium taurocholate because we had observed that the digestion by lipase of unheated yeast-EDTA was higher in the presence of sodium taurocholate.

Nevertheless, a large excess was not favourable, possibly because it denatured lipase. Sodium taurocholate has a 'detergent' nature (HASLEWOOD, 1962), and it is known that detergents can denature proteins (FOX and FOSTER, 1957).

The positive effect of sodium taurocholate on the digestion of yeast by lipase can be explained by: a. this compound affected the permeability of unheated, EDTA-treated yeast; b. it activated lipase by separating the complex triglyceride-lipase and thereby giving free molecules of lipase the opportunity to attack other molecules of triglycerides (FRITZ and MELIUS, 1963).

The facts that: 1. chymotrypsin enhances the penetration of lipase in unheated or heated soybean material (with plasmodesmata), whereas it does not do the same in the case of yeast (without plasmodesmata) and 2. lipase alone does not penetrate unheated yeast at all although it *does* penetrate unheated soybean sections, strongly support the assumption that plasmodesmata could be a way of penetration for chymotrypsin and lipase.

As just mentioned chymotrypsin enhances the penetration of lipase in soybean material with the exception, however, of unheated sections digested by both enzymes simultaneously. This can be accounted for as follows. We have

seen that chymotrypsin penetrated unheated sections only very slightly (page 24, paragraph 2.2.3.). Consequently, when both enzymes were added simultaneously, chymotrypsin did not have sufficient time to open a way for lipase. Moreover, it may be that when chymotrypsin was not immediately able to enter the unheated sections it tended to digest the lipase. We have demonstrated that lipase in the presence of chymotrypsin is digested after 40–50 minutes (page 62, paragraph 5.1.2.). Therefore, it is possible that chymotrypsin gradually opens a way, after a kind of lag phase. It might be expected that lipase would have entered from that moment. However, most of the lipase would be digested by then.

Lipase alone digested heated sections to a greater extent than unheated sections. Since heated substrate does not become more susceptible to lipase than unheated substrate (page 70, paragraph 5.2.3.1.) we conclude that the higher digestibility of heated sections is due to an increased permeability caused by heating.

Unheated and heated soybean cells present the same results. This can be explained if it is assumed that the barrier of these cells was strongly affected by maceration of the cotyledons with EDTA. However, chymotrypsin still enhances the penetration of lipase. This means that apart from other pores plasmodesmatal pores are important for the entry of enzymes. Furthermore, we must consider that lipid drops (spherosomes) in cells are surrounded by biological membranes and it may be that chymotrypsin, in addition to opening a way through the cell wall also contributes to degradation of the spherosome membrane, thus contributing to the accessibility of the substrate for lipase.

Chymotrypsin enhances the penetration of lipase into soybean cells whether the two enzymes are incubated simultaneously or not. This is because in the case of soybean cells (unheated or heated) chymotrypsin enters them from the very outset.

In none of the experiments with yeast does chymotrypsin enhance the penetration of lipase. We have seen on page 29, paragraph 2.2.5. that chymotrypsin penetrated unheated yeast-EDTA to a similar degree as it did heated yeast. Nevertheless, the penetration of lipase into the former is not similar to the latter. This shows that in contrast with the soybean there is in yeast no correlation between the entry of chymotrypsin and the entry of lipase. We assume, by way of explanation, that sites other than plasmodesmata are the way of penetration for enzymes into yeast. It may be that penetration occurs through different sites for the different enzymes.

By heating the yeast we reached the maximum penetration of lipase, and since heating does not make the substrate more susceptible to lipase (page 70, paragraph 5.2.3.1.) we concluded that heating *does* affect the permeability and thereby allows a better passage of lipase.

The fact that Helix-enzyme-treated unheated yeast was digested, demonstrates that the failure of unheated yeast to be digested by lipase was due to the barrier formed by its cell wall, and not to a hypothetical inhibitor of lipase being present within the yeast cells.

It is possible that the action of Helix enzyme helps in the penetration of lipase in one of the following ways: a. the cell wall of yeast is degraded and as a result lipase is able to penetrate; b. the cell wall of yeast is degraded and the sphero-plasts emerge. As the medium is not isotonic the spheroplasts burst and lipase digests the subdivided spheroplasts; c. both possibilities occur. Another possibility would be that the Helix enzyme destroys the hypothetical inhibitor of lipase present in the yeast. However, so far we have not found in the literature any reference to inhibitors of pancreatic lipase in baker's yeast. Therefore it is most probable that the failure of unheated yeast to be digested by lipase is indeed due to the barrier of the cell wall and the cell membrane.

Before discussing the analysis of fatty acids by GLC it should be remarked that these determinations are a complement to the potentiometric method, and it was not the main objective of our work.

We have said that the ether extract obtained by continuous extraction for two days contained 80% of the acid determined by the potentiometric method; and for the extract prepared in 1–2 hours with the separating funnel it was 50% of that obtained by the potentiometric method. At first sight it would seem that extraction of the fatty acids was a function of time or that with the separatory process we lost half of the material. However, the gas-chromatogram shows an amount of fatty acids that is 50% of the amount obtained by the potentiometric method. Therefore it is fairly certain that approximately half of the potentio-metrically determined quantity of acids is not derived from fats and does not consist of fatty acids. It may be that the lipase solution contained impurities of phosphatase, which acting on phosphatides present in the medium, could give rise to phosphoric acid which was not extracted in the separating funnel, but which was extracted with the continuous method. Phosphoric acid is not registered in the gas-chromatogram. Moreover, phosphomonoesterases are not in-activated by diisopropylfluorophosphate[1], which was present in our lipase solution. Furthermore, it is possible that a fraction of the neutralized fatty acids liberated by lipase remained adsorbed in the soybean cells.

We assumed that part of the liberated unsaturated fatty acids in unheated cells had been subject to enzymic oxidation as the peaks of the oleic, linoleic and linolenic fatty acids in the gas-chromatogram of the products of digestion from unheated cells were small and irregular, whereas those from the gas-chromatogram of the products of digestion of heated cells were larger and more perfectly traced.

This assumption grew stronger because: 1. Lipoxygenase[2], which is a natural

[1] Quoted by SCHMIDT (1967).
[2] Lipoxygenase is the modern name of lipoxidase.

enzyme in soybean, catalyses the oxidation of cis-linoleic acid and cis-linolenic acid (TAPPEL, 1963). Oleic acid might have been chemically oxidized by the hydroperoxides of linoleic and linolenic acid formed. This oxidation might be similar to that of monounsaturated fatty acids (oleic) caused by hydroperoxide-organic acids (SWERN, 1964). The hydroperoxides of linoleic and linolenic acids probably underwent further decomposition. Although hydroperoxides are quite stable at low temperatures they are more readily decomposed at temperatures above 80°C (DUGAN and SLOVER, 1960). It is very probable that during the methylation process (STOFFEL et al., loc. cit.) hydroperoxides were decomposed. The products of decomposition might cause the small and irregular peaks with retention times not corresponding with those of the unsaturated fatty acid methyl esters. 2. Although lecithin and tocopherols, both of which are also naturally present in soybean, have a synergistic effect as antioxidants, they cannot always inhibit the activity of soybean lipoxygenase (SÜLLMANN, 1941). Moreover, pancreatic lipase can attack lecithin (DE HAAS et al., 1965). Perhaps the degraded lecithin has no antioxidant activity. 3. Soybean lipoxygenase can be active under those conditions of pH and temperature which are optimal for pancreatic lipase.

Therefore, the previous considerations led us to suggest that lipoxygenase activity was the principal cause of the poor gas-chromatogram obtained from products of digestion of unheated cells. Furthermore, they made it probable that lipoxygenase was still active after the soybean cotyledons had been treated with EDTA in order to obtain isolated cells. The latter is supported by the quotation of TAPPEL (loc. cit.) that soybean lipoxygenase is not inhibited by metal complexing agents like EDTA.

6. GENERAL DISCUSSION

Our main object was to learn whether the heating of dead plant cells at 100°C in distilled water affected especially the permeability of the cell wall and the cell membrane, thereby permitting a better penetration of digesting enzymes and consequently making the contents of the cells more accessible to the digesting enzymes.

Heating not only alters the cell wall structure but in addition denatures proteins inside the plant cells and inactivates thermolabile inhibitors of digesting enzymes.

To ascertain to what extent heating affected the barrier of the cells of the different substrates (soybean, yeast, algae) we treated them with a substance (EDTA) that affected only permeability, without denaturing the proteins.
If unheated, EDTA-treated substrate were to be digested to a similar degree as occurs with substrate which was merely heated, this would demonstrate that heating affects mainly the barrier of the substrate formed by the cell wall and the cell membrane.

The EDTA-treatment was effected with 3.8 % (approx. 0.1M) EDTA solution pH 10 at 37°C for two days. The EDTA solution was afterwards removed by successive washings with 0.1M borate buffer pH 8 for soybean and algae and with 0.1 M phosphate buffer pH 7.6 for yeast.
These buffers were used because the substrates were suspended with the same buffer for the incubation with chymotrypsin. Borate buffer is recommended because it does not bind the calcium ions of the chymotrypsin solution. Phosphate buffer was employed in the case of yeast, as the borate buffer caused flocculation.

It is known that treatment with EDTA increases the permeability of the cell wall and cell membrane (LEIVE, loc. cit.; HEATH and CLARK, 1956). These authors, however, used EDTA solution of a lower concentration, viz., in the range of $10^{-4}M–10^{-5}M$, and for a shorter time than we did, because they still wanted to obtain viable cells. For us this was not important, because we used dead plant cells (page 4, paragraph 1.2.). Moreover our purpose was not exclusively to increase the permeability of the substrates by EDTA-treatment, but primarily to obtain isolated cells from soybean cotyledons (page 4, paragraph 1.2.).

The treatment with EDTA did not denature proteins. This has been demonstrated, since β-amylase, urease and lipoxygenase were still active after the treatment of soybean cotyledons with EDTA. Therefore by taking the activity of enzymes as criteria for the denaturing of proteins, we concluded that EDTA was not a protein denaturing agent. Moreover, this conclusion was corroborated,

because subcellular unheated powder did not become more susceptible to chymotrypsin after EDTA treatment.

This also means that when once the cell wall has been broken EDTA does not improve the digestibility of the substrate, which indicates that EDTA only affects permeability. This is also substantiated when one considers that EDTA did not improve the digestibility of substrate that had already been heated (for chymotrypsin compare heated and heated-EDTA of the different substrates in table 12 (page 81); and for lipase compare heated yeast and heated yeast-EDTA in table 11, page 68).

Furthermore, the fact that β-amylase (mol. weight 61,700) leaked from soybean cotyledons only after they had been treated with EDTA and not when they had been previously treated with 0.1 M glycine or borate buffers pH 10, demonstrated that the effect of EDTA in increasing permeability for big molecules is specific. The alkaline medium (pH 10) alone did not cause an increment in permeability.

It appeared, however, that the solubilizing action of sequestering cations on the cementing material of soybean cotyledon cells occurs exclusively in an alkaline medium (pH 8–10), and not, for example at pH 6, since with EDTA at pH 6 we failed to obtain isolated cells.

As the maceration of soybean cotyledons was obtained with unheated cotyledons as well as with cotyledons from heated seeds it was concluded that EDTA did indeed effect the maceration and that enzymes from soybean were not responsible for this action.

We studied the penetration of α-chymotrypsin and pancreatic lipase into the various substrates by scanning the products of hydrolysis produced within the cells and diffusing from them. To this end we made digestions in vitro of the substrates soybean, yeast and algae.

If the enzymes were inhibited within the cells of the substrates it would be most difficult to detect their entry. Consequently this factor had to be taken into consideration when carrying out our experiments. From a nutritional point of view proteolytic inhibitors from raw soybean meal are more important because they cause hypertrophia of the pancreas, rather than because they inhibit digestive proteinases (SAMBETH et al., 1967; BIRK, 1968).

We decided to use α-chymotrypsin as a proteolytic enzyme, because this enzyme is inhibited only very slightly by the Kunitz inhibitor present in soybean material, whereas trypsin is inhibited to a far greater extent.

The saponins from soybean can also act as inhibitors of proteolytic enzymes. However, as we have seen in the case of subcellular soybean powder, when enough proteins are present in the reaction mixture the saponins are not inhi-

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bitory. Therefore this type of inhibitor was no problem for our experiments.

BIRK et al. (1963a) described the purification of another inhibitor, designated *AA*, from defatted soybean meal. BIRK et al. (1967) confirmed that the AA inhibitor has a dual, independent activity against trypsin and α-chymotrypsin. This inhibitor loses its activity completely when it is autoclaved, but is stable when heated to 105 °C or boiled in an aqueous solution (BIRK, 1961).

The question arises whether in our experiments the AA inhibitor played a role in decreasing the detection of the penetration of α-chymotrypsin into soybean substrate.

We think the answer must be in the negative because of the following:
1. According to BIRK (1968) 100 g soybean flour yields 0.2 g of pure inhibitor AA.

Our reaction mixture (80 ml) contains approximately 15 g of soybean material. Therefore, there is 30 mg of AA inhibitor in it, which means roughly 0.4 mg per ml.

A close look at the publication of BIRK et. al. (1963a), in which the data of α-chymotrypsin inhibition (μg per ml) are recorded as a function of the concentration of purified inhibitor AA (μg per ml) led us to deduce that 0.4 mg inhibitor AA inhibits practically none of the α-chymotrypsin.

2. When once the inhibitor has reacted with some molecules of α-chymotrypsin it is not able to react further with other molecules of chymotrypsin, but it does do so with trypsin. Moreover, the reaction with α-chymotrypsin affects the stability of the inhibitor against this enzyme (BIRK et al., 1967).

3. Another probability, which also applies to the Kunitz inhibitor, is that by suspending the soybean substrates in 0.1M borate buffer pH 8 with 0.1% Preventol 115, and by successive washings with borate buffer pH 8 before incubation with α-chymotrypsin these inhibitors were removed. The molecular weight of the Kunitz inhibitor is 21,700 and of the AA inhibitor 20,435, as quoted by BIRK (1968).

In the case of chymotrypsin the soluble products of proteolysis were determined spectrophotometrically at 280 mμ and, in addition, by nitrogen determinations. A rough estimate showed that the amount of nitrogen released from the substrates by chymotrypsin was larger than the quantity of cell wall nitrogen in the reaction mixture. This means that the nitrogen in the samples did arise at least partly from proteins within the cells and not exclusively from proteins from the cell wall proper. Therefore chymotrypsin must have entered the substrates.

Furthermore, we confirmed this evidence immunologically. Thus, the biochemical method was judged sufficient to demonstrate that chymotrypsin and/ or lipase had entered the other substrates.

On observing table 12 we see that, generally speaking and with the exception of algae, unheated, EDTA-treated substrate is digested by chymotrypsin to a

similar degree as heated substrate. This means that heating the substrate affects mainly the permeability of the cell wall and cell membrane thereby allowing a better penetration of the enzyme. This conclusion agrees with the work of LANGRIDGE et al. (1968). These authors increased the leakage of malic dehydrogenase from cells of *Vibrio marinus* by heat treatment at 30°C. Since we were looking for the penetration of enzymes into a substrate and not those that were inherent in them we could study the effect of heating at 100°C.

As we have just stated, the only exception in our conclusion that substrate-EDTA is digested to a similar degree as heated is in the case of algae. This agrees with the reports of other authors that algae possess resistant membranes (TAMURA et al., 1958) and could explain why the coefficient of digestibility of algae is low (DAM et al., loc. cit.).

The penetration of lipase was studied with soybean, which possesses plasmodesmata, and with yeast, which has no plasmodesmata. We excluded algae (without plasmodesmata) from this study, because as will be seen later we wanted to learn mainly whether chymotrypsin would enhance the penetration of lipase in substrates with plasmodesmata. If the latter should occur it would support the theory that plasmodesmata form a pathway for the entry of enzymes into dead plant cells.

Before considering the penetration of lipase alone (without chymotrypsin) into soybean and yeast, we should like to mention that heated fat does not become more susceptible to lipase than when it is unheated (page 70, paragraph 5.2.3.1.). Therefore if heated substrate (soybean or yeast) is digested to a greater extent than unheated, it means that heating decreased its barrier, since the fats do not become more susceptible to lipase.

On observing table 11 (page 68) (lipase alone) we see that heated sections are penetrated to a greater extent than unheated. There is no difference in the case of heated or unheated isolated soybean cells. This is logical because these cells have been strongly affected in their permeability (page 38, paragraph 2.3.5.1.).

When considering the yeast substrate digested by lipase alone (table 11) we deduce that by heating the yeast we obtain the maximum penetration of lipase.

Therefore we conclude that in soybean sections as well as in yeast heating affects permeability, thereby permitting a better entry of lipase.

Unheated yeast is not penetrated at all, whereas unheated yeast-Helix or unheated yeast-EDTA are entered by lipase.

The fact that unheated yeast-Helix is digested by lipase shows that the lack of digestion of unheated yeast is due to the barrier formed by the cell wall, since the enzyme system of Helix is known to bring about the lysis of the yeast cell wall.

Unheated yeast-EDTA is more permeable to lipase than unheated yeast, but to a less extent to chymotrypsin. With chymotrypsin, unheated yeast-EDTA was

digested to a similar degree as heated yeast. This could be explained as follows. The penetration of enzymes is not an entirely inert process, because enzymes have catalytic activity. It is very likely that chymotrypsin can hydrolyse part of the proteins of the 'protein envelope' pertaining to the several biological membranes present in the cells (plasmalemma, protein bodies, spherosomes). Lipase is not able to attack the phospholipids of these membranes easily, probably because the latter are protected by the 'protein envelope'. Presumably the proteolytic activity of the lipase solution was too weak to degrade the 'protein envelope'. (DFP could not inhibit the proteolytic activity of the commercial lipase altogether, but only to the extent of approx. 74 % $\pm$ 4 % (page 62, paragraph 5.1.2.)).

Apart from the problem of the influence of heating on the permeability of the cell wall and cell membranes the possibility of penetration of digestion enzymes through plasmodesmata in dead plant cells was studied. Again we used soybean, yeast and algae as substrates. The first of these has plasmodesmata, while the last two have none.

With isolated soybean cells, as well as with soybean sections, we were able to reveal pits by staining them with zinc chloride iodine (page 47, paragraph 3.3.). Furthermore, when submitting isolated cell wall from soybean cells to electron microscopy, we photographed plasmodesmata. The minimum diameter of a plasmodesmatal pore was 150–200 Å.

In view of: 1. the fact that chymotrypsin penetrated unheated soybean sections only very slightly, whereas it did not penetrate unheated yeast or unheated algae at all; 2. that lipase when alone penetrated unheated soybean sections, although it did not enter unheated yeast – we had evidence that there was some path for the penetration of enzymes into soybean material, while such a path did not exist in yeast. We assumed that this path might be by way of plasmodesmata, as these are present in soybean, but not in yeast or algae.

Immunologically we could not detect the path by which chymotrypsin penetrated soybean cells and sections, because there was no difference between the preparations in which chymotrypsin was incubated for different periods of time. We assume that the reason for this was that the reaction of chymotrypsin (antigen) with its corresponding antibody does not always bring about a cessation of enzyme activity. Sometimes the reaction of the enzyme-molecule with the antibody occurs with a part of the enzyme molecule which is not the enzymically active centre (URIEL, loc. cit.) which results in an unimpaired catalytic activity.

Moreover, the chymotrypsin molecule is an ellipsoid with axes of 45Å, 35Å and 38Å (MATTHEWS et al., loc. cit.). The minimum diameter of plasmodesmatal pore is 150–200 Å. Therefore it is highly probable that chymotrypsin can penetrate the plasmodesmatal pore.

The probability that plasmodesmata form a pathway by which enzymes can enter was supported by the finding that chymotrypsin enhanced the penetration of lipase in the soybean substrates, which have plasmodesmata, but not in the yeast substrates, which do not possess plasmodesmata.

The experimental evidence lends support to the following hypothesis for the mechanism of penetration. Firstly, in the case of sections, chymotrypsin digests the protoplasmatic threads and thereby opens a way for itself and for lipase. Thus lipase, when incubated with chymotrypsin, would have facilitated its passage through the plasmodesmatal canal. The exception occurs when the two enzymes are incubated simultaneously with unheated sections. This is, however, accountable, because chymotrypsin can enter unheated sections only slowly, in the meantime having an opportunity for digesting lipase. We have demonstrated that lipase activity in the presence of chymotrypsin decreases after 40–50 minutes, which would imply that chymotrypsin *can* digest lipase. Consequently the said exception does not contradict the fact that chymotrypsin enhances the penetration of lipase in all types of substrates with plasmodesmata.

In the case of isolated soybean cells (unheated or heated) we observed that chymotrypsin began to enter them right from the outset. Therefore the enhancement of the penetration of lipase by chymotrypsin occurs whether the two enzymes are incubated simultaneously or not. This also shows that despite the fact that the permeability of the walls of soybean cells were strongly affected by the maceration of the cotyledons with EDTA (see page 38, paragraph 2.3.5.1.) plasmodesmatal pores are still more important than other pores for the entry of enzymes.

As chymotrypsin enhances the penetration of lipase into soybean material, whether it is heated or EDTA-treated, this would seem to indicate that the two enzymes enter through the same sites. In the case of sections we assume that these are the plasmodesmata and in the case of isolated soybean cells these could be either the plasmodesmata or the plasmodesmatal pores. Perhaps the stringent treatment of soybean cotyledons with EDTA for the preparation of isolated soybean cells caused a contraction of the cytoplasmatic threads, thereby leaving the plasmodesmatal pores open for the enzymes.

At first sight this might appear to be a contradiction, in the sense that lipase alone penetrated unheated sections through protoplasmatic threads. But we must not forget that in the protoplasmatic threads there might be present lipids as well as proteins.

Inasmuch as chymotrypsin does not enhance the penetration of lipase in the case of yeast, the penetration of the two enzymes seems to occur through different sites.

TABLE 12. Compilation of the values corresponding to the amount of nitrogen released from the different types of substrates by α-chymotrypsin. The values were obtained after 120 minutes of digestion, except with isolated soybean cells, which correspond to 80 minutes digestion.

SUBSTRATE	Amount of nitrogen (%) released from the the substrate by α-chymotrypsin
Soybean	
Unheated cells	9.8
Heated cells	22.2
Unheated sections	1.7
Unheated sections-EDTA	18.
Heated sections	23.9
Heated sections-EDTA	23
Yeast	
Unheated (STS)	0
Unheated (LTS)	0.3
Heated (STS)	3.2
Heated (LTS)	10.6
Unheated (STS or LTS)-EDTA	10.1
Heated (STS)-EDTA	8.9
Unheated (STS or LTS)-EDTA-heated	14.7
Algae	
Scenedesmus spec.: Unheated	0
Unheated-EDTA	4.6
Heated	14.3
Heated-EDTA	12.3
Unheated-EDTA-heated	18.6
Chlorella spec.: Unheated	0
Unheated-EDTA	3.3
Heated	13.5
Heated-EDTA	7.0
Unheated-EDTA-heated	13.6

Before ending this general discussion we think it worth while to stress the point that the entry of proteolytic enzymes is not a completely inert process, because these molecules have inherent catalytic activity and can penetrate the protein-filled plasmodesmatal canal by digesting the protein content. This conclusion was arrived at because although chymotrypsin and lipase did indeed penetrate into unheated sections, albeit to only a slight degree, amylopectin without catalytic activity did not enter unheated cotyledons at all. Unheated soybean sections and unheated soybean cotyledons may be compared, because neither of them had been EDTA-treated; and the conclusion is only of a qualitative nature, namely; whether the said molecules penetrated the materials or not.

It might be argued that amylopectin did not enter unheated soybean cotyledons because it is a larger molecule than α-chymotrypsin and pancreatic lipase.

The molecular weight of amylopectin is 50,000 – 1,000,000 as quoted by FOSTER (loc. cit.), while that of α-chymotrypsin is 24,000 as quoted by LASKOWSKI (loc. cit.) and that of pancreatic lipase is 38,000 as determined by SARDA et al. (1964).

However, when we applied the fluorescent antibody technique to localize α-chymotrypsin within the cells from unheated soybean sections, the antibodies which are γ-globulins with a mol. weight of 150,000 (as quoted by WEST et al., 1966) entered the unheated sections. Therefore the size of the molecules as indicated by the molecular weight, is not the reason why amylopectin did not enter unheated cotyledons. Logically, this reaffirms that chymotrypsin might very well facilitate the entry of the γ-globulins.

SUMMARY

The penetration of α-chymotrypsin and/or pancreatic lipase into dead cells of soybean cotyledons, of yeast and of algae was studied using the enzymic activity as a parameter. In addition a fluorescent antibody technique was applied for the localization of α-chymotrypsin within the soybean cells.

The digestibility of unheated, EDTA-treated substrate was similar to that of the heated substrate. Since EDTA-treatment increases the permeability of the cell wall and cell membrane of the plant cell without denaturing proteins contained in the cells, we concluded that heating affected mainly the barrier formed by the cell wall, thus permitting a better passage of big molecules (enzymes).

Leakage of β-amylase (mol. weight 61,700) from soybean occurred only after treatment with EDTA. This confirmed that EDTA enhances the permeability of the cell wall.

α-Chymotrypsin (mol. weight 24,000) and pancreatic lipase (mol. weight 38,000) penetrated unheated sections, whereas amylopectin (mol. weight 50,000–1,000,000) did not penetrate unheated cotyledons. Apparently the greater dimension of amylopectin compared with α-chymotrypsin and lipase accounts for its lack of entry into unheated soybean cotyledons. However, when we applied the fluorescent antibody technique to localize α-chymotrypsin within the cells from unheated sections, we used antibodies (γ-globulins) having a mol. weight of 150,000. They entered the unheated sections and produced a positive result of the experiment. Consequently the size of the molecules as indicated by the molecular weight, is not the reason why amylopectin did not penetrate the unheated cotyledons. Moreover, this shows that α-chymotrypsin probably opens a way for the entry of globulins. Therefore the entry of proteolytic enzymes is not an inert process. This is conceivable as the enzymes are big molecules with catalytic activity.

Unheated non–EDTA-treated soybean (with plasmodesmata) was slightly penetrated by α-chymotrypsin, whereas unheated non-EDTA-treated yeast and algae (both without plasmodesmata) were not penetrated at all. Furthermore, in soybean material (unheated or heated) α-chymotrypsin enhanced the penetration of lipase; this effect was absent in the case of yeast. The results obtained strongly suggest that plasmodesmata are a way in dead plant cells for penetration of enzymes.

SAMENVATTING

Het binnendringen van α-chymotrypsine en/of pancreas-lipase in dode cellen van soyaboon cotylen, gistcellen en algen werd bestudeerd door de enzym activiteit als een parameter te gebruiken. Bovendien werd een fluorescerende antilichamen techniek toegepast om het α-chymotrypsine in de cel te kunnen localiseren.

De mate van aantasting van het onverhitte met EDTA behandelde substraat was gelijk aan die voor verhit substraat. Aangezien de behandeling met EDTA de permeabiliteit van de wand en de membranen van de plantecel verhoogt zonder de cel-eiwitten te denatureren, concluderen wij hieruit, dat het verhitten hoofdzakelijk de hindernis, die door de celwand wordt gevormd, zodanig beïnvloedt, dat de enzymmoleculen gemakkelijker de celwand kunnen passeren.

Het verdwijnen van β-amylase (mol. gewicht 61.700) uit de soyaboon geschiedde alleen na behandeling met EDTA. Dit bevestigde dat EDTA specifiek de permeabiliteit van de celwand deed toenemen.

α-Chymotrypsine (mol. gewicht 24.000) en pancreas-lipase (mol. gewicht 38.000) drongen door in onverhitte coupes van soya cotylen, terwijl amylopectine (mol. gewicht 50.000–1.000.000) niet in onverhitte cotylen binnendrong. Het lijkt daarom dat de grotere afmeting van amylopectine vergeleken met α-chymotrypsine en lipase er de oorzaak van is dat eerstgenoemde stof niet kan binnendringen.

Bij het toepassen van de fluorescerende antilichamen techniek om α-chymotrypsine in de cellen van onverhitte coupes te localiseren, gebruikten wij echter antistoffen (γ-globulinen) die een mol. gewicht van 150.000 hebben. Deze antistoffen kwamen wel in de onverhitte coupes binnen en gaven een positief resultaat van het experiment. Dientengevolge is de afmeting der moleculen, zoals wordt aangegeven door het moleculgewicht, niet de reden waarom amylopectine de onverhitte cotylen niet binnendringt. Bovendien toont dit aan, dat α-chymotrypsine waarschijnlijk een weg opent voor de binnenkomst van de globulinen. Derhalve is het binnendringen van proteolytische enzymen geen inert proces. Dit is logisch daar enzymen grote moleculen zijn met katalytische activiteit.

α-Chymotrypsine drong slechts weinig binnen in niet met EDTA behandelde, onverhitte soyabooncellen (met plasmodesmata), terwijl α-chymotrypsine in onverhitte, niet met EDTA behandelde gistcellen en algen (beide zonder plasmodesmata) helemaal niet binnendrong. Verder vergrootte α-chymotrypsine het binnendringen van lipase in soyaboonmateriaal, zowel verhit als onverhit; dit effect bleef bij gistcellen achterwege. De verkregen resultaten steunen zeer sterk de veronderstelling dat de plasmodesmata wegen zijn waarlangs enzymen in dode plantecellen kunnen binnendringen.

RESUMEN

Usando la actividad enzimática como parámetro, se ha estudiado la penetracion de la α-quimotripsina y/o la lipasa pancreática en las células muertas pertenecientes a cotiledones de soja, a levadura y a algas. Se aplicó, además, una técnica inmunológica (anticuerpo fluorescente) para la localización de la α-quimotripsina dentro de las células de soja.

La digestibilidad de substrato crudo, tratado con etilendiaminotetraacetato (EDTA) fue similar a aquélla de substrato únicamente cocido. Debido a que el tratamiento con EDTA aumenta la permeabilidad de la membrana celular (exterior), como así también de otras membranas celulares interiores, sin desnaturalizar las proteínas contenidas en la célula, conclúyese que la acción de cocer afectó sobre todo a la resistencia que presenta la membrana celular, permitiendo de esta manera una mayor penetración de moléculas grandes (enzimas).

La pérdida de β-amilasa (peso molecular 61.700) por parte del substrato crudo de soja ocurrió solamente después que éste fue tratado con EDTA. Esto confirmó que EDTA aumentó la permeabilidad de la membrana celular.

La α-quimotripsina (peso molecular 24.000) y la lipasa pancreática (peso molecular 38.000) penetraron en las secciones crudas provenientes de cotiledones de soja, mientras que la amilopectina (peso molecular 50.000–1.000.000) no penetró en los cotiledones crudos de soja. Aparentemente el mayor tamaño de la amilopectina comparado con el de la α-quimotripsina y la lipasa, explicaría la no penetración por parte de la primera en los cotiledones crudos de soja. Sin embargo, cuando se ha aplicado una técnica inmunológica con el fin de localizar la α-quimotripsina dentro de las células pertenecientes a las secciones crudas de soja, se han usado anticuerpos. Estos son γ-globulinas y tienen un peso molecular de aproximadamente 150.000. Los anticuerpos penetraron en las secciones crudas de soja, produciendo un resultado positivo. En consecuencia, el tamaño de las moléculas, manifestado por el peso molecular, no es la causa por la cual la amilopectina no penetró en los cotiledones crudos de soja. Tambien, esto indicaría que la α-quimotripsina probablemente facilitó la entrada de los anticuerpos. Por lo tanto, el pasaje de las enzimas proteolíticas no es un proceso inerte; lo cual es lógico, pues las enzimas son moléculas grandes con actividad catalítica.

La α-quimotripsina penetró levemente en el substrato crudo de soja, no tratado con EDTA (con plasmodesmas), mientras que la mencionada enzima no penetró del todo en los substratos crudos de levadura y algas, no tratado con EDTA (sin plasmodesmas). Además, en el caso del substrato de soja, la α-quimotripsina contribuyó a elevar el grado de penetractión de la lipasa; esto no sucedió en el caso de la levadura. Los resultados obtenidos sugieren claramente que los plasmodesmas podrían constituir un camino para la penetración de las enzimas en células vegetales muertas.

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PRINCIPAL ABBREVIATIONS

CT	α-chymotrypsin
CT 5st	α-chymotrypsin 5 times more concentrated than CT
CTG	chymotrypsinogen
DFP	diisopropylfluorophosphate
EDTA	ethylenediaminetetraacetate
$E_{280\,m\mu}^{1\,cm}$	extinction value determined spectrophotometrically at 280 mμ with a cuvette of 1 cm light path
$E_{280\,m\mu}^{1\,mm}$	extinction value determined spectrophotometrically at 280 mμ with a cuvette of 1 mm light path
$E_{540\,m\mu}^{1\,cm}$	extinction value determined spectrophotometrically at 540 mμ with a cuvette of 1 cm light path
FA	fluorescent antibody
GLC	gas-liquid chromatography
HP	*Helix pomatia*
L	lipase
LTS	long time storage
MCD	modified casein digestion method
MF	mounting fluid
N	nitrogen
NaT	sodium taurocholate
PSB	phosphate saline buffer pH 7.2
STS	short time storage
TG	trypsinogen

(CT 80' + L)	substrate incubated for 80 minutes with chymotrypsin and then with lipase
(CT 80'–)	substrate incubated for 80 minutes with chymotrypsin and then with 10^{-3}M DFP (without lipase)
(–80'L)	substrate incubated for 80 minutes with NaCl (without chymotrypsin) and then with lipase
(–80'–)	substrate incubated for 80 minutes with NaCl (without chymotrypsin) and then with 10^{-3}M DFP (without lipase)
(CT + L)	substrate incubated with chymotrypsin and lipase simultaneously
(CT–)	substrate incubated with chymotrypsin and 10^{-3}M DFP simultaneously
(–L)	substrate incubated with NaCl (without chymotrypsin) and lipase simultaneously
(– –)	substrate incubated with NaCl (without chymotrypsin) and with 10^{-3}M DFP (without lipase) simultaneously

algae-EDTA	algae treated with EDTA
cotyledons-borate	soybean cotyledons treated with 0.1M borate buffer pH 10
cotyledons-borate-borate	soybean cotyledons treated twice with 0.1M borate buffer pH 10
cotyledons-EDTA	soybean cotyledons treated with EDTA
cotyledons-glycine	soybean cotyledons treated with 0.1M glycine buffer pH 10
sections-EDTA	soybean sections treated with EDTA
unheated algae-EDTA-heated	unheated algae treated with EDTA and then heated
unheated yeast-EDTA-heated	unheated yeast treated with EDTA and then heated
unheated yeast-HP	unheated yeast treated with *Helix pomatia*
yeast-EDTA	yeast treated with EDTA
yeast-LTS	yeast stored for approx. one month
yeast-STS	yeast stored for approx. one week