A BIOCHEMICAL AND PHYSIOLOGICAL STUDY OF THE INSOLUBLE PECTIC MATERIALS IN VEGETABLES AND FRUITS.

by

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INTRODUCTION.

A number of recent investigations on the chemistry of the pectic substances have renewed an interest in these plant constituents. Their importance in various physiological processes has also been recently emphasized by several investigators. However, very little study has been devoted to these materials in vegetables. This paper deals chiefly with the occurrence, extraction, and quantitative determination of the insoluble pectic substances in typical vegetables. A comparative study was also extended to a typical fruit, where the transformation was studied.

HISTORICAL REVIEW.

The important contributions of previous work on the pectic constituents will be emphasized and grouped under four general aspects of the subject.

1. Physical and Chemical Properties.

The older workers named and described a number of pectic substances, but at the present time only the following compounds are recognized and well defined: pectin, protopectin, pectic acid, and the pectates. Fellenberg (18) describes the physical properties of the pectins as follows: They are reversible colloids which upon addition of water swell into pasty masses that finally go

into solution. The opalescent solution is clear in transmitted light, turbid in reflected light, and shows under the ultra-microscope numerous particles in continual motion. If alcohol is added to a concentrated pectin solution, usually, though not always, the pectin is precipitated as a jelly while from a dilute solution it is precipitated as a flocculant mass. Not all pectins have the same properties. For example, pectin from Swedish beets dissolved much more readily, and gave less viscous solutions than pectins from fruits.

The specific rotation of pectin varies with its source and method of extraction from the tissues. Scheibler (39) found a specific rotation of $(A)_d = +200$ for pectin from beets. Extracting the same material with dilute hydrochloric acid Andrlik (1) found $A_d = +214-220$ Javillier (28) found for quince pectin $(A)_d = +188.2$, while Bourquelot (6) found $(A)_d = +82.3$ in pectin from Gentian root. Ehrlich (15) obtained pectin from sugar beets giving $(A)_d = +150 - 155$. Clayson Norris and Schryver (13) extracting various vegetables with 0.5 per cent ammonium oxalate solution obtained pectins ranging from $(A)_d = +240$ to 284. Charpentier (11) found a rotation of $(A)_d = +154.5$ in pectin from the Celery root, 119.8 in that of Japanese artichoke, and 170.5 in Orange peel pectin. Poore (36) found specific rotations of $(A)_d = +217.1$, 229.7, and 214 for the pectins from ap-

ples, lemons, and oranges, respectively.

Fremy (20) believed the ratio of H:O in pectins was much greater than 1:8 and therefore that the pectins could not be carbohydrates. Giraud (21) classed the pectins with the mucilages. Martin (32) showed that great fluctuations in the ratio of H:O from 1:8 was due principally to impurities. Tollens and Trompe de Haas (45) prepared pectins from a number of sources and purified them as much as possible, before determining the ratio of 1:8 and suggested the presence of carboxyl groups, possibly joined in the manner of lactones. Scheibler (39) obtained arabinose from metapectic acid, a decomposition product of pectin. Evidences of a galactose radical in pectins was subsequently found by Herzfeld (25). More recently Fellenberg (17) found besides pentose and galactose, evidence for methyl pentose. Ehrlich (15) claims to have actually isolated galactose. Charpentier (11) isolated it in the form of the B-ethyl galactoside.

Fellenberg (17) investigating the source of methyl alcohol in wines discovered that it came from the pectins. He showed that this group is easily split off by dilute alkali and very slowly by dilute acid. He recognized the pectins as true esters, and postulated pectins with a number of carboxyl groups and more or less of these methylated. His work has been verified by Ehrlich (15) Sucharipa (44), Poore (36) and Tutin (48). The latter reported besides methyl alcohol the constant occurrence of acetone, which he assumes to be combined in the form

of isopropyl alcohol.

Fellenberg assumed that no new constituent of the pectins would be found since when he added up the constituents in the form of their anhydrides he obtained approximately 100 per cent. Thus he obtained for orange pectin:

41.0% arabinose	=	36.1% araban
6.7% methyl pentose	=	6.0% methyl pentosan
54.8%galactose	=	49.3% galactosan
11.5% methyl alcohol	=	11.5% methyl alcohol

Total 102.9%

As a result of his investigations Fellenberg predicted that the acid character of pectins was due to galactose which was probably present in the form of the corresponding acid.

Ehrlich several years later actually isolated d-galacturonic acid from pectin. By carefully controlled hydrolysis he obtained two different compounds, one, a galactose-galacturonic acid, and the other, d-tetra-galacturonic acid. He considered pectin to be the calcium-magnesium salt of anhydro-arabino-galactose-methoxy tetra-galacturonic acid.

Fremy (20) called the insoluble pectic substance which during the ripening of fruits gave rise to pectin, "pectose". Tschirch (47) much later called it protopectin. Fellenberg states that no way is yet known of extracting protopectin from tissues. Bechamp (2) also suggests that we can scarcely expect to do this since it is changed not only by all chemical agents, but by hot water itself.

The theory that protopectin is some sort of cal-

cium compound was held first by Fremy. On the other hand it has been considered by Mangin (30) to be a compound with cellulose. Fellenberg claims to have proven conclusively that protopectin is not calcium pectate. Finally Smolenski (42) suggests that protopectin may be a compound between pectin and a pentosan.

Braconnot (8) first extracted pectin acid. He noted its acid properties, its ready solubility in alkali and its ability to form an abundant jelly when precipitated with hydrochloric acid. He also prepared the ammonium and potassium salts and determined the ratio of potash to acid by incineration to be 15 parts of the former to 85 parts of the latter. Ullik (49) described pectic acid as being a white amorphous jelly not soluble in water, alcohol, or ether, but easily in alkalies. Its specific rotation varies from $(A)_d = +186$ to 300, and is not altered by conversion into salts. Chodnew (12) describes pectic acid as colorless and when dry easily powdered. It is slightly soluble in hot water, and can be obtained almost free from ash by repeated precipitation from water solution with alcohol containing a small amount of nitric acid.

Other salts have been prepared by Fremy, Chodnew, and Fellenberg. Only ammonium and the alkali salts are soluble in water. Fremy discovered that pectic acid and the pectates were soluble in the neutral solutions

of ammonium oxalate, tartrate, and citrate. Mangin believed that these formed double salts with pectic acid.

The acid character of pectic acid was recognized by Braconnot. Herzfeld (25) states that at high temperature it produces a slow inversion of cane sugar. Fellenberg states that it will slowly expel carbon dioxide from its salts. He showed that one gram of orange pectic acid neutralized 43 c.c. of N/10 NaOH; of apple pectin acid 46 cc. N/10 NaOH. Erhlich found one gram of beet pectin equivalent to 15 c.c. N/10 NaOH. Schryver and Haynes (40) found a capacity for 44.2 c.c. of N/10 NaOH, while Poore found for apple, lemon, and orange pectins a requirement of 36.4, 37.8, and 36.9 c.c. N/10 NaOH respectively.

Fellenberg showed by saponification and neutralization experiments that pectic acid differs from pectin only by a CH_2 group for each carboxyl group present. This results from the fact that the group $COOCH_3$ in pectin becomes COOH in pectic acid. He postulates eight carboxyl groups in pectic acid. In pectins these are more or less methylated, the more carboxyl groups being methylated the more soluble being the resulting pectin. Pectic acid according to him contains two moles of arabinose, one mole of methyl pentose, one mole of galactose, and eight moles of galacturonic acid, the latter containing the carboxyl groups. Sucharipa has suggested

that pectic acids may be joined to cellulose through carboxyl groups and the remaining carboxyl groups may be methylated.

2. Extraction from Tissues and Quantitative Determination.

Braconnot obtained pectin by filtering fruit juice and precipitating with two volumes of alcohol. Bourquelot and Herissey (7) introduced a material improvement into the preparation of pure pectins by first extracting all alcohol-soluble materials with boiling alcohol. Besides removing considerable material this at the same time converted much protopectin into pectin. The latter was then removed by heating in an autoclave under pressure.

Fremy found that by cooking fruit he obtained much larger quantities of pectin and believed that this was due to the transformation of protopectin into pectin under the influence of acid. He produced pectin artificially by heating the insoluble fruit residues with dilute organic or mineral acids. Similarly, Chodnew (12) obtained a pectin from beet marc by heating with dilute hydrochloric acid. He called it "pectinige acid" and thought it might be the same as Fremy obtained from fruit although it had a slightly acid reaction. Tschirch (47) on the other hand, advocated the use of concentrated sugar solutions to extract the pectin; the insoluble pectin was not disturbed as was the case in

heating with dilute acid.

That protopectin is not hydrolyzed by alkali has been shown recently by Farnell (16) and Hardy (23).

Fremy states that no means is known for isolating protopectin in pure form, and Fellenberg much later still corroborates this statement. However Sucharipa claims to have dissolved away a part of the cellulose of the cell walls of orange peel and obtained a residue of pure protopectin. When the protopectin was hydrolyzed with ammonium oxalate solution some cellulose was released but this had been chemically united to the pectin.

Pectic acid was extracted by the older workers with dilute alkali. Chodnew used dilute ammonium hydroxide solution and believed this dissolved the "free" pectic acid. After making an acid extraction of the beet pulp, he was able to extract, by means of sodium hydroxide, additional pectic acid. Mangin working microchemically, used ammonium oxalate, tartrate, and citrate solutions to extract pectic acid and the pectates.

The early investigators (29) estimated the pectin content by precipitating with alcohol, drying and weighing. A rather detailed method for the analysis of various pectic compounds is given in Grandeau's handbook (22). Pectin is removed from tissues by extraction with water. Its quantity is determined by converting to pectic acid with alkali, dissolving in a

solution of ammonium oxalate, and precipitating as calcium oxalo-pectate with a solution of calcium acetate. Calcium pectate on the other hand, is determined by first extracting the calcium with alcoholic hydrochloric acid, and then extracting the pectic acid in the residue with ammonium oxalate solution. Pectose is first converted into pectic acid by weak alkaline hydrolysis and the latter is determined as in the previous cases. Fellenberg (17) developed a method for determination of pectin based on the quantity of methyl alcohol liberated in a tissue by alkali, for he found that methyl alcohol was not split off of any other group there, under the conditions of the determination. Carre and Haynes (9) developed a method for soluble pectin based on its conversion into calcium pectate. Carre (10) worked out a method for protopectin. She first converted the protopectin into pectin by dilute acid hydrolysis and then determined the latter by the Carre and Haynes method just mentioned. Finally Wichman and Chernoff (50) have developed a method for pectin in connection with jelly-manufacture. In this method the pectin is converted into pectic acid, dried, and weighed as such. It is then ignited and the weight of ash subtracted.

6. Occurrence in the Tissues.

The exact location of the pectic material in tissues has not caused much confusion for it was either considered to exist principally in the middle

lamella or uniformly throughout the cell wall layers. However, two very divergent schools have arisen as to the nature of the substance existing there. One school holds that an insoluble substance exists in the tissues and is changed by dilute acids into pectin. Among the advocates of this theory were first Fremy, then Mulder (33), Poumarede (37), Tschirch and later Fellenberg. Fremy noted that this substance was closely associated with the cellulose. Mulder thought that it was equally distributed throughout the cell wall layers. Tschirch studied it by means of sugar solutions and stains, especially in ripening fruits. However, he did not deny that calcium pectate could ~~not~~ exist in certain parts of the cell wall.

The other school consisting of Payen (35), Chodnew, Stude (43), Devaux (14), and more recently Clayson, Norris and Schryver, ~~have~~ has held to the view that the pectin is in some way tied to calcium. Payen believed that the pectic substances were localized in the middle lamella, where as calcium and potassium pectate they acted as binding materials for adjacent cell walls. Chodnew believed because part of the pectic acid in beets could only be extracted with potassium hydroxide, that this pectic acid must be there in combined form, perhaps with calcium. Stude thought the action of dilute acid was to break off the calcium radical from the insoluble compound. Devaux believed that the prin-

cipal constituent of the middle lamella was calcium pectate, for the solubility of the changed pectose was entirely the same as for pectic acid.

Mangin studied very carefully the cell walls of a large variety of plants. By means of differential stains and solvents he showed that the middle lamella consists of pectic acid and pectates chief among which latter was calcium pectate. The cellulose layer on the other hand, was intimately associated or chemically united with another insoluble substance, the pectose. The ~~view~~ fact that pectates also occur in cell walls has more recently been shown by Oden (34).

4. Transformations and Physiological Importance.

Fremy first called attention to the transformation that occurs in the pectic materials of fruit during ripening. The insoluble pectic material, under the influence of the acid contained in the cell sap, is broken down into pectin. Tschirch further confirmed the work of Fremy. He followed the course of the pectic transformations microscopically and called them "pectin metamorphosis". Later Carre (10) actually determined the amount of soluble pectin and protopectin in apples during storage. She found that, roughly, as the protopectin decreased in amount the pectin increased.

The transformation of the pectic materials in vegetable tissues has not been studied as thoroughly as in fruits. Mangin, after detailed study of many species

of plants concluded that in the aging of tissues and the formation of intercellular spaces the calcium pectate increases more and more, the middle lamella loses its cellulose content, and stores up calcium pectate in the form of balls and rod-like masses. At the same time the intercellular spaces become coated with a layer of calcium pectate. These changes are brought about through the combined action of the protoplasm and the osmotic pressure of the cell sap. Pectose, first laid down in the young membrane is changed to pectic acid and pressed by the osmotic pressure little by little through the molecular interstices of the membrane toward the exterior where it accumulates in the middle lamella.

Fremy first discovered the enzyme pectase which could act on pectin and convert it into pectic acid. Bertrand and Mallevre (4) found pectase to be very widely distributed. They held that pectase did not act except in the presence of a calcium salt and that the product formed was not pectic acid but calcium pectate. Tutin showed that the pectases produced the same effect upon pectin as did alkalies. Bourquelot and Herissey discovered pectinase, an enzyme in barley malt which splits pectins, as well as calcium pectate into reducing sugars. This enzyme prevents the action of pectase. Sideris (41) has recently studied

the effect of hydrogen ion concentration upon the pectinase from Fusarium cromyphthoron. Beijerinck and Delden (3) discovered an anzyme which they called pectosinase, and which was excreted by a large number of bacteria. During the retting of flax this enzyme attacked the pectose of the middle lamella, changing it first into pectin, and then into sugars. This transformation permitted the fibers to be separated.

Tschirch states that the significance of the pectic transformation is to loosen the tissue, and prepare the way for the decomposition of the fruit, as well as the isolation of seeds. True (46) believes the permeability of the cell walls is much modified by the nature of the metal combined with the pectic acid. Howe (27) suggests that the acidity of root hairs, and their consequent solvent action on the soil minerals may depend on the pectic material contained there. Oden believes that the pectic substances in cell wall membranes acts "as a regulator for the hydrogen and hydroxyl ion content" of the cells. Sampson (38) accounts for abscission and leaf fall on the basis that chemical changes occur in the pectic constituents at the abscission layer. Hooker (26) has ascribed the hardiness in certain vegetables to the accumulation of a pentosan which can be made soluble by boiling with water, and which is therefore undoubtedly protopectin. Finally, Harter (24) and Blackman (5) emphasize the importance of the pectic substances

in determining the resistance or susceptibility of various plants to fungous diseases.

EXPERIMENTAL.

I. Quantitative Determination of the Insoluble Pectic Materials in Vegetables.

. . The Carre Method for Protopectin.

The only method for the quantitative determination of protopectin which has been proposed up to the present is that of Carre (10). As worked out by her for apples it is, briefly, as follows: The finely ground material is covered with 100 c.c. of N/20 hydrochloric acid, placed in an autoclave, and heated at 110°C for one hour. This process hydrolyses the protopectin, which thereby yields pectin. The latter is washed out of the residue and determined by the Carre and Haynes method for soluble pectin (9).

In attempting to adapt the Carre method to vegetable tissues it was first necessary to know whether the concentration of acid, and temperature and time of heating would give the best results. Consequently these conditions were systematically varied and applied to different vegetable material. First some potato pulp, thoroughly washed with water and dried, was heated under the conditions of the Carre method with different concentrations of hydrochloric acid. Next, using the optimum acidity thus found, the temperature was varied. With these two conditions fixed, the period of heating was varied. Finally, using potatoes, parsnips and onions

the effect of different acid concentrations under the conditions of temperature and time of heating already found most suitable, was determined. The results obtained in this series of experiments are shown in tables I - IV, and in figures 1 - 4.

TABLE I. Amounts of Calcium pectate obtained by application of the Carre method to potato, using different concentrations of acid.

Calcium Pectate	Concentration of Hydrochloric Acid				
	Distilled water	N/50	N/30	N/20	N/10
Per cent	2.91	4.33	4.30	2.86	2.46

TABLE II. Effect of varying the temperature in the Carre Method. N/30 acid was used in all cases.

Calcium Pectate	Temperature of heating in autoclave °C			
	Boiling (100)	109.5	115.0	126.0
Per cent	4.46	4.26	2.47	1.97
	4.27	4.28	2.40	.69

TABLE III. Effect of varying time of heating in the Carre method, using boiling temperature and N/30 acid.

Calcium Pectate	Minutes of boiling				
	15	30	60	90	150
Per cent	3.18	3.97	4.46	4.18	3.93
	3.32	4.01	4.27	4.15	3.55

TABLE IV. Effect of varying the acidity at boiling temperature for one hour.

Concentration of Hydrochloric Acid	Calcium Pectate Per cent		
	Potato	Parsnip	Onion
Distilled water	....	4.82	2.15
N/50	3.39	7.95	5.39
N/40	3.47	9.11	5.53
N/30	3.48	8.53	5.60
N/20	2.85	8.22	5.49
N/10	2.67	6.99	4.55

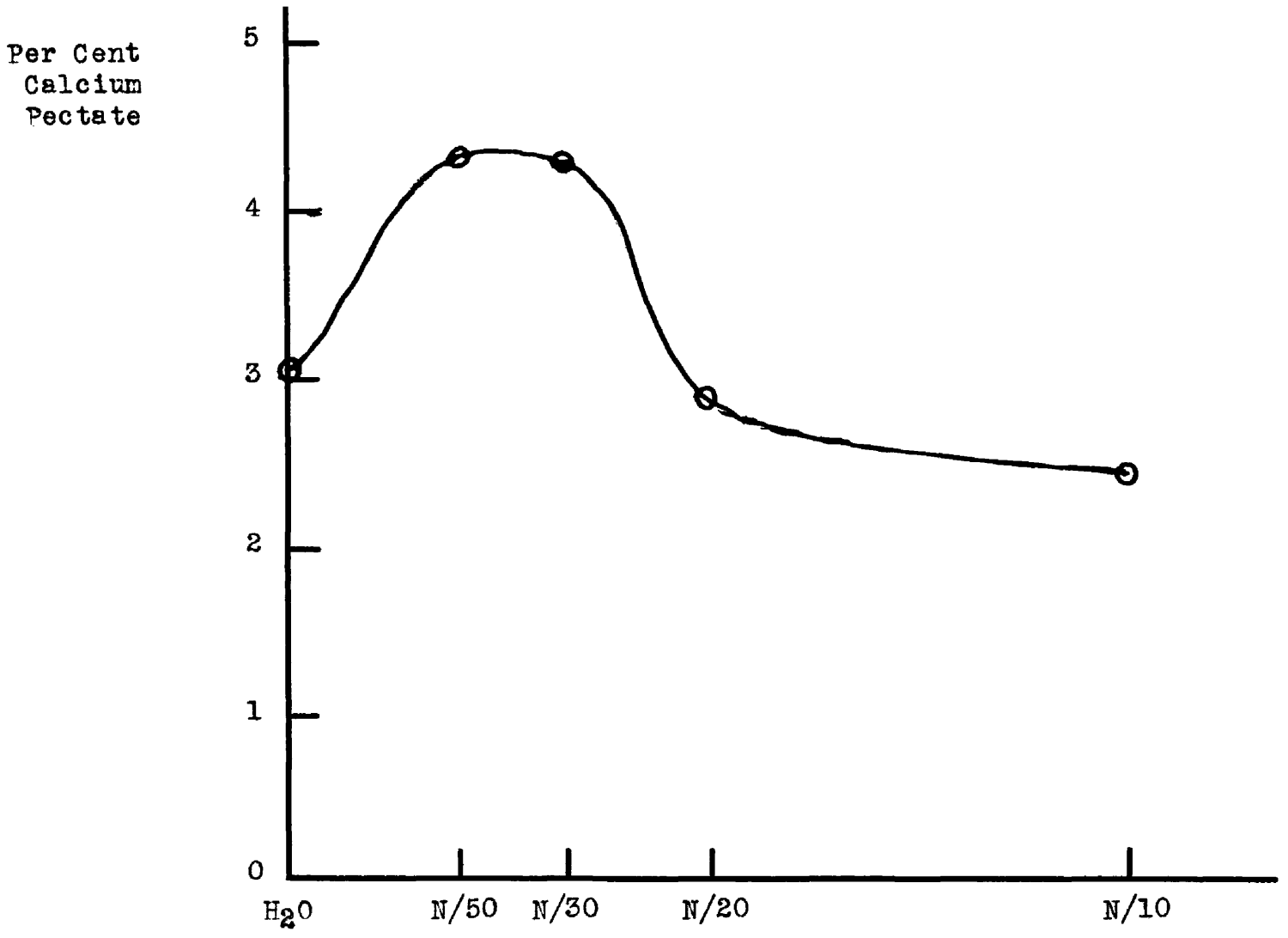

Figure 1. Effect of varying the acid concentration in the Carre method on the liberation of pectin in the potato.

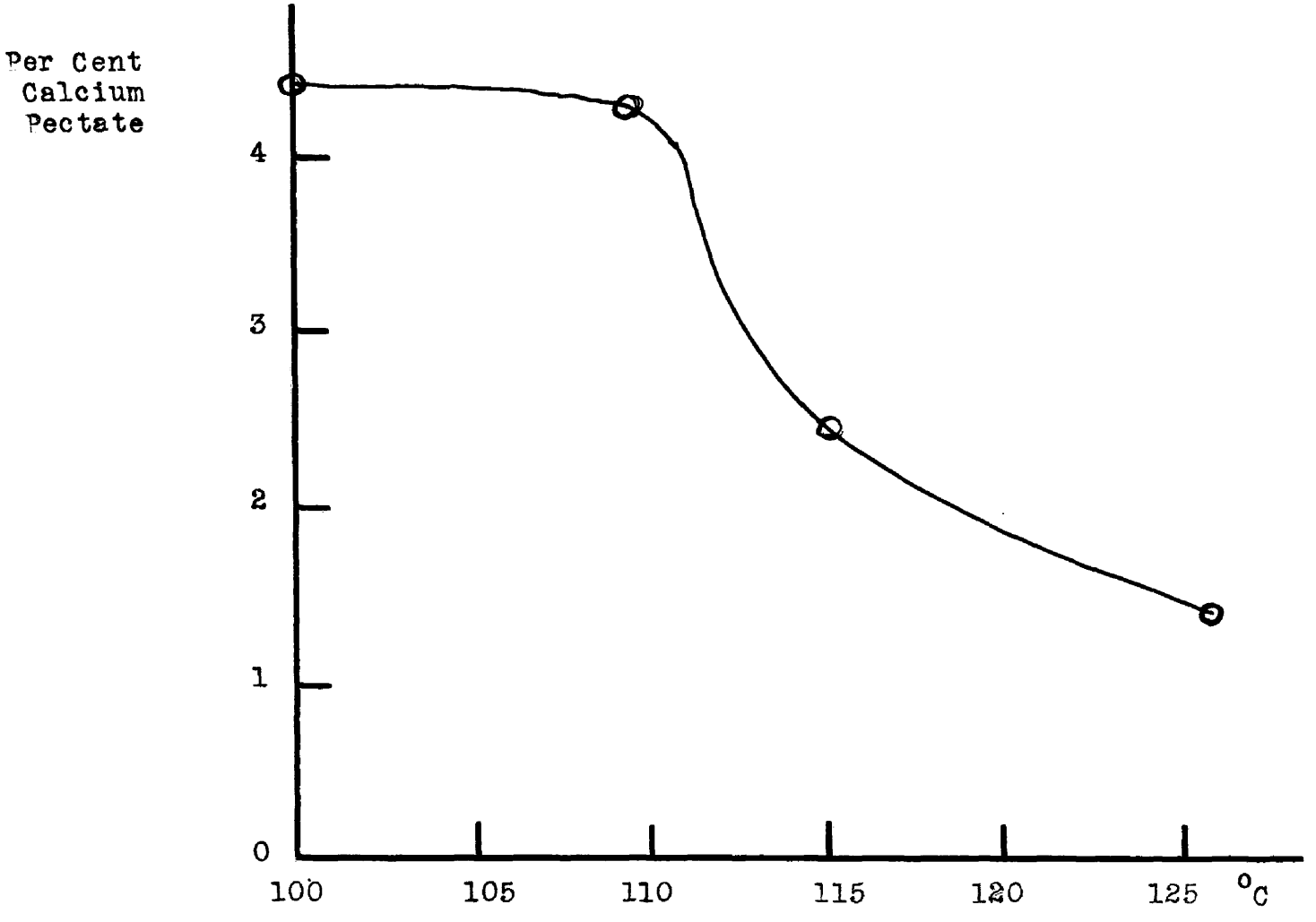

Figure 2. Effect of varying the temperature on the amount of pectin obtained from potato with N/30 hydrochloric acid.

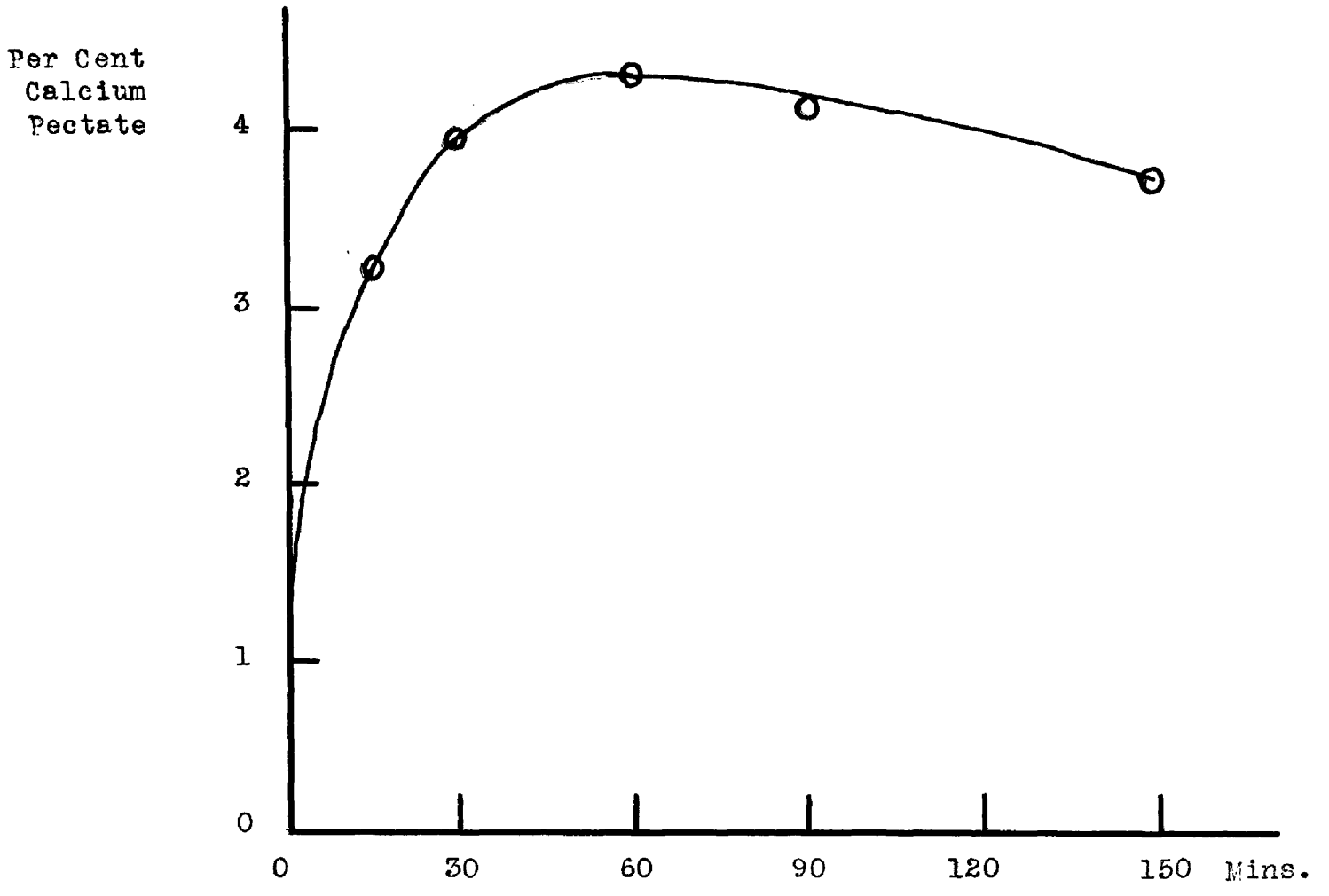

Figure 3. Influence of period of heating with N/30 HCl at boiling temperature on the liberation of pectin in the potato.

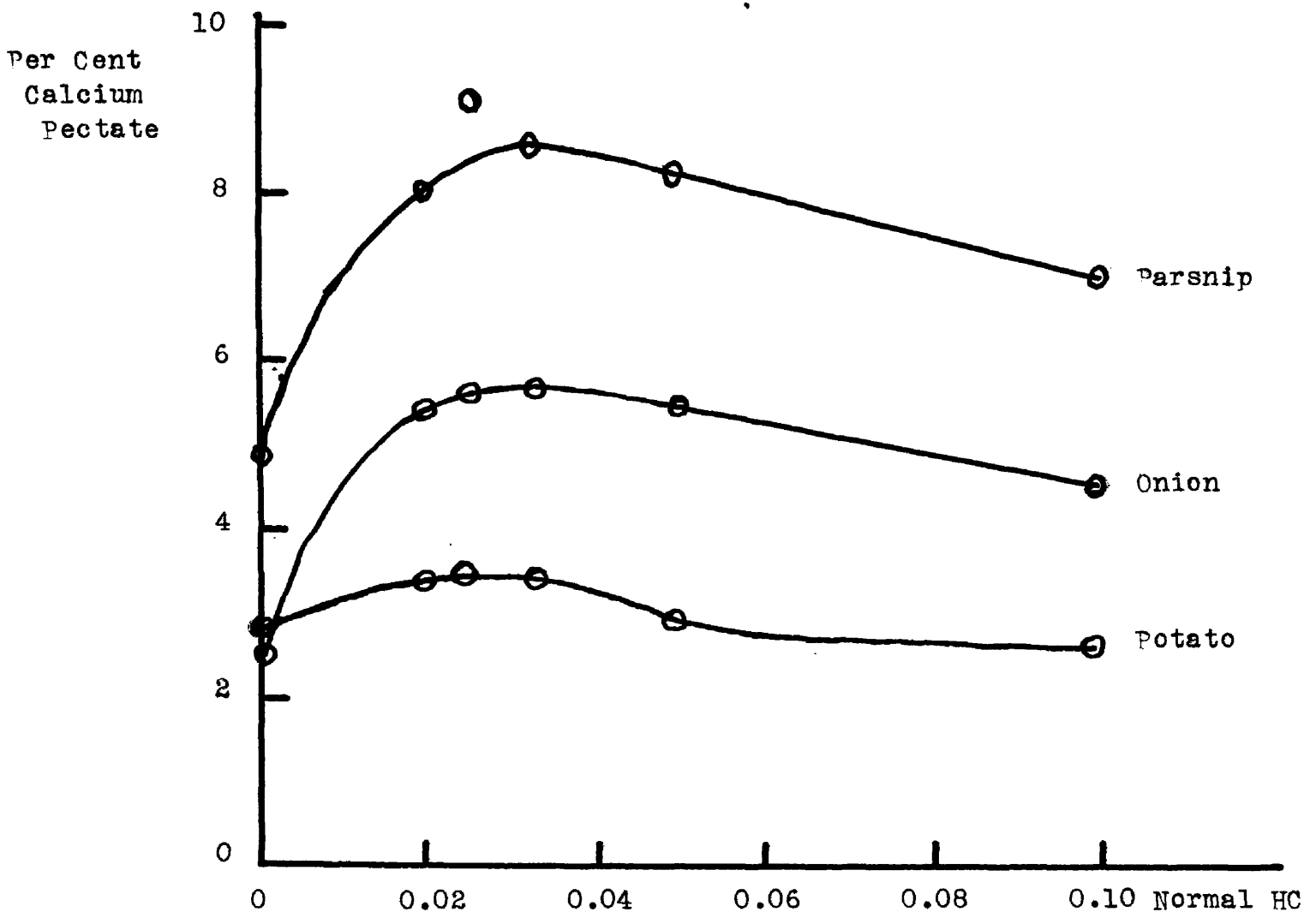

Figure 4. Effect of concentration of acid at boiling temperature for one hour on liberation of pectin from potato, onion, and parsnip.

In carrying out the preceeding analyses with the view of standardizing the Carre method for use with vegetable tissues, several difficulties were encountered. One of these was the difficulty of filtering viscous starch solutions, such as are obtained, particularly in the case of potatoes. Trials with malt and taka-diastase were made, but while these solved the problem of viscosity, the diastase attacked and destroyed the pectin. However, this difficulty was completely solved by the use of saliva, which quickly reduced the viscosity and did not attack the pectin. Difficulty in filtering was also encountered in the case of onions. This was not due to starch but to some other constituent contained in the material. This difficulty was fairly well solved by a preliminary extraction of the onion material with alcohol until most of the yellow coloring matter was removed. This apparently removed a gummy constituent which quickly clogged the filter paper.

Finally it was found that the protein in potatoes gave trouble. A certain amount of it was carried down with the calcium pectate precipitate, from which it could not be removed by washing. It was therefore dried and weighed with the pectate. This did not occur to an appreciable extent with either parsnips or onions. It is/rather difficult matter to get rid of

these proteins because the ordinary metal protein precipitants also precipitate the pectin. Some work with trichloracetic acid indicated that this reagent might be effective in removing the protein. However, because of the unknown action of such reagents on the pectin, it was believed better to develop the method first on materials in which the protein did not interfere. In the case of potato the protein was determined and subtracted from the weight of calcium pectate.

Returning, now, to the tables and figures, we see that by varying the acidity, temperature, and time of heating a great variation in the amounts of calcium pectate was obtained. By varying the acid concentration in the Carre method it was found that N/30 concentration gave the highest amount of calcium pectate. By using N/30 hydrochloric acid as much calcium pectate was obtained at ordinary boiling temperature (about 100°C) as at a higher temperature. The average amount varied from 4.36 per cent at boiling temperature to an uncertain 1.33 at 126°C. The higher temperature began to be destructive to the pectin at slightly above 110°C. Heating at boiling temperature with N/30 acid for a period longer than one hour also seemed to destroy some of the liberated pectin. Finally, boiling one hour at ordinary pressure, the acidity was varied on potatoes, parsnips, and onions, Table IV. Again the amounts were quite variable, being least for distilled water, and great-

est for N/30 to N/40 concentration of acid.

The cause of the great variability in amounts of calcium pectate obtained with different concentrations of acid merits further consideration.

Is it due to the fact that the pectin liberated is attacked by the dilute acid and partially decomposed so that it is not estimated by the Carre and Haynes method? This might account for the smaller amounts of calcium pectate obtained with the more concentrated acid solutions. It would also account for the decreased amounts obtained with longer periods of heating or at higher temperatures. In order to get some light upon this question, an experiment was carried out on apple pectin. Aliquot portions of a solution of commercial apple pectin (Certo) were heated for an hour with different concentrations of acid. The solutions were then filtered and the pectin in the filtrate determined. The per cents of calcium pectate thus obtained, based on the solids in the commercial product, are shown in table V.

TABLE V. Destruction of apple pectin at different acid concentrations.

Pectin as Calcium Pectate	Concentration of Hydrochloric Acid					
	N/30		N/20		N/10	
	20.42		19.69		18.02	
Per cent	20.42	20.42	20.28	19.98	17.66	17.84

An inspection of table V shows that increasing concentration of acid has a destructive effect upon apple pectin. The effect, barely appreciable for N/20 over N/30, becomes very pronounced with N/10th. It would seem, therefore, that the decrease in calcium pectate obtained with potatoes, parsnips, and onions by increasing acid concentration was due to the destruction of the pectin by the stronger acids. These experiments accordingly indicate that N/20 concentration of hydrochloric acid of the Carre method is slightly too concentrated for hydrolysis of the protopectin in the vegetables employed in these experiments. On the other hand, table IV shows that the greatest amount of calcium pectate is obtained with about N/30 concentration.

The foregoing experiments would suggest that the best procedure for the acid hydrolysis of protopectin in vegetables is to boil with N/30 to N/40th hydrochloric acid at atmospheric pressure for one hour. Longer periods of boiling, or higher temperatures, or greater concentrations of acid all lead to destruction of a part of the pectin which has been liberated during the process of hydrolysis. This method will probably be applicable, therefore, to all vegetable materials which contain only protopectin but we shall now show its limitations when applied to materials which contain other insoluble pectic compounds.

As previously pointed out, many of the earlier workers believed that calcium pectate or pectic acid occurred in plant tissues. Mangin (30), particularly, developed this idea, and carried out many experiments, which he believed showed that the middle lamella of most soft parenchymous tissues consisted of pectic acid and pectates, chief among which was calcium. The cellulose layer of the cell wall, on the other hand, had closely associated with it, another insoluble pectic substance, pectose, which was distinctly different from pectic acid and the pectates. It could be made soluble by boiling with dilute acid, while the pectates could not. In the course of his investigations he examined both potatoes and onions, and found evidence in them of calcium pectate and pectic acid. However, Mangin's work, carried out by microchemical methods, seems to have been almost completely ignored by recent investigators, with the exception of the microchemists. While Mangin used solutions of ammonium oxalate, tartrate, and citrate, to dissolve out the middle lamella, he apparently never examined his extract for the material removed. While Schryver and Haynes (40) have used ammonium oxalate solution to extract pectic materials, they have on the other hand ignored the protopectin.

Examination of potatoes, parsnips and onions with cold solutions of ammonium oxalate showed at once that these materials contained pectic material in abundance

which could be extracted with this reagent. The substance extracted could be precipitated in the form of a transparent gel upon adding a small amount of hydrochloric acid, and this gel had all the physical and chemical properties of ordinary pectic acid. After being filtered off and washed it could be dissolved in dilute alkali and precipitated in the form of insoluble calcium pectate upon addition of acetic acid and calcium chloride, as in the Carre and Haynes method for soluble pectin. The solubility of calcium pectate and pectic acid in ammonium oxalate solution was readily demonstrated. The evidence seemed beyond dispute, therefore, that the vegetables contained large quantities of pectates.

In order to obtain more definite information as to the dual nature of the insoluble pectic materials in vegetables, it was decided to first hydrolyse the protopectin and then examine the residue for pectates. One gram samples of parsnips were heated with 100 c.c. of N/30 hydrochloric acid for one hour, then filtered and thoroughly washed. The residues were now washed back into the flasks and one lot treated with 100 c.c. of 1% ammonium oxalate solution. The other lot was again extracted with 100 c.c. of N/30 hydrochloric acid. The mixtures were all heated at boiling for an hour, after which solutions were filtered off and the residues washed.

The filtrates from the second acid extraction were treated by the Carre and Haynes method for pectin. The filtrates from the ammonium oxalate extraction were acidified with concentrated HCl and the pectic acid, thus precipitated was filtered and washed. When the oxalate ion had been removed the pectic acid was dissolved through the paper with 100 c.c. of 0.1 normal sodium hydroxide solution, and the filtrate was diluted to 400 c.c. Calcium pectate was precipitated by the Carre and Haynes procedure. The amounts of calcium pectate obtained are given in table VI.

TABLE VI. Pectic material remaining in parsnips after removal of the protopectin.

Second Extraction by	Calcium Pectate	
	Weight	Per Cent
1% Ammonium oxalate	0.0276	2.76
	0.0262	2.62
N/30 HCl (second time)	.0111	1.11
	.0116	1.16

Upon examination of the table we see that both ammonium oxalate and acid removed additional pectin material, although the ammonium oxalate solution removed the most. We may account for the pectic material

removed by the ammonium oxalate solution as coming from the pectates. However we are at a loss as to how to account for the pectic material extracted by the second application of N/30 hydrochloric acid. From the results shown in tables III and IV we should have expected the protopectin to have been completely hydrolysed. What then is the source of this pectic material?

In attempting to answer the question of the previous paragraph, it was recalled that frequent reference is made in the literature to a slight solubility of pectic acid. The thought occurred that the action of the dilute acid might be to split the calcium off of any calcium pectate contained in the tissues and dissolve a portion of the resulting pectic acid. Or pectic acid itself, if it occurred as such, could be dissolved. In order to establish this point the following experiment was carried out: 0.1 gram samples of calcium pectate, prepared by the Carre and Haynes method and therefore fairly pure, were covered with 100 c.c. of N/30 hydrochloric acid and heated at the boiling point for one hour. The solutions were filtered and the filtrates treated according to the Carre and Haynes method for soluble pectin. In every case a visible gel separated out. In a couple of cases the residues from this extraction were washed from the filter paper into a small beaker dried, and weighed. The amounts of calcium pectate thus recovered together with the amounts

of residue remaining are given in table VII.

TABLE VII. Effect of heating calcium pectate with N/30 HCl for one hour.

Source of Calcium Pectate	Calcium Pectate from filtrate		Residue after treatment	
	Actual Wt.	Per Cent	Actual Wt.	Per Cent
Peach pectin	0.0133	13.3	.....	....
Peach pectin	.0099	9.9	.....	....
Peach pectin	.0121	12.1	0.0610	61.0
Onion	.0170	17.0	.....	....
Onion	.0175	17.5	.....	....
Onion	.0098	9.8	0.0512	51.2

From this table it is evident that the procedure for hydrolysing protopectin will extract very appreciable quantities of calcium pectate. This is shown not only by the amounts of calcium pectate recovered from the filtrate, but also from the fact that from 39 to 49 per cent of the calcium pectate is lost from the insoluble residue during the process. The actual weight of calcium pectate dissolved by 100 c.c. of N/30 hydrochloric acid varies from 0.0098 to .0175 grams. Therefore, the amount of calcium pectate obtained from the parsnip in Table VI can easily be accounted for as coming from the pectates, for which the ammonium oxalate was the more efficient solvent.

The results of the experiment just described have an

important bearing on the Carre method for determination of protopectin in case pectates are present. They would indicate that in such cases, as for example in vegetables, the protopectin determination would be too large because of the pectates dissolved at the same time.

Extraction of Pectates.

The following experiments are intended to show that the pectates cannot be extracted quantitatively before removal of the protopectin. In using ammonium oxalate solution to remove the pectates it is, of course, necessary to have the solution cold, since hot solutions readily hydrolyse protopectin. This fact is shown by the results of Tables I and IV where distilled water gave from 2.15 to 4.82 per cents of calcium pectate in onion and parsnip respectively. One gram samples of parsnips were extracted alternately with one per cent ammonium oxalate and N/30 hydrochloric acid. The order of the extractions and the amounts of insoluble pectic compounds removed by each extraction are shown in table VIII.

TABLE VIII. Insoluble pectic compounds removed from vegetables by alternate extractions with ammonium oxalate and hydrochloric acid. Determined as Calcium pectate.

Order of Extraction	Calcium Pectate Per cent	
	Onion	Parsnip
Ammonium oxalate 44 hours	2.33	4.77
Second extraction with ammonium oxalate	0.00	0.00
N/30 Hydrochloric acid	1.21	2.09
Third extraction with ammonium oxalate	3.63	3.14

This table shows that one extraction with ammonium oxalate solution removed all the pectates that can be removed until the protopectin is gotten rid of. After the protopectin is removed with N/30 HCl a second large quantity of pectates can be extracted with ammonium oxalate. In the case of the onions this was greater than the amount obtained by the first extraction. The explanation of this result is not at present evident. Possibly the pectates, obtained by the last extraction, were held from the first extraction by impermeable walls, composed partly of cellulose, partly of protopectin. On the other hand it may be possible that the pectates obtained in this last extraction were chemically united in some way and were only released by the treatment for protopectin. In any case it is shown that the first extraction with ammonium oxalate is insufficient to remove all the pectate. We are again confronted with the difficulty of removing the protopectin without dissolving the residual pectates. Therefore, a separate quantitative removal of either protopectin or pectates appears to be impossible.

Combined Determination of Protopectin and Pectates.

An attempt was now made to overcome the objections to the removal of either of the insoluble pectic compounds separately by developing a method which extracts both constituents simultaneously. In combining the extraction of the two it is possible to use a hot solution of ammonium oxalate since in this case the hydrolysis of

protopectin is desired. Furthermore a hot solvent may be expected to be much more efficient as an extracting agent.

The first combined solvent tried was composed of a 1 per cent solution of ammonium oxalate in N/30 hydrochloric acid. Samples of parsnip were heated under a reflux condensor with this solution for an hour. The mixtures were allowed to cool, filtered, and the residues thoroughly washed with water. The filtrates were just neutralized with normal sodium hydroxide. Pectic acid was precipitated by addition of sufficient hydrochloric acid to give a 1 per cent solution. Pectin, from protopectin, was not precipitated by this treatment. The pectic acid was filtered off. The filtrate was first neutralized and then treated with sodium hydroxide to saponify the pectin. After standing for 24 hours the pectic acid was precipitated with 1 per cent hydrochloric acid and converted into calcium pectate. At the same time some samples of parsnips were extracted as above and the filtrates subjected at once to alkali hydrolysis without attempting to separate the pectates and pectin from protopectin. The total amount of pectic material thus obtained is to be compared with the sum of the pectin and pectates, determined separately. See table IX.

TABLE IX. Quantitative separation of the insoluble pectic materials in parsnips by the method of combined extraction. Determined as calcium pectate.

	Calcium Pectate Per cent
Pectates	8.02
Pectin from protopectin	1.75
Pectates and Pectin, not separated	9.71

In the experiment just described the pectic acid has in every case been precipitated with 1 per cent hydrochloric acid. While precipitation by this method seems to be quite complete, still this procedure is open to two serious objections. First, the strong acid may chemically alter the pectic acid in some way as has been contended recently by Wichmann (50). In the second place it is necessary to wash the pectic acid considerably in order to remove the oxalate ion and during this washing a certain amount of pectic acid is dissolved and lost in the wash water. On the other hand a procedure which would decrease the long process of converting the pectic material into calcium pectate would be a distinct advantage, since the procedure given in the preceding experiment is quite long and tedious. All these difficulties and objections were happily solved through the use of

ammonium citrate solution in place of ammonium oxalate.

Ammonium citrate is an efficient solvent for calcium pectate and pectic acid. On the other hand, it has a peculiar property in that it does not give a precipitate, in the cold, with solutions of calcium chloride, even in concentrated solution, and in spite of the fact that calcium citrate is very insoluble. However, when solutions of ammonium citrate and calcium chloride are boiled a precipitate of calcium citrate separates out immediately. These facts permit of an important improvement in the procedure for the combined determination of protopectin and pectates. The following experiment was planned to determine the time of extraction with ammonium citrate to effect complete extraction of the pectates following the acid treatment of the tissue.

Samples of the same parsnips used in the previous experiments were heated with N/30 hydrochloric acid for one hour. The mixture was then neutralized with sodium hydroxide, and treated with 1 per cent ammonium citrate solution. It was again boiled for different periods, allowed to cool, and filtered. The residues were thoroughly washed with hot water. The filtrate and washings were allowed to cool and made to mark in a volumetric flask. Two aliquots were removed from each flask. One was treated with sodium hydroxide for a

period of about 24 hours to saponify the pectin. It was then treated as in the Carre and Haynes method for soluble pectin. The other aliquot was treated directly with acetic acid and calcium chloride. In this latter, only the pectate precipitated, the pectin remaining in solution since calcium chloride does not precipitate pectin. The calcium pectates were filtered off, and thoroughly washed with cold water in order to remove the citrate ion which would otherwise be precipitated as calcium citrate in the subsequent heating. Finally the gel was washed back into the original beaker and boiled with a considerable volume of distilled water for several minutes to extract any remaining soluble salts. The calcium pectate was filtered hot, washed until free from chlorides, and finally transferred to a small beaker where the water was evaporated off, and the gel dried. The results obtained by this procedure are given in table X. The pectic materials are calculated as calcium pectate. Pectin is obtained by difference of the saponified and non-saponified determinations.

TABLE X. Results obtained by varying the time of heating with ammonium citrate.

Time of Boiling with ammonium citrate solution Mins.	Pectic Materials as Calcium Pectate		
	Pectin and Pectates	Pectates only	Pectin (by difference)
15	9.60	6.79	2.81
30	9.92	7.24	2.68
70	9.40	7.04	2.36

This table brings out several interesting facts. It shows that 15 minutes boiling with ammonium citrate solution is not sufficient to give the maximum amount of total pectic material. (Pectin and Pectates). It shows that as the boiling is continued there is a steady decrease in the amount of pectin. About 16 per cent of the pectin is lost between the periods of 15 minutes and 70 minutes heating. This loss suggests the similar result that was noted in Table III. Since, however, the solution here was neutral, it is indicated that a loss of pectin occurs even when boiled in neutral solution. The total amount of pectic material obtained here agrees very closely with the amount obtained and shown in Table IX and X. The amounts of pectin obtained are considerably greater than shown in those tables. It would seem likely that the lower amounts of pectin obtained previously was due to the necessity of precipitating the pectic acid with strong acid which splits off some of the alcohol from the pectin. Fellenberg (18) states that acid does this slowly.

In view of the fact that heating slowly destroys the pectin it seems advisable to make the heating period as short as possible. By reference to Table III, it will be noted that almost the maximum amount of pectin was obtained after heating for thirty minutes. If now, the heating with acid were stopped, the acid neutralized, ammonium citrate solution added, and the boil-

ing again continued for thirty minutes, the total time of heating could be considerably shortened without shortening the time of heating with the citrate. Bearing in mind all these considerations, a method for the quantitative separation of protopectin and pectates, using a combined extraction is suggested as follows: From 1 to 3 grams of dry, finely ground material (or 5 to 20 grams of fresh material), depending on the pectic content, is boiled in a flask fitted with a reflux condenser with 100 c.c. of N/30 hydrochloric acid for 30 minutes. (In case fresh material is used the approximate moisture content of the material should be taken into account.) Boiling is then stopped and the mixture allowed to cool somewhat. It is then neutralized by the addition of 3.33 c.c. of N/1 sodium hydroxide solution. Next 10 c.c. of 11 per cent neutral ammonium citrate solution is added and the mixture is boiled for an additional 30 minutes. It is again allowed to cool and transferred to 250 c.c. volumetric flasks. The cooled solution is made to volume with distilled water, mixed, and filtered through loose filter paper. Whatman No. 4 has been found very suitable. Pectates are now determined in this filtrate by transferring a 100 c.c. aliquot to an 800 c.c. beaker, diluting to 400 c.c. with water and adding 50 c.c. of each N/1 acetic acid and M/1 calcium chloride.

The calcium pectate so produced after standing several hours is filtered off cold, washed with distilled water a number of times, and returned to the beaker. It is suspended in about 300 c.c. of water and boiled a few minutes. It is again filtered, washed until free from chlorides, transferred to a small beaker with a little water, dried for 6 to 8 hours at 103°C, and weighed.

Pectin is determined in the filtrate by determining the total pectic material and subtracting the pectates. The total pectic material is determined by adding 10 c.c. of N/1 sodium hydroxide solution to 100 c.c. of the filtrate and allowing to stand over night. This saponifies the pectin fraction. Next day 50 c.c. each of N/1 acetic acid and M/1 calcium chloride is added and the calcium pectate determined as in the preceding case.

The preliminary treatment of material to be analysed probably should be such as to eliminate certain enzymes, as for instance pectase. While it is not known at present just what interference such enzymes might introduce, it is known that pectase is very widely distributed in vegetable materials and certain conditions of preparation of the material might permit considerable alteration to occur in the relative amount of protopectin and pectates. A suggested method of preserving the sample would be to place the mascerated material quickly

into boiling alcohol. This should destroy enzymes, and at the same time permits the sample to be stored until it is convenient to complete the analysis.

II. Transformation of the Pectic Materials in Peaches.

The methods employed to extract pectates from vegetables were applied to peaches at different stages of ripening, but at no time was it possible to demonstrate the presence of pectates. This fact lead to a further study of the pectic transformations in this typical fruit during ripening and storage. In contrast to vegetables the insoluble pectic material in peaches appears to consist only of protopectin. Therefore, the chief transformation of this material in the tissue would be its conversion into pectin.

Method of Analysis.

The method used for determining the pectin and protopectin in peach tissue was as follows: from 4 to 6 peaches, as nearly representative and uniform as possible, were used for a sample. These were halved and the stones removed. One half of each peach was then passed through a food chopper to pulp the tissue. Samples of 50 grams were then extracted in large mortars by mascerating the pulp with successive 50 c.c. of water until tests showed the extraction of pectin to be practically complete. Four extractions of two minutes each was found sufficient. Almost

none of the tissue passed through the cheesecloth used for filtering off the solution after each extraction. The collected extracts were transferred to a 250 c.c. volumetric flask and made to mark with distilled water. After thoroughly mixing 100 c.c. portions of the solution were centrifuged for about 20 minutes. The clear supernatant liquid was then filtered and 50 c.c. of the filtrate used for determination of soluble pectin, by the Carre and Haynes method.

The residue of peach tissue after the final extraction was transferred to a small beaker and covered with 50 c.c. of 95% alcohol. After standing a day or more this was poured off and a new 50 c.c. volume added. The latter was finally drained off and the tissue was dried in a vacuum oven at 70°C. After grinding the dry material to pass a 40 mesh sieve, the sample which amounted to from 0.8 to 1.2 grams, was heated with 100 c.c. of N/30 HCl for one hour under a reflux condenser. The hydrolysed mixture was transferred to a 250 c.c. volumetric flask, neutralized by adding 3.30 c.c. of N/1 NaOH solution, and made to mark. The mixture was filtered and an aliquot of the filtrate taken for determination of pectin according to the Carre and Haynes method.

<u>Transformation of protopectin into pectin during ripening of peaches in storage.</u>

In order to find what relation existed between the

protopectin and the pectin during ripening in storage, a half bushel of Crawford peaches were picked from the tree "hard ripe" and stored in the laboratory at an average temperature of approximately 65°F. One sample was taken immediately and others at subsequent periods as the ripening of the fruit progressed. The results obtained are given in table XI.

TABLE XI. Changes in relative amounts of pectin and protopectin in Crawford Peaches during ripening in storage.

	Soluble pectin %	Protopectin %
Sampled at once	0.142	0.610
Stored at 65°F for 3 days	.363	.452
Stored at 65°F for 7 days	.656	.260
Stored at 65°F for 13 days	.639	.143

The close relationship between the increase in amount of soluble pectin and decrease of protopectin is brought out more strikingly by Figure 5. It will be noted that the curves are almost exactly inverse.

In this series of analyses, the softening of the fruit went hand in hand with the increase in soluble pectin and the decrease of protopectin. At the beginning of the experiment the tissue would not give upon pressure from the thumb. After 7 days the tissue

was uniformly soft, while at the end of 13 days it was somewhat more firm and tough, considerable water having been lost.

Pectic changes in peaches during ripening on the tree and in storage at different temperatures.

A number of hard ripe peaches as nearly uniform as possible, were selected on the tree. While they were still hard they had begun to show development of color. One lot of fruits was tagged and left on the tree. This was designated Lot I. A second lot was picked and sampled immediately while a third lot was placed in a refrigerator at 37.6°F, and a fourth lot stored in a constant temperature chamber at 72°F. After three days, samples were taken from lots 1, 3 and 4 for analysis. The results are given in table XII. and Figure 6.

TABLE XII. Pectic changes in peaches during ripening.

	Soluble Pectin %	Proto-pectin %
Lot I left on tree, daily mean 60°F	0.196	0.535
Lot II "Hard-ripe" sampled at once	.241	.543
Lot III Stored at 37.6°F	.202	.587
Lot IV Kept at 72°F	.620	.277

At the end of three days the peaches on the tree

were still hard. Those kept at the cold temperature (37.6°F) were also still hard. However those kept at the high temperature (72°F) were extremely soft at the end of this time. By inspection of the table it will be seen that in every case where the peaches were still hard the soluble pectin was low and the protopectin high, while in the one case where the peaches were soft the soluble pectin was high and the protopectin low. This indicates very conclusively the relationship between the insoluble or protopectin and the soluble pectin. The softening of the tissue is really the result of this transformation. An interesting observation was the fact that the peaches left on the tree did not soften although the average daily temperature was fairly high (60°F). The first experiment showed that peaches kept at 65° off the tree softened quite rapidly. The picking of the fruit, therefore, apparently initiated changes which hastened the softening.

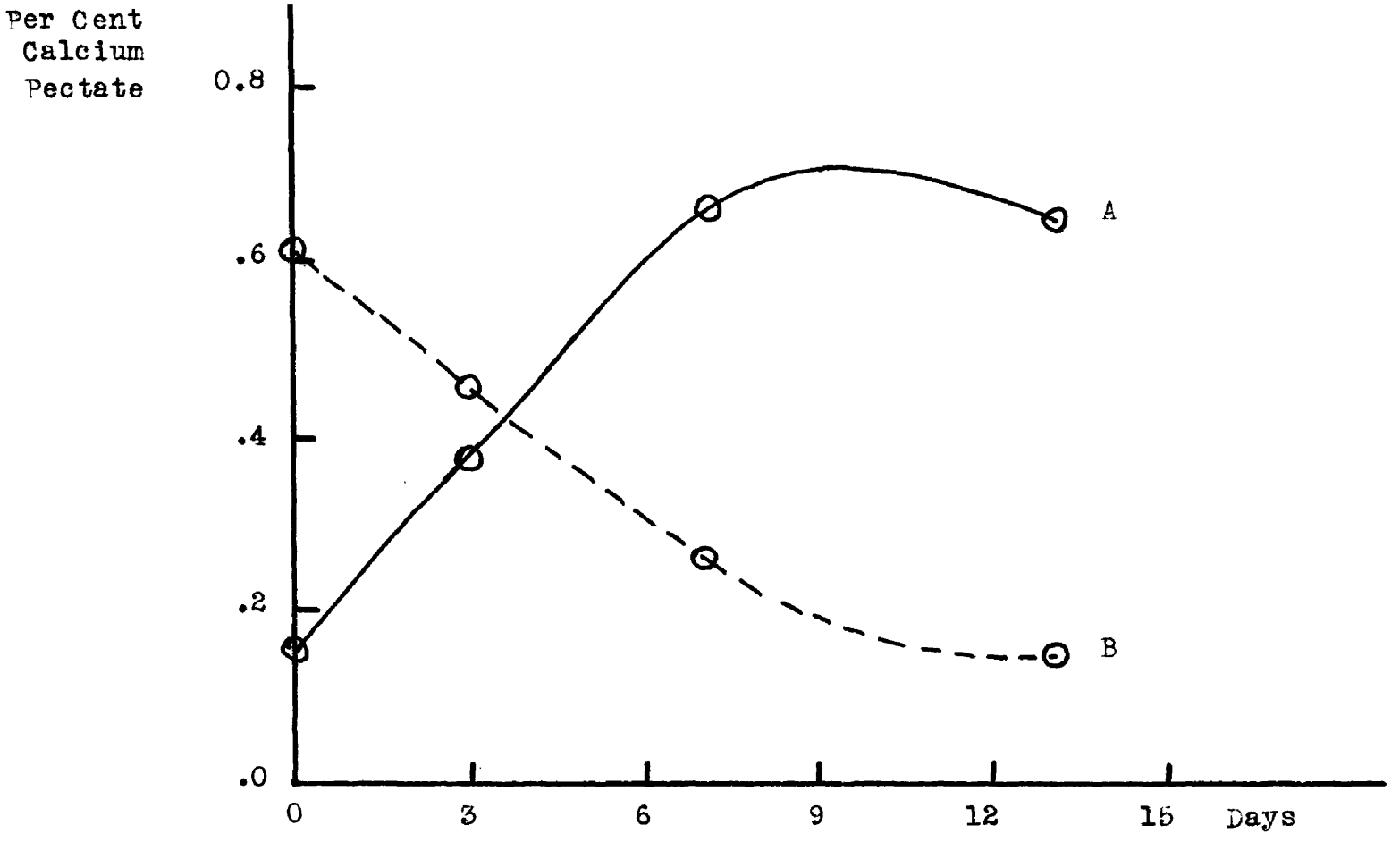

Figure 5. The course of pectic transformation in Crawford peaches during ripening. A, curve for pectin; B, for protopectin.

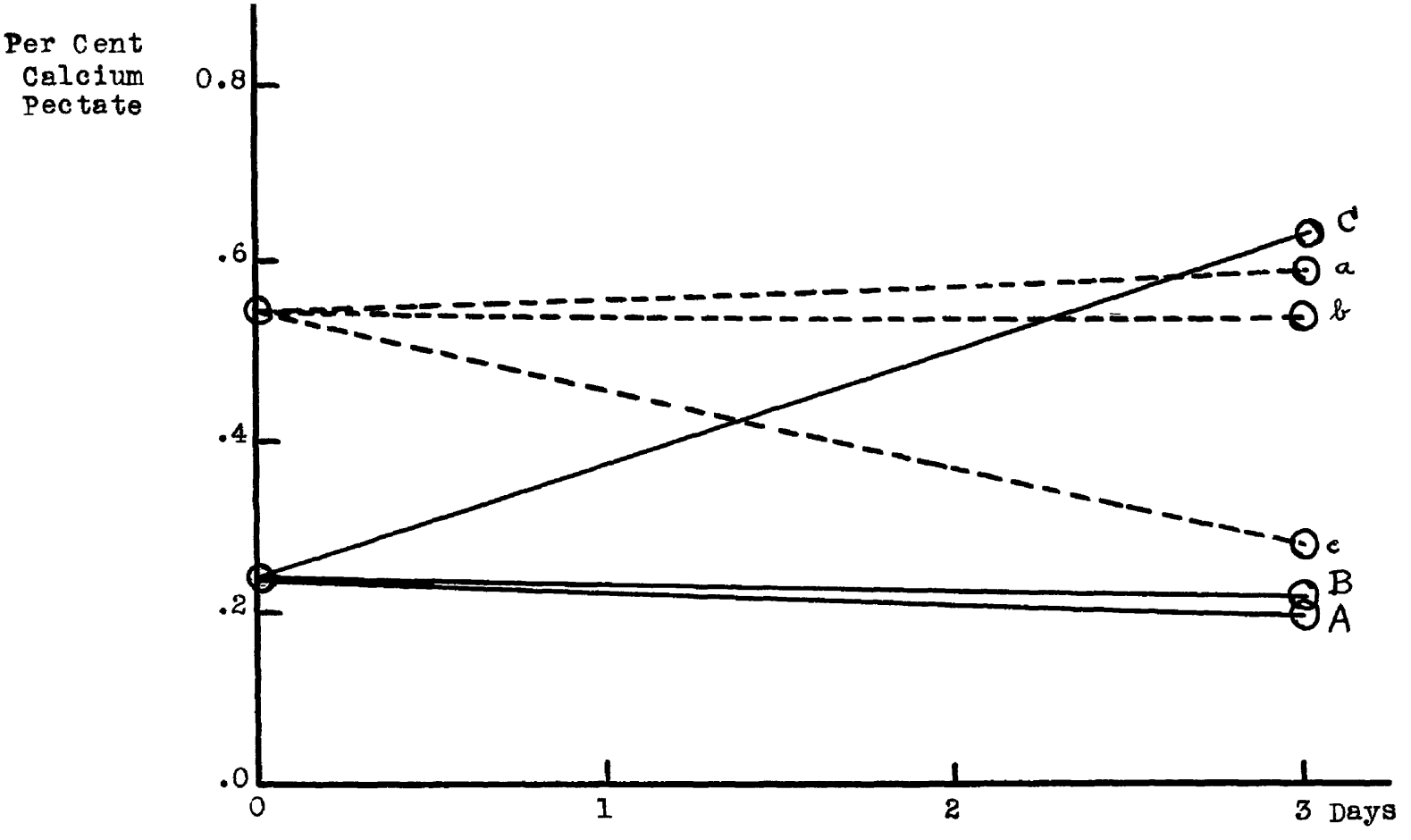

Figure 6. Effect of temperature and removal from tree on rate of pectin transformation in Crawford peaches. A, left on tree; B, stored at 37.6°F; C, stored at 72°F. Capital letter indicate pectin; small letters, protopectin.

III. Amounts of Protopectin and Pectates in Different Plant Tissues.

During the course of the previous work analyses of the pectic constituents were collected for a variety of tissues. While these were not always determined in exactly the same manner and therefore cannot be considered as entirely comparative, it is believed worth while to include them here. In a few cases the protopectin and pectates were not determined individually but were determined as a whole, and in those cases the figures for protopectin and pectin are absent. Protopectin is, of course, estimated on the basis of the pectin which it will yield, as must be done until its molecular nature is made known. All values are calculated as calcium pectate. These results are given in table XIII.

TABLE XIII. Per cents of protopectin, pectate, and total pectic material in various tissues, all calculated in terms of calcium pectate.

Material (Air dry)	Protopectin	Pectate	Total Pectic Material
Peach	28.18	0.00	28.18
Onion	1.21	5.96	7.17
Parsnip	2.81	7.24	10.05
Potato	2.31	1.20	3.51
Pea roots	....	1.99	....
Clover hay	....	....	5.03

SUMMARY AND CONCLUSIONS.

1. Mangin's claim that the insoluble pectic constituents occur in plants in two forms has been completely verified.

2. An important difference between the insoluble pectic constituents has been indicated. Protopectin, which by dilute acid hydrolysis gives rise to pectin, and pectates, which comprise pectic acid and its metallic salts, were both demonstrated in a number of vegetables. But only protopectin was found in peaches at any period of ripening. This was found in large amount in contrast to vegetables, which are characterized by relatively small amounts of protopectin and large amounts of pectates. This would lead us to expect that the pectic transformations in vegetables and fruits would be very different. The protopectin in fruits changes during the process of ripening into pectin, - a substance having little adhesive power. Protopectin seems to be the sole "cement" which holds the cells together. In vegetables, on the other hand, the protopectin is rather insignificant in comparison to the pectates, and even though it should be converted into pectin we should scarcely expect the cell walls to separate, because of the abundance of pectate which likewise acts as a cement and keeps the cells united.

Finally it seems reasonable to expect that cases will be found where the conversion of protopectin into pectin will occur in cell walls containing the pectates, and that a tissue thus constituted will be soft and tender yet cohering because of the adhesive nature of the pectates.

3. A detailed study was made of the Carre method for the quantitative determination of protopectin in fruits, with the view of standardizing it for use with vegetables. On account of the abundant pectates found in vegetables the method was not applicable to these tissues.

4. Neither protopectin nor pectate can be removed from vegetables separately but they can be removed simultaneously and the resulting pectin and pectate separated afterwards.

5. A method has been proposed for the simultaneous removal and quantitative separation of the insoluble pectic aonstituents of vegetables.

6. A study was made of the pectic transformation in peaches, a typical fruit, during ripening on the tree and during storage at different temperatures. All of the pectin found was accounted for by the transformation of protopectin. Temperature was found to have a profound influence on the rate of pectic changes dur-

ing ripening in storage. They were also accelerated after removal of the fruit from the tree, even though the temperature of storage was no higher than the air temperature around the tree.

7. Finally, a comparison was made of a variety of plant tissues in respect to the amounts of the different pectic constituents which they contain.

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