LXXVI. STUDIES ON PECTIN. PART II.

THE ESTIMATION OF THE INDIVIDUAL PECTIC SUBSTANCES IN NATURE.

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INTRODUCTION.

THE object of the present work was to study the distribution of the individual pectic substances in nature. Whereas the total pectic content of such materials as apples, exhausted beet slices, etc., is well known, since they are of commercial value by reason of their relatively high content of pectic substances, no determinations have as yet been published on the distribution of these substances generally in the vegetable kingdom. An attempt in this direction was commenced by Hornby [1920] on fruits and roots, but the method of estimation adopted by him is open to criticism as will be indicated later.

In addition to determining the total and regional contents of the material under investigation, a separation into the various types of pectic substance has been effected by differential extraction. Histological investigations have been carried out on similar lines, and these will be published elsewhere.

In the preliminary work involved, which need not be described here, two main difficulties had to be overcome. First, a method of extraction which was quantitative and yet suitable for routine work had to be devised, and secondly, it was necessary to elaborate an accurate method of estimation. The principle involved in the estimation is that put forward by Carré and Haynes [1922]. In brief, it consists in precipitating the pectin as calcium pectate, after hydrolysis by alkali, and weighing as such. Although rather involved, the method, with due precautions, is accurate.

It is proposed eventually to follow by this method the regional and seasonal changes in some selected types of plant or plants, at the same time proceeding with histological investigations on the same material.

Extraction.

Carré and Haynes [1922] first removed the soluble pectin by extraction with cold water, and then the form insoluble in water by another method. It is obvious that any incompleteness in this initial extraction would render

the later results unreliable. In addition a cold water extract would in all probability remove little more than the pectin in solution in the cell sap. The method they adopt is to freeze the material for some hours to ensure that it is completely killed, after which it is brought to the ordinary temperature and pressed through cloth with the solvent in a hand press. The residue is ground with sand, washed with the extracting liquid and pressed. Sixty to eighty extractions were usually required. So laborious a method is obviously unsuitable for a large series of estimations. In addition it is doubtful whether such repeated extraction with large volumes of solvent is sound in principle, as it is conceivable that an insoluble colloidal substance, such as pectic acid, might become dispersed, and passing into apparent solution vitiate the results.

Carré [1922] estimated the insoluble pectin or protopectin by heating the residue after extraction of the water-soluble pectin with $N/10$ HCl in an autoclave for 1 hour at a temperature of 110° . The protopectin, she states, is converted into the soluble form "presumably by hydrolysis." Such a method, however, cannot be considered accurate, as acid treatment in the autoclave produces very considerable hydrolysis of the pectin itself beyond the pectic acid stage.

Tutin [1925], for the estimation of pectin in certain pathological leaves, first cut them into strips and immersed them in boiling alcohol. After several treatments with alcohol they were dried and weighed. The pectin was then extracted with boiling water.

It is evident that the chief difficulty to be met with is that of obtaining uniformly complete extraction. The difficulty is purely a structural, not a chemical, one, *i.e.* the inability of the extracting agent to penetrate properly into all the cells of the tissue, and its failure to reach and dissolve out those insoluble pectic substances intimately connected with the cell-wall, either as an incrusting substance or in the form of the middle lamella. Thus it would appear that for complete extraction the material in question must be in a very fine state of division. Link and Tottingham [1923] have studied the effects of various methods of desiccation of plant materials, and they come to the conclusion that drying at 95° is satisfactory if the starch or sugar content of the tissue is not too high. This method accordingly was adopted for all tissues. In the case of one or two fruits the sugar appeared to caramelise to a certain extent, but there is reason to believe that very little error is caused through this.

The materials were taken in as fresh a condition as possible and placed in thin layers in a hot steam-oven, so that the enzymes were destroyed almost immediately. Seeds were first ground up, and fruits cut into thin slices. Moisture estimations were made. When completely dry the materials were ground as finely as possible, sieved in graded sieves, and re-ground to obtain a uniformly fine powder, which was used for the extractions.

The optimum time and temperature of extraction had next to be determined. It is clear that a mean period had to be found at which extraction would be complete, yet not so prolonged that hydrolysis might take place

beyond the pectic acid stage. For this reason actual boiling was avoided, the flasks being placed on a copper water-bath about 2 inches above the level of the water, the temperature of the extracting liquid being then 85°. The following table represents the yield of calcium pectate obtained per 100 g. of dried sycamore leaf powder by extraction with water at 85° for various periods.

Table I. *Extraction of sycamore leaf powder with water.*

Time of extraction in hours	Percentage yield of calcium pectate
6	1.42
12	2.30
18	3.30
24	3.72
36	3.70

As a result of this and many similar experiments, extraction was considered complete at the end of 24 hours. The residue after washing was re-extracted with water for a considerable period, but no further trace of pectin could be found.

A comparison was made using the above method of extraction for 24 hours on the same leaves taken fresh but ground with sand and water very finely. The calcium pectate yield per 100 g. calculated on the dry weight was only 0.9 in this case, compared with 3.7 on the powdered leaf.

The solutions used in addition to water for extraction in the routine investigations were oxalic acid and ammonium oxalate. It was indicated by Norris and Schryver [1925] that the best concentration both of oxalic acid and of ammonium oxalate for the extraction of pectic substances is 0.5 %. This observation was confirmed and this concentration only employed in each case.

Estimation.

The earliest method of estimation adopted was that indicated by von Fellenberg [1918] in which the pectin is precipitated by alcohol and weighed. It is obvious that this method is inaccurate, inasmuch as any other substances in the extract precipitable by alcohol would be weighed as pectin. In addition, pectin, on precipitation, almost invariably carries down with it considerable quantities of any other substances present in the solution, which are very difficult to wash out subsequently. Also, as the dilution of the pectin increases, precipitation becomes increasingly difficult. Nevertheless, this method was advocated by Wichmann [1922, 1923]. Hardy [1924] has given figures for both calcium pectate yield and alcohol precipitate of the same extract. In every case the alcohol precipitate was found to be much higher owing to the presence of impurities. Hornby [1920] employed a method of estimation suggested by the work of von Fellenberg [1918]. In this case the methoxyl yield of the tissue is determined directly, and pectin obtained by calculation on the von Fellenberg formula. This presupposes pectin in all natural sources to have the same methoxyl content, and further, assumes that pectin is the sole source of methoxyl groups in vegetable tissues.

The calcium pectate method of estimation referred to previously was described by Carré and Haynes [1922]. Briefly it consists of adding to the pectin solution $N/10$ sodium hydroxide to convert the pectin to the soluble sodium pectate. After acidification with acetic acid, calcium chloride is added, and the pectate deposited as the insoluble calcium salt. They state that acetic acid in excess removes practically all impurities. Tutin [1923] criticises this on the grounds that any acid yielding a calcium salt insoluble in acetic acid would be weighed as pectin. It is obvious that this method would be impossible using oxalic acid or ammonium oxalate as the extracting agent.

The method finally adopted involves precipitation by alcohol, re-solution of the precipitate, and subsequent hydrolysis and precipitation as calcium pectate.

Method.

A suitable amount of the dried and powdered tissue is extracted for 24 hours at 85° with about 200 cc. of the extracting solution. At the end of that period it is filtered and the residue washed with more solvent. The filtrate and washings are made up to 250 cc. 100 cc. of this extract are gently concentrated to about 25 cc. on a hot plate. When the extracting fluid is oxalic acid, it should be just neutralised before concentration to obviate any possibility of hydrolysis. After cooling, $3\frac{1}{2}$ volumes of 95 % alcohol, acidified with 5 or 6 drops of concentrated hydrochloric acid, are added. From a series of experiments it would appear that the concentration of acid in the alcohol does not affect the yield, as long as the reaction is quite definitely acid. The final concentration of alcohol should be not less than 70 %, and should be kept the same in all estimations. The object of the concentration of the extract is merely to reduce the quantity of alcohol used and to expedite the subsequent filtration. The precipitate is allowed to stand for several hours, and is then filtered off on a fluted filter-paper and washed with acid alcohol until the washings show no trace of oxalate. The filter-paper with the alcohol precipitate is then placed in a beaker and the precipitate dissolved by boiling with about 40 to 50 cc. distilled water. In the case of the ammonium oxalate extracts very dilute ammonia is employed instead of water at this stage. The use of ammonia is necessary because if any salt of pectic acid were present in the tissue, it would now, as a result of the acid-alcohol treatment, be present as free pectic acid, which is insoluble in water but soluble in dilute ammonia. The solution is filtered off, and the filter-paper boiled again, this time with dilute ammonia in the case of aqueous or oxalic acid extracts, and with water in the case of ammonium oxalate extracts. Ammonia is employed in the former cases to ensure that any pectic acid which may have been formed by partial hydrolysis during the concentration or extraction will not be lost. Finally, a further quantity of water is added to the filter-paper, which is then triturated with a stirring rod and boiled. This is filtered, and washed with hot water and hot dilute ammonia. The combined filtrate and washings, usually a little over

100 cc., are treated after cooling with 100 cc. 0.4 % NaOH and allowed to stand overnight. To precipitate the pectin, now in solution as sodium pectate, the calcium gel method is employed. 50 cc. normal acetic acid are added together with an equal volume of 11.1 % calcium chloride and the whole is allowed to boil for at least 5 minutes. It is then filtered as hot as possible through a tared filter-paper—a good Swedish type being the most satisfactory—and the precipitated calcium pectate gel washed with boiling water until the washings are quite free from chloride. As much as 400 cc. of boiling water may be required, but filtration is usually extremely rapid. The precipitate is then dried at 100° and weighed.

The method recently described by Emmett and Carré [1926] resembles the above, which, however, was in use for this work at the time. These workers do not add ammonia when dissolving up the pectin precipitated by alcohol, and in consequence lose any pectic acid which may be present. Also they filter and weigh the calcium pectate gel in a Gooch crucible, a method not found convenient as filtration is slow and washing appears to be unsatisfactory.

Differentiation of extracts.

It is now necessary to consider the specific nature of the pectic substances removed by each of the extracting agents employed, namely water, 0.5 % oxalic acid, and 0.5 % ammonium oxalate. It is generally accepted that three types of pectic substances exist in the plant; protopectin or pectose, the form insoluble in water, which appears to give rise to pectin on hydrolysis, and is often stated, without evidence, to be in combination with cellulose; pectin, the form soluble in water; and pectic acid, insoluble in water, consisting of de-esterified pectin.

Tutin [1923] questioned the existence of protopectin, stating that the persistent retention of pectin in a form apparently insoluble in water in the tissue of fruits is due to the great difficulty of obtaining by mechanical means really efficient disintegration of the tissue. This is partly true, as the degree of penetration of the extracting fluid depends on the state of division, which may consequently be regarded, up to a point, as a limiting factor. Carré [1925] replied to this criticism, producing evidence which seems conclusive for the presence of some pectic substance insoluble in water. This substance could be removed to a certain extent by very prolonged boiling with water or completely by treatment with dilute acids. She states definitely that this is "pectose"—pectin in combination with cellulose, the pectin of which could be removed completely by boiling for a few hours with acid as dilute as *N*/75 HCl.

In 1925, both Kopaczewski [1925] and Nanji, Paton and Ling [1925] noted that pectin gives, with a solution of an iron salt, a gelatinous precipitate which is insoluble in water. It is but slowly dissolved on prolonged treatment with water at 100°, but is easily soluble in dilute oxalic and hydrochloric acids. These observations caused the latter workers to suggest that pectin exists in a condition insoluble in water in the plant in loose combination with metallic

ions, such as iron. If pectin is precipitated with ferric chloride, the iron content of the precipitate is found to vary very considerably, as iron is carried down by the gel formed. Consequently, it is impossible to determine by simple analysis whether or not simple salt formation has taken place. This is, however, very unlikely, as it would involve the losing of 75 % of the theoretical methoxyl content at least, a change not observed. It seems more likely that the insoluble form is a polymer, consisting of several molecules of the tetramethylpectic acid, associated with one or two partially de-esterified molecules, the free carboxyl groups of which are linked to the metallic ion. Ehrlich [1917] regarded it originally as an insoluble calcium-magnesium salt, but there is evidence from the ash of pectin preparations that it is a mixed calcium-iron salt. Thus it would appear that the insoluble form of pectic substance, hitherto known as "protopectin," is to be regarded as being but the normal form in combination with certain metallic ions as just indicated, and it is this substance that is removed by treatment with 0.5 % oxalic acid or 0.5 % ammonium oxalate.

But it must not be forgotten that this is not the only pectic substance insoluble in water. It is well established that the middle lamella is composed of combined pectic acid. It has been suggested that it is combined with cellulose, but this is very unlikely in the mature plant, as it is easily removed by 0.5 % ammonium oxalate, or by very dilute sodium hydroxide. In addition to its occurrence in the middle lamella, it seems probable from a consideration of the experimental work later to be described that combined pectic acid, *i.e.* pectates, occurs to a much greater degree in some tissues than can be accounted for by supposing that it forms the middle lamella only. This is particularly the case in the rind of the lemon and orange. Traces of free pectic acid have also been found in some tissues. This would also be extracted by 0.5 % ammonium oxalate.

From a consideration of the data above it would seem that pectic acid is the central pectic substance. Which, however, is the precursor, and the mode of its formation, can at present be a matter for conjecture only. Carré [1925] makes the interesting suggestion that the pectic acid may play a useful part as a fixing agent for the methyl alcohol produced as a by-product in metabolism. The relations of these substances are shown in the following scheme:

The solubilities of these substances may be summarised as follows.

Soluble in water—free pectin.

Insoluble in water, but soluble in dilute acids—pectin in combination with metallic ions.

Insoluble in water, soluble in 0.5 % ammonium oxalate—pectin in combination with metallic ions, plus pectic acid, both free and combined.

Therefore in the scheme of extraction followed in this investigation:

A. water—removes free pectin:

B. 0.5 % oxalic acid—removes free pectin, plus pectin in combination with metallic ions:

C. 0.5 % ammonium oxalate—removes free pectin, plus pectin in combination with metallic ions, plus pectic acid, both free and combined.

Consequently, it follows that

A = Free pectin.

$B - A$ = Pectin in combination with metallic ions.

$C - B$ = Pectic acid: free and combined.

The results of this differential estimation of the pectic substance in various samples of leaves, cereals, and fruits are given in Table II. It

Table II. *Distribution of the individual pectic substances in nature.*

	Calcium pectate number*						Moisture %
	Expressed on dry powder			Expressed on dry powder			
	A	B	C	A	B - A	C - B	
	Water extract	Ox. acid extract	Amm. ox. extract				
<i>Leaves.</i>							
I. Sycamore	3.87	5.24	7.90	3.87	1.37	2.66	64.25
II. Alder	1.63	4.12	4.83	1.63	2.49	0.71	82.45
III. Potato	2.10	6.26	7.32	2.10	4.16	1.06	87.96
IV. Sumach	1.82	4.91	5.52	1.82	3.09	0.61	62.91
V. Lilac	2.51	3.70	6.11	2.51	1.19	2.41	66.46
VI. Laurel	2.87	4.52	4.95	2.87	1.65	0.43	77.87
VII. Ivy	3.41	4.96	7.46	3.41	1.55	2.51	70.92
<i>Cereal grains.</i>							
VIII. Barley	0.071	1.02	2.10	0.071	0.95	1.08	12.30
IX. Malt†	0.094	0.158	0.311	0.094	0.06	0.153	5.96
X. Wheat	Nil	0.404	0.606	Nil	0.404	0.202	11.65
XI. Rye	Nil	0.130	1.003	Nil	0.130	0.87	17.55
XII. Maize	Nil	Nil	0.590	Nil	Nil	0.59	11.87
XIII. Oats	Trace	0.43	0.651	Trace	0.43	0.221	14.93
XIV. Rice	Nil	Trace	1.13	Nil	Trace	1.13	13.35
<i>Fruits</i>							
XV. Apple—							
Peel	9.15	11.89	17.44	9.15	2.74	5.55	80.30
Pulp	8.92	11.94	17.63	8.92	3.02	5.69	88.00
Aver. section	8.96	11.92	17.60	8.96	2.96	5.68	87.84
XVI. Orange—							
Yellow rind	7.54	11.09	15.92	7.54	3.55	4.83	73.18
White peel	18.53	20.59	38.75	18.53	2.06	18.16	76.28
Pulp	10.45	12.06	12.40	10.45	1.61	0.34	89.08
Aver. section	14.34	15.50	24.54	14.34	1.15	9.04	84.65
XVII. Lemon—							
Peel	15.92	17.31	35.45	15.92	1.39	18.14	76.62
Pulp	11.31	13.74	14.10	11.31	2.45	0.36	83.39
Aver. section	14.92	15.49	23.31	14.92	0.57	7.82	83.12

* "Calcium pectate number" may be defined as yield of calcium pectate per 100 g. material.

† No. IX was a special research specimen prepared from Barley No. VIII.

should be emphasised here that the results in these tables are all expressed as yield of calcium pectate per 100 g. material. All the figures recorded are the average of at least two, and often more, closely agreeing determinations. Expression of the results as calcium pectate is at present the only sound method, until we know definitely the exact constitution of the substance extracted in each case. The figures for aqueous extract can be expressed if desired as tetramethylpectic acid by the use of the factor 0.9813, which represents the molecular weight of the tetramethyl ester divided by that of calcium pectate. It is by no means certain that free pectin in the plant is in the tetramethyl condition. Norris and Schryver [1925] state that it is uniformly in the tri-esterified condition. Some evidence on this point will appear in a later communication.

DISCUSSION OF RESULTS.

Leaves. Though a fairly representative selection of leaves was taken, there do not appear to be many points which stand out. Nos. II and III, alder and potato respectively, give very similar figures when expressed on the fresh leaf. Both these plants are comparatively quick growing and sappy in nature as shown by the moisture contents, which are both over 80 %. Whether or not this similarity in content of pectic substances is accidental it must remain for further work to show. There does not appear to be any close similarity between the two representative evergreens selected, ivy and laurel.

Cereal grains. The results show clearly that pectic substances are present in cereal grains only to a small extent—the highest figure recorded for total content being only just over 2 % of the dry weight, in the case of barley. It may be stated that there is practically no free pectin in any cereal, and the pectic substance present is nearly all in the form of pectate. Histological work, to be published elsewhere, shows this to lie almost exclusively in the middle lamella and cell-wall of the aleurone layer. Nos. VIII and IX are interesting, inasmuch as they were special research samples, the malt, No. IX, being obtained from the barley, No. VIII. It would seem that during the process of germination in malting the free pectin increases slightly, probably at the expense of the pectin in combination with metallic ions, while the latter and the pectates decrease enormously. In this connection the work of Tollens [1898], Schöne and Tollens [1901], and of Windisch and Hasse [1901], is very significant. These workers state that during germination an increase of pentosan takes place, and, from the observations mentioned above, there can be little doubt but that this increase is at the expense of the pectic material.

Fruits. It should be noted that the results given under the heading of "average section" were obtained from wedge-shaped slices from the fruits. Simple sections cannot be regarded as giving a true average sample. The main point of interest in the distribution in the fruits examined is the surprisingly high content of pectates in the rind of the orange and the lemon, and the low content of the same in the pulp of these fruits. The free pectin appears to be distributed through the fruits fairly evenly, the region containing the lowest

amount being the pulp. Experiments on the gently expressed juice show that it contains by no means all of the free pectin, and consequently some of this must be regarded also as forming a part of the cell-wall.

SUMMARY.

A method for the differential extraction of the individual pectic substances from plant materials, suitable for routine work, is indicated, and the estimation of each by an improved method as calcium pectate is described. Results are given in the case of certain leaves, cereal grains and fruits.

In conclusion, it is a pleasure to acknowledge the advice and encouragement given by Prof. A. R. Ling.

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