CLI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VI.

BY EDWARD HOYLE AND SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

REPORT TO THE MEDICAL RESEARCH COUNCIL.

(Received August 30th, 1927.)

THE experiments described in this communication are in continuation of a previously reported investigation on the behaviour of some of the inorganic constituents of lemon juice in the process of fractionation [Daubney and Zilva, 1926]. Nickel, cobalt, boron, iodine and phosphorus were considered in the first part of this research. The study of manganese and of iron in this connection became complicated by the presence of traces of these elements in the reagents used in the various manipulations. It was found that the mere absence of manganese and iron at the end of a blank experiment was not a fair indication that the two elements if found in the final fraction did not originate, at least in part, from the reagents used. Their presence in such fractions could only be considered of any significance if reagents free from all traces of the elements in question were used throughout the procedure. With this aim in view we have succeeded in preparing all our necessary reagents entirely free from interfering impurities. Manganese, iron, phosphorus and sulphur were investigated.

MANGANESE.

The scheme of fractionation adopted in this and in the following experiments was based on the improved method recently described by one of us [Zilva, 1927], eliminating the use of basic lead acetate. The method, although different in some details, did not differ essentially from the one employed by Daubney and Zilva in the first part of this research. The lemon juice was treated with excess of calcium carbonate, allowed to stand for about an hour and filtered. The filtrate was then concentrated by distillation in a vacuum at 50° to about a fifth of its original volume and treated with about half of its original volume of absolute alcohol. After filtering off the precipitate and removing the alcohol under reduced pressure at 50° the solution was diluted to the original volume of the juice with distilled water. After this the solution was fractionated according to the improved method mentioned above. All the reagents were previously purified.

Calcium carbonate was prepared from purified calcium chloride and sodium carbonate. The reagents were crystallised twice from distilled water. The resulting precipitate was thoroughly washed with distilled water and dried. To demonstrate that the chalk was free from manganese, 50 g. of it, *i.e.* about the amount required to neutralise 500 cc. of lemon juice, was stirred into an excess of dilute sulphuric acid. After filtering off the calcium sulphate the filtrate was concentrated to small bulk and any further precipitate of calcium sulphate removed. The liquid was then evaporated to dryness in a silica crucible and the residue tested for manganese by the method described later. Manganese was found to be totally absent. Ordinary precipitated chalk tested in the same way was found to contain appreciable quantities of manganese. The lead acetate was crystallised three times from dilute acetic acid, drained on a Büchner funnel and dried in the air. The absence of manganese from this reagent was demonstrated in the following ways.

(a) 10 g. of the salt (the quantity required for the treatment of an equivalent to 500 cc. of lemon juice) was dissolved in distilled water to which a drop of acetic acid was added to ensure perfect solution. The lead was then precipitated by the addition of an excess of sulphuric acid and the filtrate was evaporated to dryness in a silica crucible. Manganese was found to be absent from the residue.

(b) 10 g. of the lead acetate was dissolved as in (a) and magnesium sulphate added in slight excess above the quantity required to precipitate the lead. The mixture was then treated with two volumes of absolute alcohol, filtered, and the filtrate concentrated at 50° at low pressure, and eventually evaporated to dryness in a silica crucible. Manganese was absent from the residue.

No manganese was found in the filtrate when a solution of magnesium sulphate containing the quantity of the salt used in the manipulation was precipitated by the addition of two volumes of absolute alcohol, and the salt was therefore considered free from manganese.

The ammonia used in this experiment was also found to be free from all traces of manganese.

Bertrand's [1911] method, modified to suit our conditions, was employed in the estimation of manganese. In this method the manganese is oxidised to permanganate and is estimated colorimetrically. The ash of the various residues was heated with a few drops of sulphuric acid to drive off any chlorides present and after cooling was taken up in about 5 cc. of distilled water acidified with 1 cc. of concentrated nitric acid. The solution was then transferred to a specially selected test-tube suitable for colorimetric work, 5 drops of 10 % silver nitrate and about 0.1 g. of potassium persulphate were added and the mixture was boiled until the maximum pink coloration developed. Before matching, the solution was made up with distilled water to 10 cc.

A freshly made-up solution of potassium permanganate in graded dilutions and contained in suitable test-tubes was used as a standard. Manganous sulphate, which when oxidised as above yields equivalent quantities of per-

manganate, produces a solution which very often does not maintain its colour for any length of time and therefore the direct use of permanganate is more convenient.

It is possible in this way to determine manganese quantitatively within the limits of 0.005 and 0.15 mg., although the highest degree of accuracy is obtained within the range of 0.01–0.10 mg. in 10 cc. Below 0.005 mg. the coloration is perceptible but quantitative differentiation is unreliable. Above 0.10 mg. the difference in colour also gradually becomes less marked and solutions very much above this concentration show a tendency not to retain the pink colour and to deposit manganese dioxide. Although the latter compound can be reoxidised the procedure is troublesome. The sample of nitric acid used by us showed traces of manganese, but as this was the only reagent contaminated with this element, blanks, containing the quantity of nitric acid used in the entire determination including the incineration, were employed. It is to be pointed out that in the oxidation of manganese salts with nitric acid and persulphate the use of too much or too little of the acid influences the stability of the pink coloration.

Five batches of lemon juice were fractionated, two of 250 cc. each and three of 500 cc. each. In none of these could any manganese be detected in the final fraction. The decitrated lemon juice, and the supernatant liquid from which the active fraction was precipitated, contained 0.01 mg. of manganese per 500 cc. of juice.

Table I. *Mg. of Mn in fractions from 250 cc. of tomato juice.*

No. of batch	No addition of Mn			0.02 mg. Mn as MnSO ₄ added to supernatant liquid from neutral lead acetate ppt.	
	Supernatant liquid from neutral lead acetate ppt.	Supernatant liquid from basic ppt.	Basic precipitate	Supernatant liquid from neutral lead acetate ppt.	Basic precipitate
I	0.055	0.060	nil	0.080	nil
II	0.040	0.040	—	0.060	„
III	0.080	0.080	nil	0.090	„
IV	0.060	0.060	„	0.070	„
V	0.060	0.060	„	0.080	„
VI	0.060	0.055	„	0.075	„

Apart from the above experiments a preliminary investigation on tomato juice produced similarly negative results. This work was done before the new method of fractionation was introduced and consequently the fractionation was carried out by the basic lead acetate method. The latter reagent was obtained free from manganese by recrystallising a sample of ordinary basic lead acetate from a saturated boiling water solution. The non-active material was first removed from the tomato juice by precipitation with two volumes of concentrated alcohol followed after removal of the alcohol from the filtrate by treatment with purified neutral lead acetate. The fraction precipitated by the basic lead acetate was taken up in dilute acetic acid and decomposed

with sulphuric acid. On removing the lead sulphate, the filtrate was evaporated, incinerated and tested for manganese as previously described.

In this experiment the batches were divided into two series, to one of which MnSO_4 equivalent to 0.02 mg. of Mn per 250 cc. was added to the supernatant liquid from the neutral lead precipitate, in order to see whether inorganic manganese would be precipitated under these conditions. As will be seen from Table I no manganese was found in any of the final fractions. Similar fractions from tomato juice (decomposed however with MgSO_4) were tested biologically on guinea-pigs and were found to be active.

IRON.

The reagents used in the production of the active fractions for the estimation of iron were purified as in the case of the manganese estimations.

The calcium chloride and the sodium carbonate used in the preparation of the calcium carbonate were found free from iron after several crystallisations. Similarly the recrystallised neutral lead acetate on being decomposed with sulphuric acid was found to contain no iron. Nor did the ammonia and magnesium sulphate show the presence of any iron. The nitric acid contained traces of iron. This was compensated for by using blanks in the estimations. The iron was determined colorimetrically as ferric ferrocyanide, ferric alum stabilised by the addition of a few drops of hydrochloric acid being used for the standards. To the solution contained in specially selected test-tubes, 1 cc. of 0.5 % potassium ferrocyanide and a drop of concentrated hydrochloric acid were added for the production of the Prussian blue and the contents brought up to 10 cc. The optimum range was found to be between 0.02 and 0.15 mg. per 10 cc. By observing these conditions a colour of uniform tint and requisite durability was obtained. The various fractions were prepared, incinerated as for the manganese estimations and taken up in hydrochloric acid. Since about 0.15–0.26 mg. of iron per equivalent of a litre of original juice was found to be present, it was thought of interest to study the behaviour of this residual iron on dialysis through collodion membranes soaked in 88 % and in 95 % alcohol respectively [Zilva and Miura, 1921; Connell and Zilva, 1924; Daubney and Zilva, 1926]. This was carried out in the way described by

Table II. *Mg. of Fe in undialysed and dialysed fractions.*
Calculated for 1 litre of lemon juice.

No. of batch	Decitrated alcohol-treated lemon juice	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
			88 %	95 %
I	2.4	0.16	0.16	0.12
II	1.0	0.20	0.08	nil
III	1.6	0.28	0.14	"
IV	0.6	0.20	0.04	"
V	1.0	0.16	0.16	"
VI	1.3	0.16	0.12	"
VII	—	0.24	0.16	"

Daubney and Zilva. Iron was estimated in 50 cc. of decitrated juice and in equivalents of 250 cc. of the other fractions which as before were all reduced to 10 cc. for the estimation. Table II gives a summary of the results calculated for an equivalent of 1 litre of juice.

It is seen that the iron passed through an 88 % thimble only to a small extent whilst, with the exception of one case, the 95 % thimbles permitted the entire passage of the iron. This will be discussed in a later section of the paper.

PHOSPHORUS.

The reagents used in the fractionation were purified as before. The calcium carbonate was tested in the following ways. (a) The requisite quantity of chalk was treated with sulphuric acid until slightly acid, filtered, and the filtrate concentrated to about 5 cc. After filtering off any further calcium sulphate which separated out during concentration, the solution was tested for total phosphorus by the Martland and Robison modification [1926] of the Briggs method. (b) In this case the calcium carbonate was decomposed with citric acid using a quantity of the acid which was approximately equivalent to that contained in 100 cc. of lemon juice. After neutralising the acid it was treated with two volumes of alcohol, filtered, and the alcohol removed by distillation from the filtrate which was concentrated to about 5 cc. and tested for phosphorus by the above method. No traces of phosphorus could be detected by either method. Ordinary samples of precipitated chalk treated as above showed a content of 0.1 mg. of phosphorus per 10 g. of calcium carbonate.

The neutral lead acetate was tested for phosphorus after being decomposed by sulphuretted hydrogen and was found to be free, as was also the ammonia.

Lemon juice was fractionated as before, except that the final fraction was decomposed by sulphuretted hydrogen instead of by magnesium sulphate. Fractions treated in this way, as previously pointed out by one of us, lose their activity. It was found, however, that the magnesium sulphate treatment owing to the almost unavoidable presence of small quantities of this salt in the final fraction, made the ignitions of the organic matter, as carried out in the above method, troublesome. This inactivation of the fraction would not of course interfere with the inorganic content of the fraction. The analysis of six batches of the pure fraction equivalent to 100 cc. yielded 0.12, 0.13, 0.13, 0.14, 0.12, 0.11 mg. respectively and, as will be seen from Table III, the phosphorus in the final fractions dialysed only to a very small extent through collodion thimbles of 88 % alcohol index, whilst the thimbles with a 95 % index permitted the entire phosphorus to diffuse in the course of three days.

Attempts to estimate how much of the phosphorus was inorganic failed, owing to the turbidity and to the modification in the tint obtained by adding the Briggs reagent before ignition.

It may however be noted that disodium hydrogen phosphate and potassium

dihydrogen phosphate in equivalent dilutions dialysed in the same way as the phosphorus of the final fraction.

Table III. *Mg. of P in final fractions. Calculated for 1 litre of lemon juice.*

No. of batch	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
		88 %	95 %
I	1.28	1.13	nil
II	1.22	0.97	"
III	1.37	0.96	"
IV	1.17	0.95	"
V	1.10	0.74	"
VI	1.14	0.92	"
VII	1.11	1.08	"
VIII	1.20	1.00	"

The figures for total phosphorus obtained in these experiments are rather lower than those previously obtained [Zilva, 1924; Daubney and Zilva, 1926], but this no doubt is due to the use of purified reagents.

SULPHUR.

In spite of the use of reagents free from sulphur, quantities of this element were found in the final fraction which, considering the nature of the investigation, were of a high order. These figures were obtained without incinerating the preparation by direct precipitation as barium sulphate out of the solution which was previously heated with hydrochloric acid in order to convert any "ethereal" into inorganic sulphur. In nine batches, equivalent to 1 litre of original juice, 2.0, 3.6, nil, nil, 0.8, 2.3, 0.9 mg. were found. The sulphur in the active fraction diffused only partly through an "88 %" thimble, but entirely through a "95 %" collodion thimble. In view of these high and variable results the further effort of determining the total sulphur was not considered justified.

CONCLUSIONS.

In the two sections of this enquiry the active fraction from lemon juice has been examined for iron, phosphorus, sulphur, iodine, manganese, nickel, cobalt and boron. The last four elements were found to be absent and consequently, if at all associated with the antiscorbutic activity, they must be so in quantities not detectable by our methods. Of those present, iodine may also be excluded since, as has been shown by Daubney and Zilva, no evidence could be obtained that any of it diffused through a collodion thimble with a 95 % alcohol index. Antiscorbutic solutions dialysed under the same conditions lose their activity. On the other hand the iron, phosphorus and sulphur of the active fraction behaved on dialysis in the same way as the active principle. One must however be very cautious in postulating a connection between them and the antiscorbutic activity. These inorganic constituents cannot be responsible for the activity *per se* as they are almost ubiquitous in animal and plant tissues, whilst the distribution of the anti-

scorbutic factor in these tissues is limited to certain sources only. If there be any connection, these elements must be either in physical or chemical association with some other substance or radicle or themselves be in a definite physical state responsible for their physiological function. Needless to say this investigation offers no indication which would justify such hypotheses. The possibility that the presence of iron, phosphorus and sulphur in the active fraction is of significance must therefore await consideration until our knowledge of the problem is more advanced.

SUMMARY.

The concentrated antiscorbutic fraction from lemon juice does not contain any manganese but contains traces of iron, phosphorus and sulphur. These elements of the active fraction behave on dialysis like the active principle.

REFERENCES.

- Bertrand (1911). *Bull. Soc. Chim.* **9**, 361.
Connell and Zilva (1924). *Biochem. J.* **18**, 641.
Daubney and Zilva (1926). *Biochem. J.* **20**, 1055.
Martland and Robison (1926). *Biochem. J.* **20**, 847.
Zilva (1927). *Biochem. J.* **21**, 354.
Zilva and Miura (1921). *Biochem. J.* **15**, 422.