CXCIII. THE NON-SPECIFICITY OF THE PHENOL-INDOPHENOL REDUCING CAPACITY OF LEMON JUICE AND ITS FRACTIONS AS A MEASURE OF THEIR ANTISCORBUTIC ACTIVITY.

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(Received August 25th, 1932.)

EARLY attempts at the fractionation of the antiscorbutic factor in lemon juice [Zilva, 1924, 1, 2] have revealed that active fractions possessed the property of reducing ammoniacal silver nitrate in the cold and of decolorising potassium permanganate. This fact taken in conjunction with the observations made on the inactivation of the vitamin by exposure to oxygen [Zilva, 1921; 1922; 1923] led the writer to pursue the inquiry with the object of ascertaining whether the reducing capacity of antiscorbutic concentrates was directly due to the vitamin itself or to substances, protective or otherwise, associated with it. It was found that the capacity for reducing ammoniacal silver nitrate or potassium permanganate in the cold, although closely associated with the antiscorbutic factor, was destroyed at a different rate from it [Connell and Zilva, 1924]. Efforts to modify active solutions in order to destroy their capacity for reducing ammoniacal silver nitrate and at the same time to leave the antiscorbutic activity unimpaired failed. A different technique of measuring the reducing capacity of the various fractions obtained from lemon juice, namely, that of titrating them with phenolindophenol (1-dimethylaminophenylindophenol) which was found to be decolorised by some of them was, therefore, eventually adopted. In this way the reducing capacity of various fractions could be determined with a good degree of accuracy. By using this procedure two main facts were ascertained by the writer [1927], (a) that it was possible to obtain fractions possessing little antiscorbutic activity, namely those precipitated by normal lead acetate from decitrated lemon juice at p_H 5.4, which possessed a reducing capacity equal to or sometimes even higher than that of the more active fractions obtained by precipitation with the same reagent from the same source at p_H 5.4–7.2; (b) that by adding to decitrated lemon juice phenolindophenol until it was no longer reduced the antiscorbutic activity was not destroyed, when tested immediately. In view of these observations the writer advanced a hypothesis that the

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substance (or substances) reducing phenolindophenol acted protectively in relation to the antiscorbutic factor in virtue of its reducing potential and it was referred to by him for the sake of convenience in all subsequent communications as "the reducing principle or factor." Later work indicated the possibility that certain reducing substances other than those reducing phenolindophenol and capable of having a deleterious effect on the antiscorbutic activity were present in some preparations [Zilva, 1930; Johnson and Zilva, 1932].

Recently Tillmans, Hirsch and collaborators [Trillhose, 1930; Tillmans, Hirsch and Hirsch, 1932; Tillmans, Hirsch and Siebert, 1932; Tillmans, Hirsch and Jackisch, 1932, 1, 2; Tillmans, Hirsch and Dick, 1932; Tillmans and Hirsch, 1932] produced evidence which in their opinion justified the assumption that the antiscorbutic factor itself was responsible for the reduction of phenolindophenol (2: 6-dichlorophenolindophenol). The following is their evidence.

A fraction precipitated from decitrated lemon juice with normal lead acetate at p_H 5.4 (an equivalent of 1 cc. of the original juice reducing about 1.5 cc. of 0.001 M 2:6-dichlorophenolindophenol) neither prevented nor delayed death from scurvy of four guinea-pigs on a scorbutic diet, receiving a dose equivalent to 1 cc. per day, but protected another set of four guinea-pigs for 42 days when five times the dose was administered daily (the reducing capacity of the fivefold dose was 7.5 cc. 0.001 M 2:6-dichlorophenolindophenol). The fraction from the same juice which is precipitated between p_H 5.4–7.2 was not examined by them either for its reducing capacity or its antiscorbutic content. These writers consider, however, that evidence in support of the view that the vitamin itself is responsible for the reduction of the indicator is afforded by the fact that the reducing capacity of the fivefold dose of the fraction which is precipitated at p_H 5.4 is about equal to that of the protective dose of 1 cc. of decitrated lemon juice from which it is prepared, *i.e.* about 8 cc. of 0.001 M 2:6-dichlorophenolindophenol.

It is not at all surprising that by raising the dose of the fraction which is precipitated at p_H 5.4 some antiscorbutic protection was obtained. The separation of the vitamin by precipitation with normal lead acetate at various p_H values is by no means clear cut and a certain amount of the active principle is usually present in the fraction which is precipitated at p_H 5.4; sometimes this activity may be rather high [Zilva, 1929]. Had Tillmans and his collaborators tested the fraction obtained between p_H 5.4 and 7.2 prepared at the same time they would have found that the ratio of the antiscorbutic activity of this fraction to its reducing activity was decidedly higher than that found in the fraction precipitated at p_H 5.4. This is illustrated by Table I which gives the results of such a fractionation experiment performed by the writer in which the greater part of the antiscorbutic activity was contained in the p_H 5.4–7.2 fraction. These figures leave no doubt as to the lack of parallelism between the antiscorbutic activity and the reducing capacity for phenolindophenol.

Table I.

	Daily dose equivalent to original juice cc.	Reduction value of daily dose in cc. of 0.02 % phenolindophenol	Corresponding degree of protection from scurvy*. Av. score obtained from 3 animals for each test
Fraction precipitated at p_H 5.4	1.5	3	1
	3	6	2
	5	10	3
Fraction precipitated at p_H 5.4-7.2	1.5	2.6	5
	3	5.2	6
	5	8.7	7

* For an explanation of these numbers, see Table II.

Tillmans and his collaborators also claim that in the course of other investigations they found a consistent proportionality between the phenolindophenol-reducing capacity of a number of products and their antiscorbutic activity. In this connection about 114 tests comprising about 350 guinea-pigs, carried out by the writer over a period of 5 years on decitrated lemon juice and various fractions prepared from it, have been analysed by him for their capacity of reducing phenolindophenol and for their antiscorbutic potency. These tests were not specially selected but were chosen at random. Just as the biological test comprises in this case the composite effect of 60 days' dosing, so the phenolindophenol reducing capacity is the average of the daily titration values obtained for the same doses just before administration to the experimental animals. Each score in the biological tests comprises the average of three and sometimes even more guinea-pigs. As is well known, young healthy growing guinea-pigs of approximately the same weight vary very little in their response, as regards growth and development of scurvy, to the antiscorbutic factor, and consequently within the quantitative limits of the biological method the results obtained even in one test are incontrovertible. It is thus impossible to attribute a positive or negative result in the following experiments to anything but the presence or absence, as the case may be, of the antiscorbutic factor in the particular dose. The analysis of the tests is set out in Table II.

It will be seen that in five tests involving about 15 guinea-pigs, which received a daily dose with a reducing capacity of 9-10 cc. of phenolindophenol, little or no antiscorbutic protection was obtained. On the other hand a fair degree of antiscorbutic protection was obtained in three tests comprising about 9 guinea-pigs with a daily dose of a reducing capacity of only 2-3 cc. of phenolindophenol. The other doses also do not show a consistent relationship between antiscorbutic activity and reducing capacity. It will be further noted that the number of doses possessing some activity becomes greater and the doses show on the whole a higher activity when the reducing capacity reaches the figure of 4 cc. or thereabouts. This is most probably due to the fact that in any of these doses in which the vitamin was originally present, it was protected from inactivation during manipulation owing to the suitable

Table II. *The relationship of the phenolindophenol-reducing capacity of various preparations from lemon juice and their antiscorbutic activity as tested on guinea-pigs.*

Reduction value of daily dose in cc. of 0.02 % phenol- indophenol	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Corresponding degree of pro- tection from scurvy. Average score obtained from three animals for each test	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 2 . . .	0 0 0 0 0 0 0 0 0 0 0 0 1 2 2 2 5 5 6 .	0 0 0 0 0 0 0 1 1 1 2 2 3 3 5 6 7 . . .	0 0 0 0 0 1 2 2 4 5 6 7 5	0 0 0 0 1 1 2 3 5 5 6 8 5	0 0 1 1 2 3 3 5 5 6 6 6 8	0 0 2 2 4 6 8 . 5 6 6 6	1 5 6 7 8	0 0 1 1 1 5 5 5 5 7 7 8

0 means death from scurvy within 30-35 days preceded by manifestation of scorbutic symptoms after 18-21 days and subsequent decline.

- 1 „ the same as 0 but with death ensuing after 35-40 days. It is doubtful whether the survival for these few extra days signifies the presence of any vitamin.
- 2 „ death from scurvy after 40-50 days.
- 3 „ death from scurvy after 50-60 days.
- 4 „ survival of guinea-pigs for 60 days. No growth. Severe scurvy at autopsy on chloroforming animals.
- 5 „ survival of guinea-pigs for 60 days. Initial growth but subsequent decline. Severe scurvy at autopsy on chloroforming animals.
- 6 „ survival of guinea-pigs for 60 days. Some growth. Slight scurvy at autopsy on chloroforming animals.
- 7 „ survival of guinea-pigs for 60 days. Moderate growth. Very slight scurvy at autopsy on chloroforming animals.
- 8 „ survival of guinea-pigs for 60 days. Good growth. No signs of scurvy at autopsy on chloroforming animals.

reducing potential of the medium. This could not be expected to happen when the reducing value was low, which would explain why in only 2 out of 17 tests a trace of antiscorbutic activity was detected with doses having a reducing capacity of only 1-2 cc.

Tillmans and his colleagues further maintain that the antiscorbutic activity which was observed by the writer in solutions which had been oxidised with phenolindophenol and had then been administered to the experimental animals immediately after oxidation was due to a reversible oxidation of the vitamin by the dye. This thesis will be dealt with in another communication.

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