LVII. THE ALLEGED PRESENCE OF DEHYDRO-ASCORBIC ACID IN BLOOD.

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SOME years ago Van Eekelen *et al.* [1933] found that the capacity of trichloro-acetic acid filtrates from the blood of man, rabbit, pig and guinea-pig for reducing indophenol was increased on treatment with hydrogen sulphide and further that on addition of vitamin C to blood *in vitro* it quickly passed into dehydroascorbic acid. As a consequence it has apparently become generally assumed that dehydroascorbic acid is present in blood with the implication that the latter is capable of dehydrogenating ascorbic acid.

In a previous communication [Kellie and Zilva, 1935] we had already shown that plasma, intact erythrocytes and leucocytes were unable to oxidise ascorbic acid *in vitro* and had further pointed out that the general evidence available was not only against the above assumption but, on the contrary, favoured the theory that the animal organism tended to reduce dehydroascorbic acid. We are now producing further experimental evidence showing that blood does not contain the reversibly oxidised form of the vitamin. The contrary view which some workers hold has, we believe, been responsible for a considerable confusion of thought in the interpretation of results obtained in this field of research.

EXPERIMENTAL.

The capacity of blood to reduce indophenol after treatment with hydrogen sulphide.

(a) *The influence of hydrogen sulphide on whole plasma.* The results presented in this connection are representative examples of a number of observations made by one of us (S. S. Z.) in the course of an investigation of wider scope. It was found that when ascorbic acid was added to whole plasma it could be accounted for with a good degree of accuracy by direct indophenol titration in acid solution even in concentrations as low as 1–2 mg. per 100 ml. There was nothing in plasma, free from haemoglobin, to interfere with the end-point. Failure to reduce the dye, therefore, indicated the absence of ascorbic acid from the plasma. Yet such plasmata were found to acquire the capacity of reducing indophenol to a very marked extent after treatment with hydrogen sulphide.

The procedure adopted in these experiments was as follows. The blood was collected in potassium oxalate solution (1 ml. 5 % potassium oxalate for 20 ml. blood) quickly centrifuged and the plasma carefully pipetted off. About 5 ml. of plasma containing one or two drops of octyl alcohol were placed in a glass bubbler of 15 ml. capacity and hydrogen sulphide passed for 5 min. The bubbler was then sealed and allowed to remain overnight. The hydrogen sulphide was removed by passing a stream of nitrogen through the solution for 24 hours. A dry lead acetate paper then held for 5 min. close to the fine orifice of the outlet tube invariably failed to stain appreciably, thus showing the absence of significant traces of volatile sulphides in solution. The p_H of the plasma fell from 7·4 to 6·8, while saturated with hydrogen sulphide, but regained its original value after the

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latter was displaced by the nitrogen. The titrations of the plasma samples were carried out at p_H 2.5–3.0 before and after treatment. The added indophenol was decolorised quickly by the plasma as it would have been if ascorbic acid were present.

The figures in Table I show that the amounts of indophenol decolorised by the hydrogen sulphide-treated plasma both of scorbutic guinea-pigs and of guinea-pigs kept on a mixed diet containing cabbage were of the same order.

Table I.

Wt. of animal g.	Scorbutic diet days	ml. N/1000 indophenol per ml. plasma		Wt. of animal g.	Diet	ml. N/1000 indophenol per ml. plasma	
		Before H ₂ S	After H ₂ S			Before H ₂ S	After H ₂ S
285	3	0.0	3.0	250	Mixed	0.0	5.1
275	5	0.0	2.2	260	"	0.0	5.2
280	7	0.0	5.2	280	"	0.0	4.0
285	8	0.0	5.2	280	"	0.0	4.2
295	11	0.0	3.0	410	"	0.0	8.6
300	13	0.0	4.2	420	"	0.0	4.7
360	13	0.0	5.0	575	"	0.0	6.3
385	14	0.0	2.7	580	"	0.0	2.3
372	15	0.0	4.0	580	"	0.0	4.6
395	15	0.0	5.8	635	"	0.0	5.3
210	22	0.0	4.5	650	"	0.0	2.7
270	22	0.0	4.1	750	"	0.0	3.3
620	23	0.0	4.2				
270	26	0.0	3.3				
		Average	4.0			Average	4.7

Table II.

Wt. of animal g.	Scorbutic diet days	Quantity injected (mg.)	Bled after min.	ml. N/1000 indophenol per ml. plasma	
				Before H ₂ S	After H ₂ S
315	7	100	5	8.9	4.0
310	7	100	10	6.3	3.3
285	10	100	15	2.2	3.3
300	8	100	120	0.0	6.3
300	10	100	120	0.0	4.9
				Average	4.4

Table II gives the values of the reducing capacities of the untreated and treated plasma of guinea-pigs which received ascorbic acid by intravenous injection. When high doses (100 mg.) are administered in this way ascorbic acid can be detected in the blood of the animals by titration with indophenol for about an hour afterwards. Yet the samples of blood, examined at various intervals following the injection, have shown reducing capacities after treatment with hydrogen sulphide no different from those obtained in the case of scorbutic guinea-pigs and animals which received cabbage daily.

Consequently serious doubts were expressed as to whether the reduction of indophenol by the treated plasma in a way so characteristic of ascorbic acid was really due to the vitamin. If this were so they should have contained 30–40 mg. per 100 ml. of plasma. It is difficult to conceive that guinea-pigs which subsisted for periods up to 26 days on a vitamin C-deficient diet, and some of these even in the premortal phase, should all have about the same concentration of the reversibly oxidised form of the vitamin in their blood as animals which received large quantities of the reduced form either *per os* or

by injection into the blood stream. This is the more striking since dehydroascorbic acid is as active antiscorbutically as ascorbic acid. Furthermore, it has been found that solutions of ascorbic acid will not remain at p_H 6.8–7.4 without serious loss during the period of hydrogen sulphide treatment as described above. Additional, and to our mind conclusive, proof will be given in a subsequent section that the reducing substance thus formed in the plasma is indeed not ascorbic acid.

(b) *The influence of hydrogen sulphide on trichloroacetic acid filtrates of plasma, erythrocytes and whole blood.* To the plasma obtained from the blood as previously described, an equal volume of 20 % trichloroacetic acid was added. After centrifuging, the supernatant fluid was filtered and treated with calcium carbonate until no longer acid to Congo red. This partially neutralised solution was saturated with hydrogen sulphide and allowed to remain in a sealed glass bubbler for 12 hours. The hydrogen sulphide was then displaced by a current of nitrogen. Preliminary experiments have shown that the indophenol-reducing capacity acquired by the trichloroacetic acid filtrates from plasma after treatment with hydrogen sulphide varied firstly with the time allowed for the filtrate to decolorise the indicator and secondly with the thoroughness of the displacement by nitrogen of the hydrogen sulphide or other volatile substances capable of darkening dry lead acetate papers. In order to obtain a truer assessment of the character of the indophenol-reducing substance it was considered desirable to titrate the hydrogen sulphide-treated filtrates at two stages of the displacement and in each case to observe two end-points, the first the point at which 0.1 ml. *N*/1000 indophenol (dimethylaminobenzoindophenol) remained undecolorised after 10 sec. and the second that at which the same amount remained undecolorised after 30 sec. The first stage of the displacement was arbitrarily fixed at the point at which 5 min. were required for a black spot to be produced on a dry lead acetate paper held at the orifice of the bubbler. The solutions were then titrated again at a later stage when only a brown stain developed on the dry lead acetate papers after 15-min. exposure. These criteria were preferable to a time basis of comparison in the case of these experiments since they indicated more accurately corresponding states of the solutions.

The results (Table III) show that the highest figures were obtained after treatment with hydrogen sulphide when the 30-sec. end-point was used and when the extracts were titrated at the first stage of the passage of nitrogen. In the case of plasma E this figure reached an equivalent to 3.7 mg. of ascorbic acid per 100 ml. equivalent of plasma. When, on the other hand, the 10-sec. end-point was used, the equivalent fell to 0.9 mg. of ascorbic acid.¹ That this difference between the figures obtained with the two end-points was characteristic only of the reduced plasma filtrates and was not due to ascorbic acid is seen from the fact that in all the experiments in which authentic ascorbic acid was added to the original non-reducing plasma the trichloroacetic acid filtrates showed the same reducing values with both end-points.

Further examination of the results shows that even the reduction of indophenol by the samples of plasma observed when the 10-sec. end-point was employed could not be attributed to the presence of ascorbic acid. It will be seen, for instance, that when the passage of the nitrogen was prolonged to a stage at which the lead acetate paper reaction had become very weak, the indophenol reducing values of the solutions fell considerably. That the fall was not due to destruction of any formed ascorbic acid by the prolonged treatment is

¹ When 2:6-dichlorophenolindophenol is used the difference obtained between the two end-points is not so high.

Table III.

	Indophenol reduction, mg. equivalent ascorbic acid/100 ml. plasma			
	Plasma		Plasma + 2 mg. ascorbic acid/ 100 ml.	
	10 sec.	30 sec.	10 sec.	30 sec.
Plasma A.				
Before treatment with H ₂ S	0.0	0.0	2.1	2.1
After treatment with H ₂ S and displacement with nitrogen:				
(1) Blackening of dry PbAc ₂ paper after 5 min.	0.0	1.1	2.5	3.0
(2) Brown staining of dry PbAc ₂ paper after 15 min.	0.0	1.1	2.5	2.8
Plasma B.				
Before treatment with H ₂ S	0.0	0.0	2.1	2.1
After treatment with H ₂ S and displacement with nitrogen:				
(1) Blackening of dry PbAc ₂ paper after 5 min.	0.9	2.1	5.3	5.5
(2) Brown staining of dry PbAc ₂ paper after 15 min.	0.2	1.2	2.3	3.3
Plasma C.				
Before treatment with H ₂ S	0.0	0.0	2.3	2.3
After treatment with H ₂ S and displacement with nitrogen:				
(1) Blackening of dry PbAc ₂ paper after 5 min.	0.7	2.5	3.7	6.2
(2) Brown staining of dry PbAc ₂ paper after 15 min.	0.2	1.4	2.3	3.3
Plasma D.				
Before treatment with H ₂ S	0.0	0.0	2.3	2.3
After treatment with H ₂ S and displacement with nitrogen:				
(1) Blackening of dry PbAc ₂ paper after 5 min.	0.9	3.3	3.3	5.6
(2) Brown staining of dry PbAc ₂ paper after 15 min.	0.0	1.9	2.3	4.4
Plasma E.				
Before treatment with H ₂ S	0.0	0.0	2.1	2.1
After treatment with H ₂ S and displacement with nitrogen:				
(1) Blackening of dry PbAc ₂ paper after 5 min.	0.9	3.7	3.7	5.1
(2) Brown staining of dry PbAc ₂ paper after 15 min.	0.2	1.6	2.3	3.3

seen from the fact that in all the experiments the added ascorbic acid could be, considering the limitation of the method, satisfactorily accounted for at the second stage. This can be seen if the reducing values of the blank samples are subtracted from those obtained in the samples containing the added vitamin.

There still remained to be ascertained in this connection the behaviour of trichloroacetic acid extracts of erythrocytes and of whole blood after reduction with H₂S especially as in most if not all the experiments mentioned in the literature such extracts of whole blood were employed. The whole blood was extracted in the same way as the plasmata. In the case of the erythrocytes, on the other hand, a quantity of 20 % trichloroacetic acid equal to that amount of the plasma removed by centrifuging from the blood was added. The titrations of the extracts were also carried out as above and at two similar stages. It may, however, be noted that in these experiments the nitrogen had to be passed for a longer time in order to reduce the darkening of the lead acetate papers to the required intensities. Only one end-point, namely, the inability of the solution to decolorise 0.10 ml. of a N/1000 solution of the indicator during 10 sec., was observed in these experiments. The results are given in Table IV and need hardly any comment. As in the case of the whole plasma a capacity for reducing indophenol, but only to a very much lower extent, was acquired by the trichloroacetic acid extracts in both cases, but this reducing property, unlike that of ascorbic acid, gradually diminished during the passage of nitrogen until it totally vanished at the second stage of the displacement.

Table IV.

	Indophenol reduction, mg. equivalent ascorbic acid/100 ml. blood							
	Corpuscles				Corpuscles + 1.5 mg. ascorbic acid/100 ml. blood			
	0.0	0.0	0.0	0.0	1.5	1.5	1.4	1.4
Before treatment with H ₂ S								
After treatment with H ₂ S and displacement with nitrogen:								
Brown staining of dry PbAc ₂ paper after 5 min.	0.7	0.6	0.6	0.6	1.7	1.8	1.7	1.7
Brown staining of dry PbAc ₂ paper after 15 min.	0.0	0.0	0.0	0.0	1.7	1.5	1.5	1.4
	Whole blood				Whole blood + 1.5 mg. ascorbic acid/100 ml. blood			
	0.0		0.0		1.4		1.5	
Before treatment with H ₂ S								
After treatment with H ₂ S and displacement with nitrogen:								
Brown staining of dry PbAc ₂ paper after 5 min.		0.9	0.7			2.5	1.9	
Brown staining of dry PbAc ₂ paper after 15 min.		0.0	0.0			1.4	1.5	

Taking the above points into consideration it is concluded that the very low capacity for reducing indophenol by trichloroacetic acid filtrates of plasmata, erythrocytes and whole blood treated with hydrogen sulphide cannot be due to the presence of quantities of ascorbic acid capable of detection by this method.

(c) *The possible precursors of the indophenol-reducing substances formed on treatment of whole plasma with hydrogen sulphide.* This problem has not been studied in detail as it was considered to be too far removed from the aim of the general scope of the work which prompted the present research. It may, nevertheless, be of interest to describe here experiments which, although not complete in themselves, are likely to shed some light on the subject. It was found that when whole plasma was precipitated with absolute alcohol and the alcoholic filtrate treated with hydrogen sulphide according to the above technique it acquired a much higher reducing capacity for indophenol than the original plasma. On the other hand, after further precipitation with cadmium chloride the alcoholic filtrate did not acquire this property at all.

15 ml. of absolute alcohol were gradually added to 5 ml. of plasma from guinea-pigs on a mixed diet. After removing the precipitate by centrifuging, the supernatant liquid was divided into two equal parts. One was reduced with hydrogen sulphide without further treatment, the other was precipitated with alcoholic cadmium chloride (0.1 ml. 2.5% CdCl₂ per 1 ml. of solution). After standing for 15 min. the precipitate was removed on the centrifuge and the

Table V.

Alcohol filtrate before precipitation with CdCl ₂ , mg. ascorbic acid equivalent per 100 ml. plasma		Alcohol filtrate after precipitation with CdCl ₂ , mg. ascorbic acid equivalent per 100 ml. plasma	
Before treatment with H ₂ S	After treatment with H ₂ S	Before treatment with H ₂ S	After treatment with H ₂ S
0.0	87.5	0.0	0.0
0.0	122.8	0.0	0.0
0.0	70.0	0.0	0.0

resulting supernatant alcoholic solution was also submitted to hydrogen sulphide treatment. In Table V the reducing values obtained in these experiments are expressed for convenience only as ascorbic acid, which does not, however, imply that the reduction was due to ascorbic acid. All the evidence is conclusively against this assumption.

Spectrographic examination of plasma before and after treatment with hydrogen sulphide.

An attempt was next made to ascertain spectrographically whether ascorbic acid was present in plasma reduced with hydrogen sulphide. For this purpose both non-reducing normal samples of plasma and reduced samples of plasma each alone and also with the addition of authentic ascorbic acid (20 mg. and 2 mg. of ascorbic acid per 100 ml. of plasma respectively) were examined.

The samples free from haemoglobin were obtained from the blood by the procedure described above. The non-reducing normal and reduced plasmata were then precipitated with freshly distilled absolute alcohol which was added slowly with constant stirring until a final concentration of 80 % alcohol was reached. The corresponding preparations containing ascorbic acid were obtained by the addition of (a) 2 ml. of 98 % alcohol containing 0.4 mg. ascorbic acid, (b) 2 ml. of 98 % alcohol containing 0.04 mg. ascorbic acid respectively, to 2 ml. plasma, before precipitating the proteins with alcohol. The precipitates were removed on the centrifuge. 5 ml. of each centrifugate were diluted with 4 ml. of freshly distilled absolute alcohol and acidified with 1 ml. of 0.2 *N* HCl, thus maintaining the concentration of alcohol at 80 % in the final solutions. These solutions were compared in the spectrograph with blanks containing 0.02 *N* HCl in 80 % alcohol. The solutions prepared from plasma containing 20 mg. ascorbic acid per 100 ml. were examined in 1 cm. absorption tubes, whilst in the case of the more dilute solutions 4 cm. tubes were found to be more convenient. A Hilger quartz spectrograph with sector photometer and Ilford Q 3 plates were employed. The source of light consisted of a condensed oscillatory discharge between electrodes composed of a mixture of cadmium and iron. These electrodes gave more intense spectral lines in the lower region of the spectrum than the normal tungsten steel.

Figs. 1 and 2 are self explanatory. There is no suggestion of any significant difference in the absorption in the region of $245 m\mu$ between plasma treated with hydrogen sulphide and the normal non-reducing plasma. On the other hand, on the addition of 20 mg. of ascorbic acid to 100 ml. of the plasma, a concentration well below the calculated average obtained from the indophenol-reducing figures of all the samples of the treated whole plasma, well-marked characteristic peaks at $245 m\mu$ were obtained in both the reduced and non-reduced media. Furthermore, definite absorption in this region was established with a concentration as low as 2 mg. of ascorbic acid per 100 ml. of plasma.

DISCUSSION.

The entire evidence obtained in this investigation does not lend support to the view that blood of normal guinea-pigs contains demonstrable quantities of dehydroascorbic acid. It is true that it was found that whole plasma free from ascorbic acid could on treatment with hydrogen sulphide acquire the capacity of reducing indophenol to a very marked extent, but this was shown at the same time not to be due to the formation of ascorbic acid. The examination of trichloroacetic acid filtrates of the blood from normal guinea-pigs has revealed that they too can acquire by the same means a similar indophenol-reducing capacity, although to a very much less extent than the whole plasma. The evidence of

Fig. 1 A.

Fig. 1 B.

Fig. 1. A, $\times - \times$ Plasma of mixed diet pig + 2 mg. ascorbic acid per 100 ml.; $\circ - \circ$ plasma of mixed diet pig; $\times - - - \times$ ascorbic acid curve by subtraction. B, $\times - \times$ Plasma of mixed diet pig treated with H_2S + 2 mg. ascorbic acid per 100 ml.; $\circ - \circ$ plasma of mixed diet pig treated with H_2S ; $\times - - - \times$ ascorbic acid curve by subtraction.

Fig. 2 A.

Fig. 2 B.

Fig. 2. A, $\times - \times$ Plasma of mixed diet pig + 20 mg. ascorbic acid per 100 ml.; $\circ - \circ$ plasma of mixed diet pig; $\times - - - \times$ ascorbic acid curve by subtraction. B, $\times - \times$ Plasma of mixed diet pig, treated with H_2S + 20 mg. ascorbic acid per 100 ml. $\circ - \circ$ plasma of mixed diet pig treated with H_2S ; $\times - - - \times$ ascorbic acid curve by subtraction.

this paper shows, however, that this acquired reducing capacity is due essentially to an artefact and not, as is assumed by some workers, to the reduction of dehydroascorbic acid.

The statement of Van Eekelen *et al.* that vitamin C added to blood *in vitro* quickly passes into the reversible form must now be considered. These workers do not give any experimental details, but their observation can be readily explained on our established facts. We have already shown [Kellie and Zilva, 1935] that in the presence of laked erythrocytes ascorbic acid was in the first place oxidised, most probably by oxyhaemoglobin, to an extent which was proportional to the amount of haemolysed cells added. After this initial reaction the disorganised corpuscles exercised a protective action against the oxidation of further amounts of ascorbic acid. We, therefore, assume that the formation of dehydroascorbic acid observed by Van Eekelen *et al.* was due most probably to a partial laking of the blood employed in their experiments. It is essential to note that at the same time we found that intact erythrocytes did not manifest this initial oxidising property, but were, on the contrary, capable of preventing oxidation. The present investigation throws further light on the matter. The red corpuscles of the blood of guinea-pigs on a mixed diet with cabbage *ad lib.* do not contain any dehydroascorbic acid nor in fact do any of the other constituents of the blood. In view of these observations it is very difficult to reconcile the results of our experiments with the assumption that blood in which the erythrocytes are intact can dehydrogenate and store ascorbic acid in quantities capable of detection by our present methods.

SUMMARY.

Whole plasma of guinea-pigs subsisting on a diet containing cabbage *ad lib.*, of guinea-pigs injected intravenously with high doses of ascorbic acid about an hour before bleeding and of scorbutic guinea-pigs acquires the property of reducing indophenol after treatment with hydrogen sulphide to about the same extent. Calculated in terms of ascorbic acid, these would contain about 38 mg. of the vitamin per 100 ml. of plasma. It is shown, however, that this acquired reducing capacity is not due to ascorbic acid.

Trichloroacetic acid extracts of whole blood, plasma and erythrocytes also reduce indophenol after treatment with hydrogen sulphide to a very much smaller extent, *i.e.* to an equivalent of 1–3 mg. per 100 ml. of blood. In this case also the acquired reducing capacity is not due to ascorbic acid but is shown to be an artefact. Unlike the reduction of the indicator by ascorbic acid of similar concentration it is influenced by the thoroughness of the displacement by nitrogen of the hydrogen sulphide and other volatile substances capable of staining dry lead acetate paper and by the time taken to decolorise the indicator (dimethylaminobenzoinidophenol) by the solutions within 10–30 sec.

This evidence lends further support to the authors' assertion that blood with its erythrocytes intact is incapable of dehydrogenating ascorbic acid *in vivo* and storing it in quantities capable of being detected by the existing methods and that there is a tendency of the animal organism to keep the vitamin in a reduced stage.

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