The Effect of Cortisone and Adrenocorticotrophic Hormone on the Dehydroascorbic Acid of Human Plasma

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It is well known that ascorbic acid in aqueous solution is easily and reversibly oxidized to dehydroascorbic acid, and it is believed that above pH 4 the latter can undergo irreversible change, possibly to diketogulonic acid, as a preliminary to further oxidation. The possibility of the existence of dehydroascorbic acid or diketogulonic acid in blood plasma, however, has been generally ignored since the time of Borsook, Davenport, Jeffreys & Warner (1937) who, with van Eekelen (1935), Kellie & Zilva (1936), Farmer & Abt (1936) and Ralli & Sherry (1941), claimed that vitamin C exists in plasma in the reduced state as ascorbic acid. The brief statement that dehydroascorbic acid exists in human blood has recently been made by Chen & Schuck (1950) but no supporting evidence was given. Apart from its academic interest, the relation between dehydroascorbic acid and experimental diabetes (Patterson, 1950) and the close association of ascorbic acid with the adrenal cortex and stress (Selye, 1946) suggest that the form in which vitamin C exists in blood is worthy of investigation. The experiments described in this paper establish the facts that a significant part of the total vitamin C present in normal human plasma exists as dehydroascorbic acid, that diketogulonic acid does not exist in plasma in significant quantities, and that the ratio between dehydroascorbic acid and ascorbic acid can be consistently influenced by adrenocortical steroids.

EXPERIMENTAL AND RESULTS

The existence of dehydroascorbic acid in human plasma

Chemical methods. Plasma from normal human subjects was submitted to the technique of Roe & Keuther (1943) and to a procedure employing 2,6-dichlorophenolindophenol. The former involves preliminary oxidation of ascorbic acid with activated charcoal and the colorimetric estimation of the reaction product with 2,4-dinitrophenylhydrazine under defined conditions. It is claimed to estimate the dehydroascorbic acid produced by oxidation of ascorbic acid plus any preformed dehydroascorbic acid and diketogulonic acid (i.e. 'total ascorbic acid'). The procedure involving 2,6-dichlorophenolindophenol was applied to a sample of the same plasma in order to estimate ascorbic acid itself. For this purpose 3.0 ml. of 3% (w/v) metaphosphoric acid were

added to 2.0 ml. of plasma, and the precipitated proteins separated by centrifugation and filtration. A known excess of standard solution of the indophenol was added to a sample of the filtrate, and the residual colour was measured in a spectrophotometer (Unicam SP 500) at a wavelength of $480\text{ m}\mu$, 30 sec. from the time of addition of the indophenol. The method is similar in principle to that of Mindlin & Butler (1937-8), except that it was found possible to dispense with the buffer, and greater accuracy was obtained by constructing a standard curve instead of using a single standard solution.

The validity of the procedure was first tested by measuring the rate of fading of the indophenol solution under varying conditions, and the results are shown in Fig. 1. The indophenol was decolorized rather slowly in the presence of aqueous metaphosphoric acid (curve *A*) and more rapidly in the presence of trichloroacetic acid (curve *B*) at the same pH. Ascorbic acid added to the metaphosphoric acid solution (curve *C*) completely stabilized the indophenol colour after the initial reduction due to oxidation of the ascorbic acid. Unfortunately, it was impossible to obtain readings earlier than 20 sec. after the addition of the indophenol, but the reduction of the dye by ascorbic acid is certainly completed within 30 sec. When the indophenol was added to a metaphosphoric acid solution containing a trace of H_2S (as an example of a substance oxidized slowly by the indophenol) fading continued to occur after 30 sec. and for as long as determinations were made (curve *D*). A similar slow reduction of the dye occurred in the presence of metaphosphoric acid and $\text{Na}_2\text{S}_2\text{O}_3$ (curve *E*) in a concentration comparable with that in plasma; the curve, however, can be superimposed on that for metaphosphoric acid alone, and it can be concluded that thiosulphate had no appreciable effect. It is of interest to note also that stabilization of the indophenol colour did not occur when ascorbic acid was added to trichloroacetic acid (curve *F*). Similar results were obtained when the indophenol was added to metaphosphoric acid filtrates of plasma. With normal plasma the reaction with the dye was complete in 30 sec. (curve *G*) and this also occurred when the plasma was treated with H_2S which was subsequently removed by N_2 (curve *H*). This curve shows that the H_2S , used in this way, does not interfere with the determination of ascorbic acid, although if the removal of H_2S is incomplete (curve *J*) stabilization of colour is delayed and estimation of ascorbic acid is impossible. The general conclusion is that with spectrophotometer readings taken 30-60 sec. after addition of the dye, with metaphosphoric acid but not trichloroacetic acid as the protein precipitant and with no residual H_2S , the readings represent ascorbic acid concentrations, and that the presence of interfering substances is indicated by a steady change of spectrophotometer readings during the chosen period.

To test the reproducibility of results, twenty determinations were made on a solution of pure ascorbic acid; the mean result was 1.81 ± 0.025 mg./100 ml., the range being 1.77–1.83; similarly, a sample of plasma with added ascorbic acid gave twenty results between 2.09 and 2.13 mg./100 ml., with a mean of 2.11 and s.d. of 0.019. At a lower ascorbic acid concentration the figures for twenty replicate determinations were: mean, 0.43 mg./100 ml.; s.d., 0.023; range, 0.40–0.48.

became 0.82 ± 0.06 (Table 3, column 6), the absolute differences remaining about the same.

There are several possible explanations of this phenomenon other than the obvious one that the difference represents dehydroascorbic acid and/or diketogulonic acid existing in the circulating plasma. Oxidation of ascorbic acid to dehydroascorbic acid and subsequent change to diketogulonic acid may

Fig. 1. The effect of time on the transmission at 480 m μ . of 1.0 ml. of the indophenol solution (4 mg./100 ml.) in the presence of 2.0 ml. of various reagents. A, metaphosphoric acid 3% (w/v); B, trichloroacetic acid 5% (w/v); C, HPO₃ 3% (w/v) plus ascorbic acid, 1 mg./100 ml.; D, HPO₃ 3% (w/v) + H₂S; E, HPO₃ 3% (w/v) + Na₂S₂O₃, 8 mg./100 ml.; F, trichloroacetic acid 5% (w/v) + ascorbic acid, 1 mg./100 ml.; G, 2 ml. plasma + 3 ml. HPO₃, 3% (w/v); H, as G, after treatment with H₂S and removal of excess H₂S by N₂; J, as H, but with H₂S incompletely removed.

The results of recovery experiments when pure ascorbic acid was added to plasma are shown in Table 1. Recoveries were always within 0.05 mg. ascorbic acid/100 ml.

Table 1. Recovery of ascorbic acid added to human plasma using the indophenol method

Ascorbic acid (mg./100 ml. plasma)			
Added	Found	Calculated	Difference
—	0.15	—	—
0.25	0.37	0.4	-0.03
0.5	0.64	0.65	-0.01
1.0	1.17	1.15	+0.02
1.5	1.70	1.65	+0.05
2.0	2.20	2.15	+0.05

Invariably the results obtained by the method of Roe & Keuther (1943) were higher than those by the method using the indophenol, although both methods were standardized against the same original solution of pure ascorbic acid. A similar difference was found in the plasma from subjects who had been given a diet rich in vitamin C for a few weeks. These results are shown in columns 4 and 5 of Tables 2 and 3. In the normal fasting subjects on an ordinary diet, the mean ratio of plasma ascorbic acid determined by the indophenol method to the 'total ascorbic acid' determined by the method of Roe & Keuther (1943) was 0.68 ± 0.15 (Table 2, column 6). After a period of diet rich in vitamin C, the ratio

occur spontaneously after withdrawal of blood or during the manipulation involved in the indophenol method prior to the estimation. Alternatively, interfering substances may be reacting with 2:4-dinitrophenylhydrazine in the method of Roe & Keuther (1943), giving a falsely high figure for the concentration of total ascorbic acid. Indeed, both possibilities may be involved.

In regard to the first of these suggestions, the stability of ascorbic acid was investigated in blood, plasma and plasma filtrate. For this purpose, venous blood was withdrawn into an oiled syringe; part of the sample was allowed to stand at room temperature with occasional mixing, the remainder being used for the immediate determination of plasma total ascorbic acid and of ascorbic acid by the method of Roe & Keuther (1943), and by the indophenol method respectively. After about 60 and 120 min., further determinations were made by both methods. Similar experiments were carried out on plasma separated immediately after the withdrawal of blood. A third series was done on plasma filtrate prepared as quickly as possible after the withdrawal of the blood. The earliest determination possible in any of these experiments was about 30 min. after withdrawal. Typical results of these experiments are shown in Fig. 2, where it is seen that from the time of the first determination there is a very slow fall in the apparent ascorbic acid con-

Table 2. *Concentration of vitamin C in the plasma of normal fasting subjects taking an ordinary diet*

Subject no. (1)	Sex (2)	Age (yr.) (3)	Indophenol method. Ascorbic acid (mg./100 ml.) (4)	Method of Roe & Keuther. 'Total ascorbic acid' (mg./100 ml.) (5)	Ratio: Indophenol method Roe & Keuther method (6)	Ratio: H ₂ S-indophenol method Roe & Keuther method (7)
1	M.	28	0.50	0.75	0.67	—
2	M.	24	0.40	0.60	0.67	—
3	M.	30	0.78	0.85	0.91	1.05
4	F.	21	0.30	0.55	0.55	1.04
5	M.	23	0.14	0.40	0.35	0.98
6	F.	28	0.35	0.50	0.70	1.04
7	F.	35	1.32	1.56	0.84	1.00
8	F.	21	0.32	0.50	0.64	1.00
9	F.	21	1.0	1.30	0.77	1.00
10	F.	23	0.50	0.70	0.71	—
Mean	—	—	—	—	0.68	1.01
S.D.	—	—	—	—	±0.15	±0.03

Table 3. *Concentration of vitamin C in the plasma of normal fasting subjects taking a diet rich in vitamin C*

Subject no. (1)	Sex (2)	Age (yr.) (3)	Indophenol method. Ascorbic acid (mg./100 ml.) (4)	Method of Roe & Keuther. 'Total ascorbic acid' (mg./100 ml.) (5)	Ratio: Indophenol method Roe & Keuther method (6)	Ratio: H ₂ S-indophenol method Roe & Keuther method (7)
1	F.	28	0.84	1.05	0.76	1.00
2	M.	23	0.44	0.60	0.73	0.96
3	M.	30	1.07	1.25	0.86	1.00
4	F.	25	0.94	1.10	0.85	1.06
5	F.	20	1.56	1.90	0.81	1.05
6	F.	21	0.94	1.10	0.85	1.00
7	M.	24	1.97	2.22	0.88	0.99
Mean	—	—	—	—	0.82	1.01
S.D.	—	—	—	—	±0.06	±0.03

centration in blood, blood plasma, and plasma filtrate under these conditions. The rate of loss of ascorbic acid is slightly greater when the plasma is left in contact with red cells, than when it has been separated. The total ascorbic acid does not alter during the period of these experiments or, indeed, for several hours afterwards.

If the decay curve before the time of the first determination is the same as it is afterwards, extrapolation to zero time should provide an estimate of ascorbic acid at that time. It is evident from Fig. 2 that the plasma concentrations of ascorbic acid at zero time obtained by such extrapolation are very little different from the concentrations 30 min. later and are still lower than those of total ascorbic acid by the method of Roe & Keuther (1943). The validity of the assumption that the rate of loss of ascorbic acid between the withdrawal time ($t=0$) and the earliest determination ($t=30$ min.) follows the same simple decay curve as it does subsequently is established by the recovery of ascorbic acid and dehydroascorbic acid added to blood plasma or plasma filtrate. Table 1 shows that such recoveries are complete. It is concluded from

these experiments that the differences between the plasma concentrations of total ascorbic acid as determined by the method of Roe & Keuther (1943) and those determined by the indophenol method are not due to the oxidation of ascorbic acid following the withdrawal of blood and during the indophenol procedure.

In regard to the second possibility, Roe & Keuther (1943) have satisfactorily excluded many potential interfering substances, either because the colour with 2:4-dinitrophenylhydrazine does not develop under the strongly acid conditions of the estimation, or because they (e.g. glucose) are not present in plasma in sufficient concentrations to interfere significantly with the results.

The nature of the substance or substances responsible for the difference obtained by the two methods was investigated by means of hydrogen sulphide.

Pure H₂S, prepared from Sb₂S₃, was passed for 10 min. through samples of filtrate prepared with metaphosphoric acid from plasma in which determinations by the two methods had already been carried out (i.e. subjects shown in Tables 2 and 3). Excess of H₂S was then removed by blowing

pure N_2 saturated with water vapour through the solution for 2-3 hr., by which time the nitroprusside and lead acetate reactions were negative.

The indophenol method was again applied (the stability of the colour showing absence of interference by hydrogen sulphide), and the ratios of these determinations to the original determinations by the method of Roe & Keuther (1943) are given in column 7 of Tables 2 and 3. In every case, the indophenol-reducing substance has increased to the value given by the Roe & Keuther (1943) method for total ascorbic acid, so that the mean ratio rises from 0.68 or 0.82 to 1.0 ± 0.03 irrespective of the original value. Control experiments showed that when a trichloroacetic acid filtrate of plasma, as used for the Roe & Keuther (1943) method, was treated with hydrogen sulphide and nitrogen prior to oxidation with charcoal, the result did not differ from that obtained by the usual procedure.

Fig. 2. The effect of time on the apparent ascorbic acid content of plasma. ○—○, plasma separated immediately after blood withdrawal, samples used for ascorbic acid determination by the indophenol method. (Plasma separated and immediately treated with metaphosphoric acid gave results indistinguishable from these.) ▲—▲, plasma allowed to stand in contact with red cells until required (indophenol method). ×—×, plasma separated immediately after blood withdrawal; 'total ascorbic acid' determined by Roe & Keuther method.

Thus the substance in plasma filtrate, which is responsible for the difference between the ascorbic acid content measured by the indophenol method and the 'total ascorbic acid' measured by the 2:4-dinitrophenylhydrazine method of Roe & Keuther (1943), (a) reacts with dinitrophenylhydrazine but not with the indophenol, (b) is reducible by hydrogen sulphide to a substance which reacts with the indophenol as rapidly as does ascorbic acid, and (c) when calculated as ascorbic acid, accounts precisely for the observed difference. Taking into account the specificities of the two reactions used in the analyses,

it is difficult to imagine such behaviour being due to anything except dehydroascorbic acid. Diketogulonic acid also reacts with dinitrophenylhydrazine under the conditions of Roe & Keuther (1943), but may be ruled out since it is not reduced by hydrogen sulphide in acid solution. It may be concluded, with a high degree of probability, that the observed differences are due to the presence of dehydroascorbic acid in the filtrates prepared from plasma by addition of metaphosphoric acid. The extension of this conclusion to native plasma rests partly on the time relationships discussed earlier and partly on the well established fact that in weakly acid solution at room temperature equilibrium between dehydroascorbic acid and diketogulonic acid (with the latter forming about 80% of the equilibrium mixture) is reached only in about 70 hr. Hence if diketogulonic acid were originally present in the plasma it should still be detectable in the acidified plasma filtrate 30 min. later (Herbert, Hirst, Percival, Reynolds & Smith, 1933). The distinction may be of some importance, since at the slightly alkaline reaction of plasma some conversion of dehydroascorbic acid to diketogulonic acid might be expected. The results of the experiments just described suggest, however, that plasma itself contains dehydroascorbic acid but no appreciable amount of diketogulonic acid.

Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following intramuscular administration of adrenocorticotrophic hormone (ACTH)

The known relationship of vitamin C to the adrenal cortex suggested that an investigation of the ratio of dehydroascorbic acid to total ascorbic acid in plasma following administration of ACTH and compound E would be of interest. Further, a consistent alteration in the ratio brought about by these means would strengthen the conclusion that dehydroascorbic acid exists in plasma.

ACTH ('Acthar', Armour Laboratories, Armour and Co., Chicago) was given by intramuscular injection in doses of 100 mg. to four normal fasting and resting subjects. Prior to the injection one or two blood samples were withdrawn. Following the injection, samples of blood were withdrawn at hourly intervals for 4-7 hr., and during this period the subjects were allowed to drink weak tea or coffee which was shown to have no effect upon the concentration of ascorbic acid or dehydroascorbic acid. The same subjects were used as controls about 1 week later, at which time samples were taken at hourly intervals for 4-6 hr. for analysis without hormone administration but otherwise under the same conditions. The activity of the ACTH used was confirmed in all cases by eosinopenic responses of at least 50% within 2-5 hr. after the injection. In addition, H_2S was passed through a sample of the metaphosphoric acid filtrate for 10 min., after which excess H_2S was removed by N_2 and a further estimation of the indophenol-reducing power was made.

Table 4. *Changes in the concentration of plasma ascorbic acid and dehydroascorbic acid following 100 mg. ACTH given by intramuscular injection*

Subject no.	Plasma ascorbic acid		Plasma dehydroascorbic acid		Plasma 'total ascorbic acid'	
	(Δ mg./100 ml.)	(Δ %)	(Δ mg./100 ml.)	(Δ %)	(Δ mg./100 ml.)	(Δ %)
1 B.S.	+0.42	+42	-0.17	-56	+0.25	+19
2 J.R.	+0.25	+37	-0.13	-65	+0.15	+13
3 M.K.	+0.25	+76	-0.18	-100	+0.05	+10
4 E.B.	+0.23	+13	0*	0	+0.25	+14
Mean	—	+42	—	-74	—	+14

* No fasting dehydroascorbic acid present.

Table 5. *The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of ACTH*

Treatment of subject	Ratio:	Ratio:	Ratio:	Ratio:
	Indophenol method Roe & Keuther method	H ₂ S-indophenol method Roe & Keuther method	Indophenol method Roe & Keuther method	H ₂ S-indophenol method Roe & Keuther method
Fasting	0.76	1.0	0.65	1.0
ACTH 1 hr. previously	0.86	0.98	1.0	1.0
ACTH 2 hr. previously	0.86	1.01	1.01	1.01
ACTH 3 hr. previously	0.87	0.95	0.95	0.96
ACTH 4 hr. previously	0.92	0.96	0.85	0.97
ACTH 5 hr. previously	0.98	0.99	—	—
Mean	—	0.98	—	0.99

The results are summarized in Table 4 and one case is shown graphically in Fig. 3. Following the administration of ACTH, a rise in total ascorbic acid occurred in all four subjects as determined by the method of Roe & Keuther (1943). The rise started

Fig. 3. The ascorbic acid (black columns) and dehydroascorbic acid (white columns) of human plasma, A, after intramuscular injection of 100 mg. ACTH (time measured from the time of ACTH injection), and B, in successive hours without ACTH administration.

within 1 hr. of the injection and reached a maximum in 2-4 hr., corresponding approximately in time to the maximum eosinopenia. The mean percentage change in total ascorbic acid concentration (ascorbic acid + dehydroascorbic acid) was +14, absolute

elevations varying from 0.05 to 0.25 mg./100 ml. plasma. Ascorbic acid itself, as determined by indophenol reduction, also showed increases of from 13 to 76 % of the fasting level, and these increases were maximal at the time corresponding to the peak of total ascorbic acid concentration. The absolute increments in ascorbic acid concentration varied from 0.25 to 0.41 mg./100 ml. plasma. The larger increments in ascorbic acid as compared with total ascorbic acid (ascorbic acid + dehydroascorbic acid) resulted in a diminution in the absolute concentration of dehydroascorbic acid amounting to 0.13-0.18 mg./100 ml. in three subjects (56-100 %). In the fourth subject no fasting dehydroascorbic acid existed prior to the injection and none appeared following it. The control experiments in which no ACTH was given showed only very slight irregular alterations in the plasma concentration of total and reduced ascorbic acid. The results obtained following the passage of hydrogen sulphide through the metaphosphoric acid plasma filtrate are shown in Table 5, which gives the ratios of ascorbic acid to total vitamin C before and after injection of ACTH for subjects B. S. and M. K. The disappearance of dehydroascorbic acid in these subjects is shown by the rise in the ratio from 0.76 to 0.98 and from 0.65 to 1.0 respectively; the reappearance of dehydroascorbic acid in M. K. is evidenced by the gradual fall of the ratio to 0.85, 4 hr. after the injection. Hydrogen sulphide had the effect of making the ratio very nearly 1.0 whatever its value in the original plasma. It is important to note that when the original ratio is 1.0, hydrogen sulphide does not increase it.

Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following oral administration of cortisone acetate

The changes in plasma ascorbic acid and dehydroascorbic acid following administration of ACTH are not easily interpreted. ACTH is known to diminish the concentration of ascorbic acid in the adrenal gland (e.g. Sayers, Sayers, Liang & Long, 1946). This might explain the increase in the plasma total ascorbic acid, but does not by itself account for the decrease in dehydroascorbic acid. In addition, however, the increased secretion of adrenocortical hormones, acting peripherally, might produce effects upon vitamin C metabolism capable of accounting for the latter phenomenon. In order to dissociate such central from peripheral effects, the changes in blood concentration following the oral administration of 350 mg. of cortisone acetate were investigated in five normal subjects, one of whom (J.R.) had already been examined following ACTH administration.

As before, the subjects were fasting and at rest, though weak tea or coffee was allowed. One or two samples of blood were withdrawn prior to giving the cortisone, after which samples were taken into oiled syringes at 30 min., 1 hr., and then at hourly intervals for 4–6 hr. The chemical determinations were made as before, and eosinophils were counted.

The results are shown graphically for two subjects in Fig. 4 and in Table 6 they are summarized for the five subjects in terms of the maximum changes occurring in the plasma concentration of ascorbic acid and dehydroascorbic acids. These occurred between 0.5 and 2 hr. after administration of cortisone and

corresponded in time to the maximal eosinopenia (i.e. 50 %). The mean increase in plasma ascorbic acid was 38 %, corresponding to an absolute rise in concentration ranging from 0.1 to 0.34 mg./100 ml. of plasma. There was no rise in the concentration of total vitamin C in any of the subjects and the

Fig. 4. The effect on the ascorbic acid (black columns) and dehydroascorbic acid (white columns) of human plasma of oral administration of 350 mg. of cortisone acetate given immediately after withdrawal of the control blood sample. Two cases.

maximum rise in concentration of ascorbic acid exactly equalled the original concentration of dehydroascorbic acid, so that all the dehydroascorbic acid in the plasma temporarily disappeared. The ratio of ascorbic acid to total vitamin C for subjects J.R. and M.McI. at various times following administration of the cortisone is given in Table 7,

Table 6. *Changes in concentration of plasma ascorbic acid and dehydroascorbic acid following 350 mg. cortisone given orally*

Subject no.	Plasma ascorbic acid		Plasma dehydroascorbic acid		Plasma 'total ascorbic acid'	
	(Δ mg./100 ml.)	(Δ %)	(Δ mg./100 ml.)	(Δ %)	(Δ mg./100 ml.)	(Δ %)
1 H.L.	+0.34	+44.0	—	—	—	—
2 J.R.	+0.20	+36.0	−0.19	−95	+0	0
3 M.McI.	+0.10	+22.0	−0.10	−100	+0	0
4 H.A.	+0.17	+33.0	−0.17	−100	+0	0
5 R.C.	+0.10	+55.0	−0.10	−100	+0	0
Mean	—	+38.0	—	−99	—	0

Table 7. *The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of 350 mg. cortisone given orally*

Treatment of subject	Subject J.R., 30 yr., M.		Subject M.McI., 45 yr., F.	
	Ratio: Indophenol method	Ratio: H ₂ S-Indophenol method	Ratio: Indophenol method	Ratio: H ₂ S-Indophenol method
	Roe & Keuther method	Roe & Keuther method	Roe & Keuther method	Roe & Keuther method
Fasting	0.74	1.00	0.82	1.02
Cortisone, 1 hr. previously	1.00	0.99	1.00	0.98
Cortisone, 2 hr. previously	0.83	1.0	0.95	—
Cortisone, 3 hr. previously	0.87	1.01	0.94	1.04
Cortisone, 4 hr. previously	0.74	0.98	0.92	1.02
Mean	—	1.0	—	1.02

which also shows the effect of hydrogen sulphide upon this ratio. Without hydrogen sulphide treatment the ratio rises to 1.0 about 1 hr. after administration of cortisone (i.e. dehydroascorbic acid has disappeared) and subsequently falls as plasma dehydroascorbic acid reappears. Treatment with hydrogen sulphide gives a ratio approximately 1.0 (but never significantly greater), irrespective of the original value, so that the changes are legitimately interpreted as indicating disappearance of dehydroascorbic acid.

DISCUSSION

The possibility of the existence of significant concentrations of dehydroascorbic acid in blood or tissues has received little attention in the past. The bare statement that both dehydroascorbic acid and diketogulonic acid occur in plasma and urine was made by Chen & Schuck (1950). Nyden (1948) studied alterations in ascorbic and dehydroascorbic acids in rat tissues and plasma following their injection with *Trypanosoma hippicum*. She used the method of Roe & Oesterling (1944), devised originally for the estimation of dehydroascorbic acid in plants, to estimate tissue and plasma dehydroascorbic acid concentrations. In our hands this method proved unsuitable for the determination of plasma dehydroascorbic acid, since reduction of dehydroascorbic acid occurred in the presence of all samples of thiourea (1%, w/v) available to us, and oxidation occurred when thiourea was omitted. The concentration of thiourea required to maintain the naturally occurring ratio between the reduced and oxidized forms of the vitamin was unpredictable. The almost complete absence in the literature of a simple comparative study of the method of Roe & Keuther (1943) and one estimating the vitamin in the reduced form (e.g. by indophenol or methylene blue) is surprising. Lowry, Lopez & Bessey (1945) compared the micro-indophenol method of Mindlin & Butler (1937-8) with their own micro-adaptation of the method of Roe & Keuther (1943), using 0.01 ml. of serum but without making any direct comparison with the micromethod of Roe & Keuther (1943). They found no significant difference between the results of the two techniques when applied to serum, but it is questionable whether their micromodification was sensitive enough to detect the small differences reported in this paper. In addition, it has been established by Friedman, Rumin & Kees (1938), Farmer & Abt (1938) and by Cushman & Butler (1938) that, as we also have found, potassium cyanide employed in the method of Mindlin & Butler (1937-8) invalidates the results; this compound was presumably used by Lowry *et al.* (1945). As long ago as 1935, Martini, Bon-signine & Pinotte claimed that dehydroascorbic acid existed in significant concentration in guinea

pig tissues, but the use of trichloroacetic acid in tissue extraction may invalidate these results, at least partly, since trichloroacetic acid itself appears to decolorize indophenol.

The source of the increment in total plasma vitamin C following intramuscular ACTH is unknown. Vogt (1948) was unable to detect significantly increased amounts of vitamin C in venous blood leaving the adrenal cortex of dogs when the gland was directly stimulated. It can be said, however, that the amount of ascorbic acid reputed to disappear from the adrenal cortex after ACTH administration is sufficient, if added to the extracellular body fluids, to increase the ascorbic acid concentration by the amounts observed.

The temporary diminution or disappearance of plasma dehydroascorbic acid is an effect common both to intramuscular ACTH and oral cortisone. The fact that total plasma vitamin C concentration does not alter significantly following cortisone administration indicates that one of the essential responses to this adrenocortical hormone is an activation of the mechanism for the reduction of dehydroascorbic acid.

Simple *in vitro* experiments in which cortisone and ACTH are incubated with dehydroascorbic acid either in solution or in plasma were carried out in the course of this work but have not been reported in detail in the experimental part. They showed clearly that the observed apparent reduction of dehydroascorbic acid is not a direct chemical effect of the circulating hormone. Borsook *et al.* (1937) have shown that normal human plasma does not possess the power of reducing dehydroascorbic acid. We have confirmed this and shown further that the power of such reduction is not acquired by plasma following ACTH or cortisone administration. The activation is, therefore, likely to occur within the cells either of the blood or of tissues and it is possible that the mechanism involves glutathione. Experiments to test this possibility are now in progress.

SUMMARY

1. A comparison of the method of Roe & Keuther (1943) with a procedure using 2,6-dichlorophenol-indophenol for determination of 'total ascorbic acid' and ascorbic acid respectively provides results which suggest that a significant part of the vitamin C in human plasma is present as dehydroascorbic acid.

2. This possibility is further substantiated by the fact that the differences obtained in the concentrations of plasma vitamin C by the two methods are completely removed by treating plasma filtrate with hydrogen sulphide prior to using the indophenol method, irrespective of the magnitude of the difference which existed before such treatment.

3. Taking into account the specificities of the two methods and the reactions they involve, it is difficult to explain such results except as being due to the presence of dehydroascorbic acid. These procedures do not suggest the presence of diketogulonic acid in human plasma.

4. The administration of adrenocorticotrophic hormone to human subjects resulted in a rise in the concentration of plasma total ascorbic acid and of plasma ascorbic acid with a diminution in the concentration of plasma dehydroascorbic acid 2-4 hr. after intramuscular injection.

5. The oral administration of cortisone to human subjects resulted in no rise in the concentration of plasma total ascorbic acid, but in a rise in ascorbic acid of such a magnitude that dehydroascorbic acid temporarily disappeared from the plasma 0.5-2 hr. after administration.

6. The significance of these alterations is discussed both with respect to the origin of the increment of total ascorbic acid appearing in the plasma and to the mechanisms involved in the reduction of dehydroascorbic acid to ascorbic acid.

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Note added in proof. In a few experiments it has now been found that, following the administration of cortisone or ACTH and coincident with the resulting diminution of the plasma dehydroascorbic acid concentration, there is a decrease in the reduced glutathione concentration of the whole blood. When, later, the original dehydroascorbic acid concentration is restored, the reduced glutathione of the blood also increases. The sum of the reduced and oxidized glutathione remains constant throughout.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors: Further Studies with *Corynebacterium* 'Q'

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Previous work (Pollock, Howard & Boughton, 1949) on an oleic acid-requiring diphtheroid bacterium (*Corynebacterium* 'Q') showed that a number of related unsaturated fatty acids from natural sources (elaidic, linoleic, linolenic, petroselinic and palmitoleic acids) would support growth as well as oleic acid, although all saturated fatty acids tested were quite inactive. During the last few years there have been a number of reports along similar lines on species of *Lactobacillus* many of which require unsaturated fatty acids related to oleic acid for growth in the absence of biotin (Williams, Broquist & Snell, 1947; Axelrod, Hofmann & Daubert, 1947; Hassinen, Durbin & Bernhart, 1950; Cheng,

Greenberg, Deuel & Melnick, 1951), although there is some variation in the extent of replaceability of oleate by analogues. Thoma & Peterson (1950) have shown that unsaturated fatty acids are similarly necessary for the growth of *Clostridium sporogenes* without biotin. Long-chain saturated fatty acids have occasionally been known to produce some stimulation of growth in the presence of unsaturated acids, but this is more likely to be simply a protective effect against toxic properties of the unsaturated acids. In the presence of an adequate source of carbon, long-chain saturated fatty acids have not so far been found essential for the growth of and known strains of micro-organisms.