The Ascorbic Acid Content of Human Blood

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Doctor of Philosophy

by

Betty J. Iggo, M.H.Sc. (N.Z.)

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CONTENTS

SECTION I.

The estimation of vitamin C

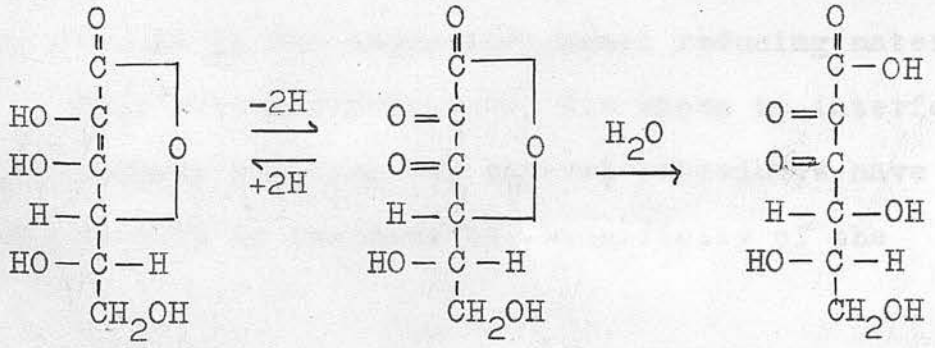
The following abbreviations have been used throughout this thesis :-

AA	=	L-ascorbic acid
DHA	=	dehydro-L-ascorbic acid
DKG	=	2:3 -diketo-L-gulonic acid
total AA	=	ascorbic acid + dehydroascorbic acid + diketogulonic acid

INTRODUCTION

The earliest methods for the estimation of vitamin C were biological and depended on either the prevention or curing of scorbutic symptoms in guinea pigs or on the rate of growth of young scorbutic animals. Tillmans in 1927 observed that fresh lemon juice strongly reduced the dye 2:6-dichlorophenol-indophenol. With his associates he later established that this reducing power of certain food-stuffs was related to their anti-scorbutic potency. At about the same time AA was isolated in the crystalline state and since then more precise chemical methods for its estimation have been devised to replace the tedious biological ones. The biological methods continued to be used for some time as a check on the new chemical methods.

The structure and inter-relations of the compounds of interest in the chemical methods for estimating vitamin C are given below.



L-ascorbic acid Dehydro-L-ascorbic acid 2:3-diketo-L-gulonic acid

Because of the configuration at carbon atoms 2 and 3 AA can be oxidised to DHA and it is a strongly reducing substance. This property has formed the basis of many methods for its estimation. Since the oxidation is readily reversible, techniques which use the reduction of DHA have also been developed. The two keto groups formed by the oxidation of AA to DHA or DKG may be coupled with 2:4-dinitrophenylhydrazine to form a bis-2:4-dinitrophenylhydrazone.. This latter property provides the principle underlying another group of methods used for the estimation of AA.

Substances such as indophenol and methylene blue have been used to measure the reducing property of AA; the most satisfactory of the oxidation-reduction methods are those based on the use of 2:6-dichlorophenol indophenol (indophenol). In acid medium indophenol is pink and on reduction it fades to the colourless leuco form. The first methods based on this reaction were visual titration procedures and titration into a known amount of indophenol is still used for the estimation of AA in urine. A necessary assumption when using these methods is that AA is the major indophenol reducing material present. Many substances however, are known to interfere with the indophenol reaction and several procedures have been devised in attempts to increase the specificity of the method.

Spectrophotometry has been a useful tool in increasing the specificity and accuracy of the indophenol methods. A quantity of the solution containing AA which is sufficient

to partially decolourise the indophenol is used. The decrease in colour gives an estimate of the amount of AA present. Three advantages of this procedure over visual titration procedures are (a) the elimination of inaccuracies due to the difficulties in judging the titration end-point, (b) the preparation of a blank sample by complete decolourisation of the original solution, so correcting any turbidity or colour in the original solution and (c) the possibility of detecting the presence of slowly reacting interfering substances. Some of the more notable modifications of this method have been suggested by Evelyn, Malloy & Rosen (1938), Mindlin & Butler (1937-38), Loeffler & Ponting (1942), Hochberg, Melnick & Oser (1943) and Stewart, Horn & Robson (1953).

That some vitamin C may exist as DHA in tissues has been the subject of investigation since 1932. Application of the indophenol technique to tissue extracts after treatment with a reducing agent, usually H_2S, has been used as a measure of the total vitamin C content (AA & DHA). The kinetics of the reduction of DHA to AA by H_2S have been thoroughly investigated by Levenson, Rosen & Hitchings (1951) who showed that the rate of reduction was related to the pH of the solution and that reduction was complete only over the pH range 1.2 to 4.7. Although the excess of H_2S can be removed from metaphosoric acid solutions by N_2, many workers (Johnson, 1933; Mack & Tressler, 1937; King, 1936; Bessey, 1938a) have noted that even after prolonged treatment with N_2, traces of H_2S may still be present in tissue extracts. This fact casts serious doubt on the validity of the H_2S method since H_2S itself

reduces indophenol and furthermore it may reduce or combine with other compounds present in tissues and these materials may later react with the dye. Lugg (1942), Smythe & King (1942) and Hochberg et al. (1943) have shown that this occurs with solutions of aldehydes, ketones and quinones and the presence of increased amounts of interfering substances has been reported after H_2S treatment of whole blood filtrates (Butler & Cushman, 1940) and of urine (Scarborough & Stewart, 1937). Kellie & Zilva (1936) on the basis of ultraviolet absorption spectra analyses suggested that this increase was not due to the formation of AA from DHA in trichloroacetic acid filtrates of guinea pig plasma treated with H_2S.

Certain micro-organisms reduce DHA. Mapson & Ingram (1951) have used the ability of Escherichia coli to convert DHA to AA as the basis of a method to estimate DHA. After reduction, the total indophenol reducing capacity is measured. Such a procedure, however, requires careful manipulation of the pH of the extract and of the temperature of incubation for the method to be quantitative. The method has not found wide use.

Two methods have been proposed in which a serious attempt has been made to remove some of the substances which interfere with the indophenol reaction. In 1934 Emmerie & Van Eekelen suggested the removal of thiol compounds in tissue extracts by precipitation with mercuric acetate. Excess mercuric acetate was removed by treatment with H_2S. Since Hg^{++} tends to oxidise AA to DHA, the H_2S ensured the reduction of DHA to AA. The method has not been widely used

because of the laborious H_2S procedure and the uncertainties about its complete removal from the tissue extracts. The second method was designed to permit the determination of AA in the presence of sulphites, sulphides, thiol compounds and reductones. The method was proposed by Lugg (1942) who discovered that HCHO will condense with these materials and with AA to an extent depending on the pH. Condensation with HCHO destroys the indophenol reducing capacity. At pH 1.5 HCHO condenses very rapidly with sulphite, sulphide and thiol compounds but only very slowly with AA. AA is however unstable with HCHO at pH 3.5. This principle has been extended by Snow & Zilva (1943, 1944) and by Mapson (1943). The latter has used it to determine AA in urine and in certain treated foods in which reductones occur. He used pH 0.6 for condensation of the S-compounds and although he did not find any pH at which HCHO condenses with AA and not with the reductones, at pH 2 AA condensed fairly rapidly and the reductones slowly and at a linear rate. By measuring the reducing capacity at pH 2 at intervals after the addition of HCHO it was possible to construct a reductone reducing curve which on extrapolation to zero gave the indophenol reducing capacity of the reductones. The difference between this value and the value at pH 0.6 was considered to give the AA value. More recently Mapson (1953) has simplified the procedure for determining AA in urine. The difference in indophenol reducing capacity after condensation at pH 0.6 and 3.5 is taken as a measure of the AA. Unfortunately these methods are extremely laborious and the

recovery of AA added to tissue extracts is often low. Since the calculation is based on a difference, it is most unsuitable for estimating low concentrations of AA.

Methods based on the oxidation of AA to DHA and DKG and the formation of the ozazone of DKG with dinitrophenylhydrazine have been developed by Roe and his co-workers. Such methods possess a high degree of specificity and since they involve mild oxidation with charcoal the uncertainties associated with the instability of AA and indophenol are overcome, as is the risk of including interfering materials. The procedure devised by Roe & Kuether (1943) can be used satisfactorily with most biological materials. Roe, Mills, Oesterling & Damron (1948) modified the method so that AA, DHA and DKG could be estimated separately. A micro-method which requires only 0.01 ml. of blood has been developed by Bessey, Lowry & Brock (1947), and Lowry, Lopez & Bessey (1945). In all these methods the coupling reaction is carried out in a mildly reducing medium obtained by the use of thiourea or stannous chloride which is claimed to reduce interference from non-AA chromogens. The ozazone in concentrated H_2SO_4 forms a soluble, stable, red compound which has a maximum absorption at 518mμ.

Schaffert & Kingsley (1955) suggested on the basis of work by Penney & Zilva (1943a,b; 1945) that the incubation time can be reduced to 5 min. if the temperature is raised to 100°C. Separate determination of AA is achieved by omitting the charcoal oxidation stage. Geschwind, Williams & Li (1951) also modified the procedure for the estimation of total AA in adrenals by increasing

the temperature of the coupling reaction. Bolin & Book (1947) suggested a modification so that the method can be used with metaphosphoric acid extracts of tissues instead of trichloroacetic acid extracts. This allows both indophenol and phenylhydrazine procedures to be carried out on the same extract. In place of charcoal, to which some AA may be adsorbed, indophenol is used as the oxidising agent.

The choice of a method to be used for the estimation of AA in biological tissues depends on the kind of information and the degree of accuracy desired. If only the total AA is needed the Roe & Kuether method is probably the most accurate. The selection of a method suitable for the precise estimation of AA is made difficult by the number of compounds which decolourise the dye and which may be present in tissue extracts, e.g., H_2S, thiosulphate, sulphite, thiol compounds, phenols and reductones. Harris & Mapson (1947) using a "continuous flow" method found that most of these substances react more slowly than does AA at pH 3.5 and 20°C. Of the substances examined only hydroxytetronic acid reacted in the same manner as did AA. Several workers have shown that the pH at which the reaction proceeds is critical, and it is generally agreed that pH 3.5 to 4.5 is most satisfactory. The buffers, sodium acetate (Mindlin & Butler, 1937-38) or sodium citrate (Bessey, 1938a,) have been used to ensure this pH range, outside of which interfering materials react to a greater extent. Harris & Mapson (1947) showed that thiosulphate, dihydroxymalic acid and Fe^{++} react slowly at pH 3.5 and much more rapidly at pH 1.4.

On the other hand GSH, cysteine and sulphite react faster at the higher pH. The presence of many of these interfering materials has been demonstrated in biological tissues e.g. kidney (Hopkins & Slater, 1935); brain (Young & Mitolo, 1934); whole blood (Butler & Cushman, 1940); urine (Evelyn et al., 1938 and Scarborough & Stewart, 1937); semen (Mann & Leone, 1953). On the other hand Butler & Cushman (1940) reported that human plasma does not contain substances which react slowly with indophenol.

Investigations on the state in which vitamin C exists in the tissues and particularly in blood and its components have been greatly hampered by the lack of a method specific for DHA and by the unreliability of the indophenol methods. Most early workers considered that plasma vitamin C existed in the reduced state (Borsook, Davenport, Jeffreys & Warren, 1937; Kellie & Zilva, 1936; Farmer & Abt, 1936). In contrast, Van Eekelen, Emmerie, Josephy & Wolff (1933), Plaut & Bülow (1935), and Fujita & Ebihara (1937) reported that it was present entirely as DHA. Van Eekelen later considered that it was indeed in the reduced state. More recently it has been shown that not more than 0.25 mg/100 ml., and more usually between 0.05 and 0.15 mg/100 ml. of DHA is present in plasma (Daubenmerkle, 1949; Lloyd, Sinclair & Webster, 1945; Chen & Schuck, 1950; Davey, Wu & Storvick 1952; Banerjee & Belavady, 1953; Stewart, Horn & Robson, 1953; Linkswiler, 1954). No comparable analyses have been reported for erythrocytes probably because of the lack of specificity of the indophenol methods. Furthermore, acid precipitation of

oxy-haemoglobin is known to cause oxidation of AA (Lemberg, 1942). Total AA values of erythrocytes closely follow the plasma values (Lubschez, 1945).

The amount of DHA reported in plasma is very small and therefore the accuracy of the methods used for its estimation need to be examined critically. Furthermore since its estimation depends on a determination by difference the accuracy decreases alarmingly when the total AA concentration is low.

The purpose of the experiments reported in this section was the critical examination of the indophenol reaction and its suitability for determining the AA content of plasma, erythrocytes, urine, liver and kidneys. In an attempt to improve the specificity of the reaction for AA the use of a well known -SH inhibitor, p-chloromercuribenzoic acid (CMB) has been used. A method by which this material can be applied to extracts of plasma, erythrocytes, urine and tissues is described. A preliminary account of the CMB technique has been published (Owen, Iggo & Horn 1954), and a full account of the method and its applications has been accepted for publication in the Biochemical Journal (Owen & Iggo, 1955, in the press). A comparison of the indophenol and dinitrophenylhydrazine procedures applied to extracts of plasma, erythrocytes and urine is reported.

MATERIALS AND METHODS

General Reagents : The general reagents used for the estimation of vitamin C are listed below. Other reagents used in a few experiments were of AR quality and are mentioned in the appropriate places.

Metaphosphoric acid. (BDH) 20% (w/v) A stock solution was made up at intervals of not longer than four days and stored in a refrigerator. This solution was diluted as required.

2:6-dichlorophenol indophenol. Preparation A, sodium salt (L.Light & Co.) A solution containing 100 mg./100 ml. was made up at weekly intervals and diluted as required.

Preparation B, Tablets (BDH) equivalent to approximately 1 mg. AA. A solution containing 2 tablets/100 ml. was made up daily and filtered. No difference was found between these two preparations.

L-ascorbic acid (Roche).

L-dehydroascorbic acid. Prepared by oxidation of AA by quinone according to the method of Patterson (1950).

Sodium citrate (AR). 30% (w/v). This solution was diluted as required.

Sodium acetate buffer. (pH 4.0). 1.8 vol. of 0.2N sodium acetate solution was added to 8.2 vol. of 0.2N acetic acid.

Formaldehyde (AR). A 40% (w/v) solution.

p-chlormercuribenzoic acid. (CMB). Prepared according to the method of Whitmore & Woodward (1946). 200 mg./100 ml. in 0.05N NaOH.

Trichloroacetic acid (AR) 6% (w/v)

Activated charcoal (BDH). Acid washed.

Thiourea (AR). 10g. dissolved in 100 ml. 50% (v/v) alcohol.

2:4-dinitrophenylhydrazine (AR). 2g. in 100 ml. 9N H_2SO_4. This solution was filtered through Whatman No.42 filter paper and kept at 5°C.

Sulphuric acid (AR). 85% (v/v). Prepared by diluting 900 ml. conc. H_2SO_4 with 100 ml. water.

Preparation of biological extracts.

Plasma: For the indophenol method, protein-free filtrates of human plasma (oxalated or heparinised) were prepared by adding 2 vol. of plasma to three vol. of 3% (w/v) metaphosphoric acid. After 15 min. a clear solution was obtained by filtration through Whatman No 42 paper.

For total ascorbic acid 1 vol. of plasma was added to 3 vol. of 6% (w/v) trichloroacetic acid.

Erythrocytes. Whole blood (oxalated or heparinised) was centrifuged and the white cell layer removed as completely as possible. The erythrocytes were resuspended in the plasma and transferred to a tonometer. A steady stream of CO generated from sodium formate (AR) and conc. H_2SO_4 was passed into the tonometer for 15 to 20 min. Rotation of the tonometer during this time ensured complete saturation of the blood with CO. After centrifugation 2 vol. of erythrocytes were added to 3 vol. of 6% (w/v) metaphosphoric acid for the indophenol method. For total AA estimations 1 ml. of CO saturated erythrocytes was added to 3 vol. of 6% (w/v) trichloroacetic acid.

Urine. Freshly voided human urine, after filtration, was diluted to give an AA concentration of approximately 1 mg./100 ml. To 3 vol. of 3% (w/v) metaphosphoric acid were added 2 vol. of diluted urine.

For total AA determination 1 vol. diluted urine was added to 3 vol. of 4% (w/v) trichloroacetic acid.

Preparation of AA standards.

Calibration curves for all experiments were prepared with standard solutions of AA made by diluting, immediately after preparation, a 100 mg./100 ml. solution of AA to give final concentrations of 0.25, 0.5, 1.0, 1.5 and 2.0 mg. AA/100 ml. For the indophenol estimations 2 vol. of each of these solutions was immediately added to 3 vol. of 3% (w/v) metaphosphoric acid. For the total AA estimations 1 vol. of the same standard solutions was added to 3 vol. of 6% (w/v) trichloroacetic acid.

Measurement of indophenol reducing activity.

The method employed was basically that used by Evelyn, Malloy & Rosen (1938) and is termed the "buffered indophenol" method. Into a 10 ml. cuvette was pipetted 2 ml. of the test solution, 0.5 ml. of sodium citrate (concentration depending on the final pH required) and 1.0 ml. indophenol. After rapid mixing the optical density of the solution was measured at 520 mμ in a Unicam S.P.600 spectrophotometer. Any slight turbidity or colour in the test solution was allowed for by measuring the optical density after decolourising the remaining indophenol with a small amount of AA. Indophenol reducing activity measured by the "unbuffered indophenol"

method was obtained by using the same method but without the sodium citrate. The calibration curves for both methods were obtained by treating the metaphosphoric acid solutions of the standards in precisely the same manner as the test solutions.

H_2S procedure for the measurement of AA + DHA.

Pure H_2S, generated by warming Sb_2S_5 (AR tartar emetic) and conc. HCl to 68°C, was bubbled for 10 min. through metaphosphoric acid solutions of AA, DHA and filtrates of plasma and erythrocytes of pH 1.5 or adjusted to pH 3.5 with sodium citrate. The solutions were allowed to remain saturated with H_2S for a further 30 min. Excess H_2S was then removed by blowing N_2 saturated with water vapour through the solutions for at least 3 hr. The temperature of the test solutions was held at 37°C. Both lead acetate and nitroprusside were used to test for traces of H_2S. AA was then estimated by the "buffered" and "unbuffered" indophenol methods.

Formaldehyde procedure for the measurement of AA.

This determination was based on a method suggested by Mapson (1953). Condensation with HCHO at pH 0.6 was carried out by allowing 5 ml. of a metaphosphoric acid extract of urine to react with 1 ml. of 2% (v/v) H_2SO_4 and 1.5 ml. of 40% HCHO (adjusted to pH 0.6 with conc. H_2SO_4). Aliquots of 2 ml., removed at 12 min, 24 min, and 36 min, were added to tubes containing 0.3 ml. of 30% sodium citrate and 1 ml. of indophenol. After rapid mixing the material was transferred to spectrophotometric cuvettes and the optical density measured exactly 30 sec. after the addition to the indophenol. For condensation at

pH 3.5, 1 ml. of 15% (w/v) sodium citrate and 1.5 ml. of HCHO previously adjusted to pH 3.5 were added to 5 ml. of urine extract. The mixture was again allowed to react for 12, 24, and 36 min. The indophenol reducing activity was measured by taking 2 ml. of the reaction mixture, 0.3 ml. of water and 1 ml. indophenol. Blank values obtained by decolourising the remaining indophenol with a slight excess of AA were substracted to correct for the natural colour of the urine. Graphical extrapolation to zero time of the values obtained at 12, 24 and 36 min. after adding the HCHO were related to values obtained for standard solutions of AA treated in a similar manner. The difference between the values obtained by condensation with HCHO at pH 0.6 and 3.5 represents the AA content of the urine.

Procedure using p-chloromercuribenzoic acid.

To 3 vol. of a metaphosphoric acid extract of plasma, erythrocytes or urine, 1 vol. of CMB was added. Because of the low solubility of the reagent at this pH (about 1.5) most of excess reagent precipitated, together with any insoluble complex formed by the reagent with materials in the test solution. After standing 5 min. the precipitate was removed by centrifugation and the indophenol reducing activity of the clear supernatant was determined by the "buffered indophenol" method.

Procedures for the estimation of total AA.

Methods using charcoal as the oxidising agent.

The method of Roe & Kuether (1943) originally described for blood was used for estimating the total AA of plasma, erythrocytes and urine. A trichloroacetic acid extract of the test material

was shaken with 0.45 g. of acid-washed activated charcoal on a mechanical shaker for 5 min. and filtered through a No.42 filter paper. A drop of thiourea was added to 2 ml. of the filtrate, then 0.5 ml. phenylhydrazine reagent was added and mixed. The mixture was then incubated for 3 hr. at 37°C. The mixture was ice cooled and 2.5 ml. of 85% H_2SO_4 was added slowly with thorough mixing. The optical density of the red-brown colour which developed during the next 30 min. was read at 540mμ in the spectrophotometer. The Geschwind modification of this method, in which the incubation time is reduced to 45 min. at a temperature of 61°C has also been used.

Blank determinations were carried out in two ways, (a) The charcoal treated filtrates of AA standards or the test material without dinitrophenylhydrazine were incubated, and cooled. The dinitrophenylhydrazine was then added and immediately after it H_2SO_4. (b) A 4.5% (w/v) solution of trichloroacetic acid (diluted from 6%) was treated in exactly the same way as the test solutions.

Procedure using indophenol as the oxidising agent.

The method described by Bolin & Book (1947) was used on metaphosphoric acid extracts of plasma and urine. To 2 vol. of the test extract was added 1 drop of indophenol of such concentration that a faint pink colour persisted after shaking. An equal volume of a solution containing 4% (w/v) metaphosphoric acid and 2% (w/v) thiourea was added and then 1 vol. of dinitrophenylhydrazine reagent. Incubation, colour development

with 5 vol. of 85% H_2SO_4 and measurement of the optical density was as for the Roe & Kuether method. A slight modification of this method was also used. It was found that 1 drop of thiourea was sufficient to decolourise the pink colour of the indophenol so that after incubation the volume of H_2SO_4 was reduced by one half.

RESULTS

The stability of Indophenol.

The fading of indophenol, in the absence of substances known to reduce it, has been examined by following the decrease in optical density at minute intervals of a solution containing 2% (w/v) metaphosphoric acid and 1 ml. of indophenol. 0.5 ml. of sodium citrate of varying concentration or of water or of 2% metaphosphoric acid were added to adjust the pH of the final mixture to 4.2, 3.5, 2.6, 2.1, and 1.9. The rate of fading is shown in Fig.1. At pH 4.2 there was no fading and the dye in fact remained stable for at least 30 min. As the pH was reduced the rate of fading was progressively increased, When sodium acetate buffer was used in place of sodium citrate, indophenol again remained stable at pH 4.0. Mindlin & Butler (1937-38) also observed fading of indophenol at low pH using phosphate buffers of pH 1.9 and 2.0. The rate of fading is therefore largely determined by the pH and at a pH below 2.0 a small change in pH can cause a considerable change in the rate of fading. From these results a pH of 4.0 must be regarded as the most satisfactory one for measuring indophenol reducing activity since spontaneous fading of the dye is negligible at this pH.

Fading of indophenol in the presence of ascorbic acid.

Partial stabilisation of indophenol can be achieved by the addition of AA. This effect was examined by measuring the optical density of indophenol added to 2% (w/v) metaphosphoric acid solutions containing 0.25, 0.5, and 1.0 mg./100 ml. AA.

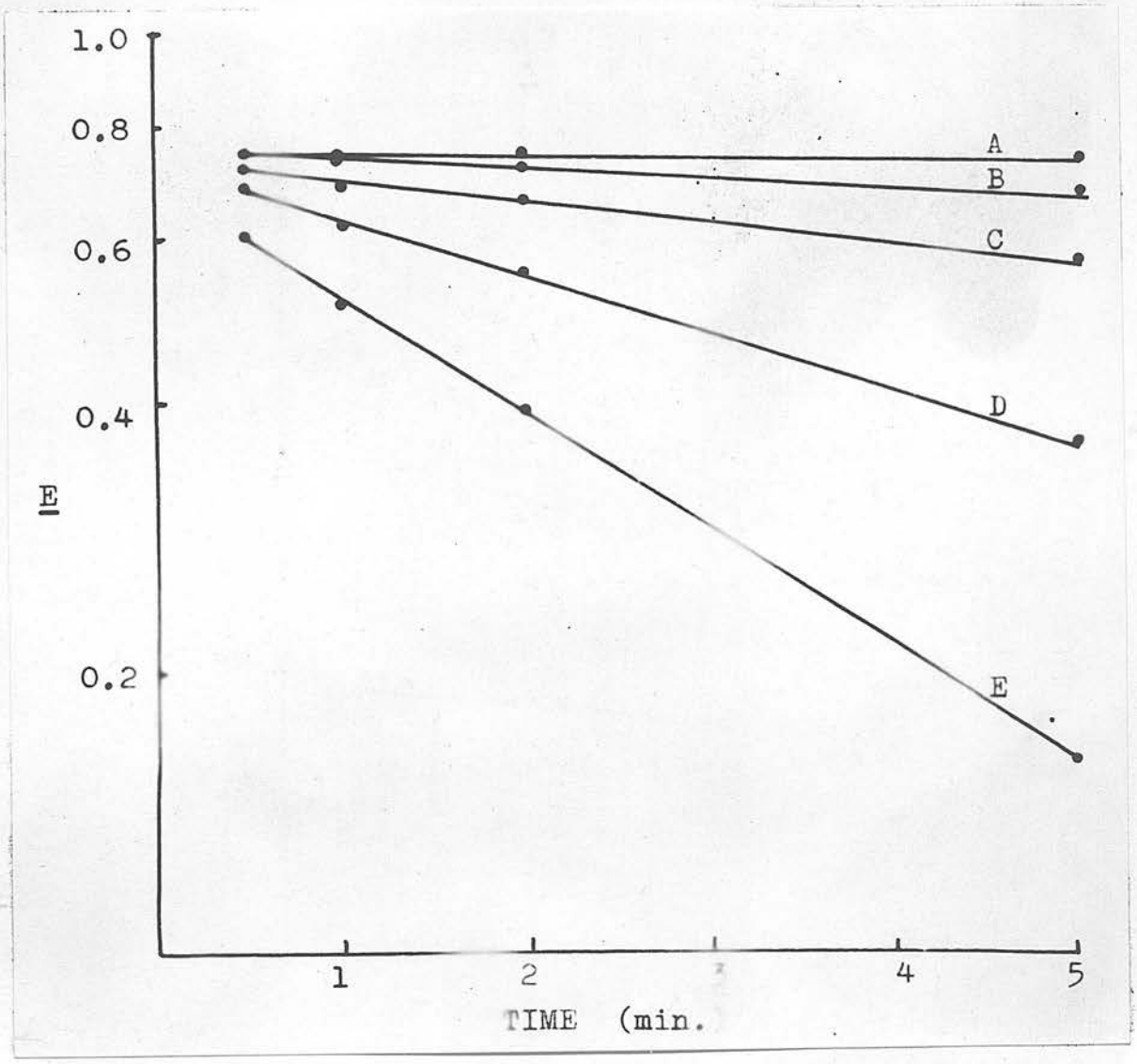


Fig.1. The effect of pH on the stability of indophenol. pH:- A, 4.2; B, 3.5; C, 2.6; D, 2.1; E, 1.9.

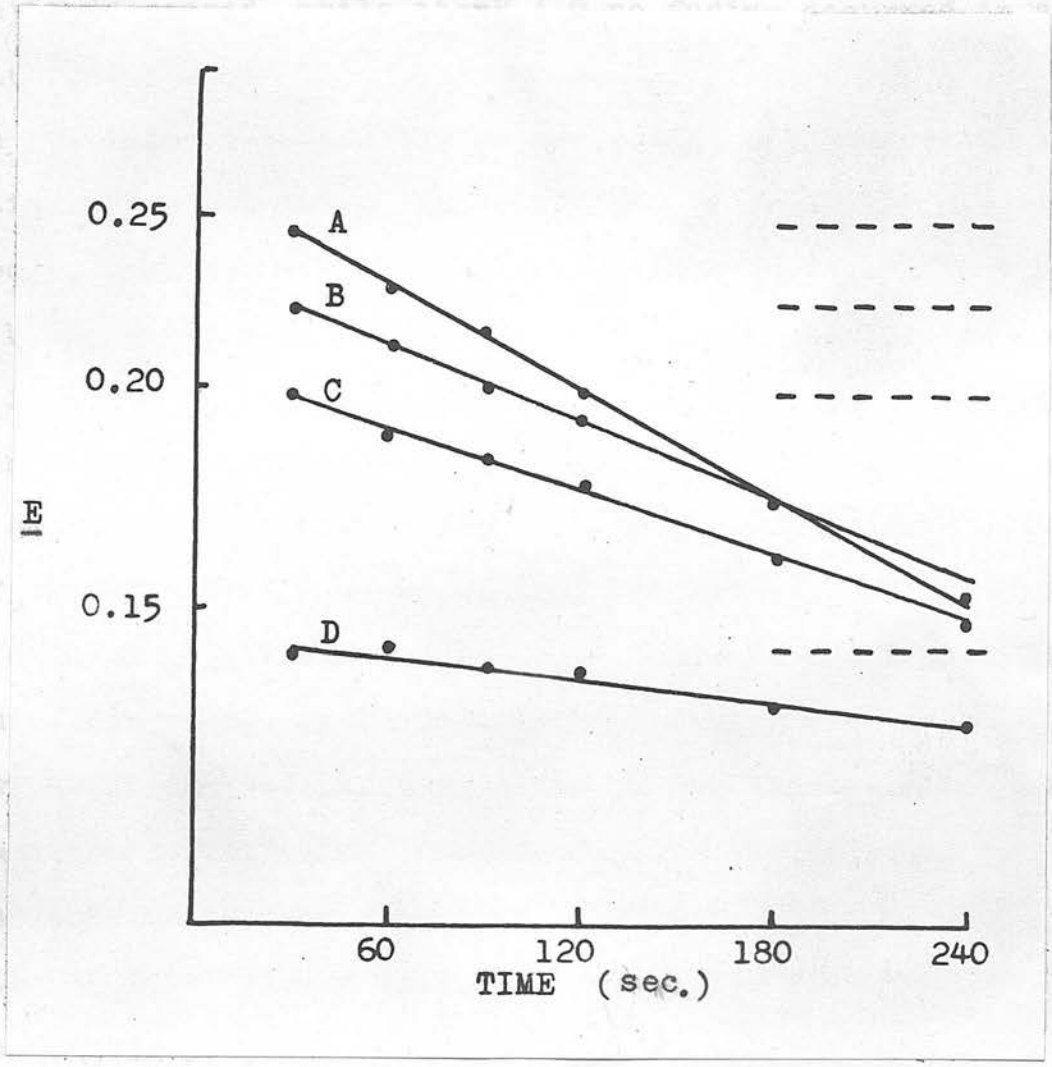


Fig.2. The effect of AA on the stability of indophenol at pH 1.5. Test solutions of HPO_3 containing AA (mg./100ml.) A, No AA; B, 0.25; C, 0.5; D, 1.0. Broken lines show that no fading of indophenol occurred in these solutions during 4 min. at pH 4.0

Fig. 2 shows the rate of fading in 4 min. of these solutions (pH 1.5) and of the same solutions adjusted to pH 4.0 with sodium citrate. With increasing concentrations of AA the rate of fading of the dye is decreased, while at pH 4.0 no fading occurred in any of the solutions.

Since the rate of fading varies according to the concentration of AA initially present it is important that the reaction mixture is buffered to about pH 4.0 when concentrations of less than 1 mg./100ml of AA are being measured. Under ordinary conditions the concentration of AA in blood is generally less than 1 mg./100ml. The increase in reducing activity of indophenol in the presence of AA expressed as "apparent ascorbic acid" is shown in Fig. 3. Sixty seconds after the addition of indophenol the reducing activity of the reaction mixture containing 0.25 mg./100ml. AA had increased by the equivalent of 0.1 mg./100ml. AA. The base line was obtained with all the solutions when the pH was increased to 4.0, showing that the reduction of indophenol by AA was complete in 30 sec. and that no further reduction occurred.

In view of this finding it is not surprising that AA calibration curves prepared at low pH e.g. by the "unbuffered indophenol" method deviate from linearity below 0.5 mg./100ml. (Fig.4) In contrast, calibration curves prepared by the "buffered indophenol" method i.e. at pH 4.0, are strictly linear.

Fading of indophenol by various sulphur-containing compounds.

Thiols and some other compounds containing sulphur can reduce indophenol. The rate of fading of indophenol in 2%

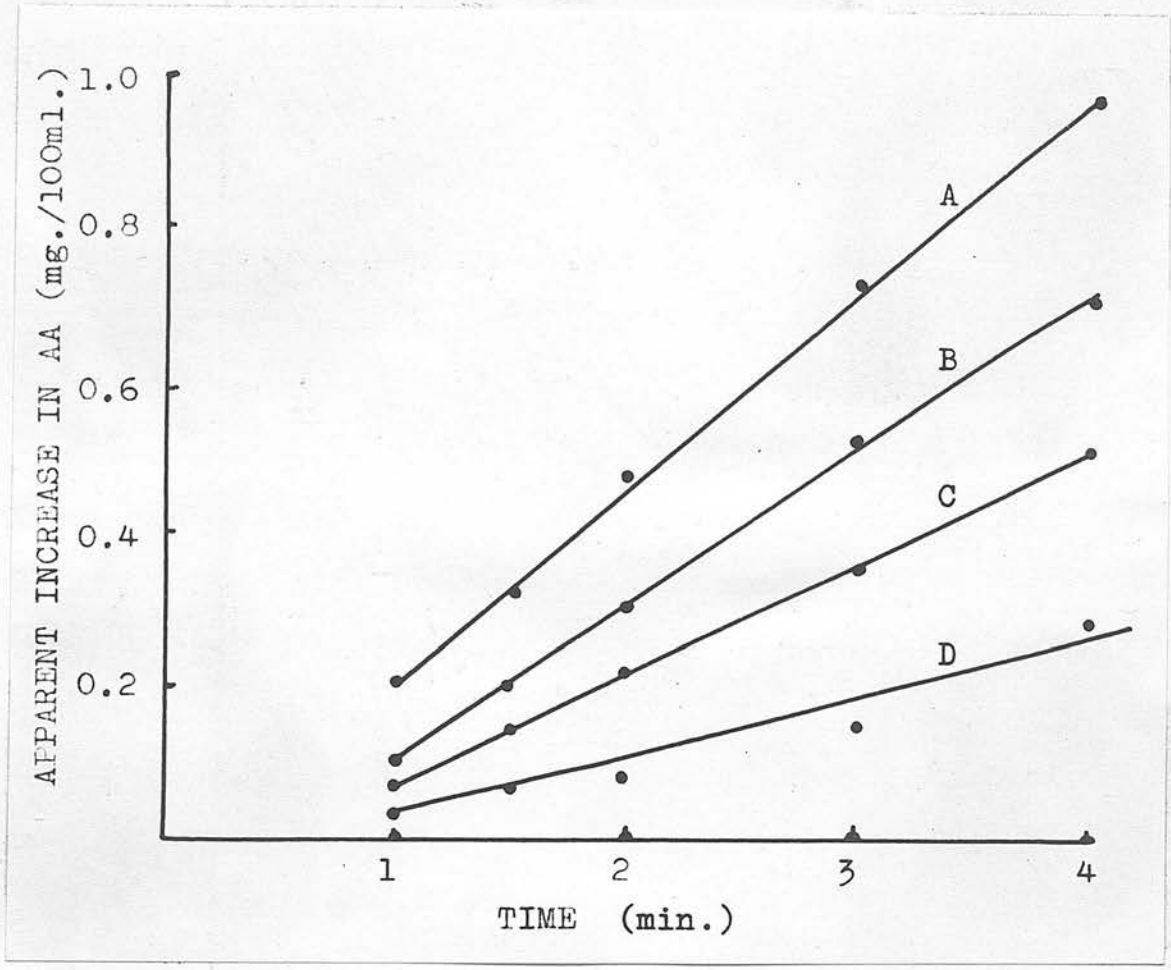


Fig.3. The apparent increase in ascorbic acid content of standard solutions of AA in HPO_3 at pH 1.5. Concentration of AA (mg./100ml.) A, No AA; B, 0.25; C, 0.5; D, 1.0. The base line shows no increase in AA in all the solutions at pH 4.0.

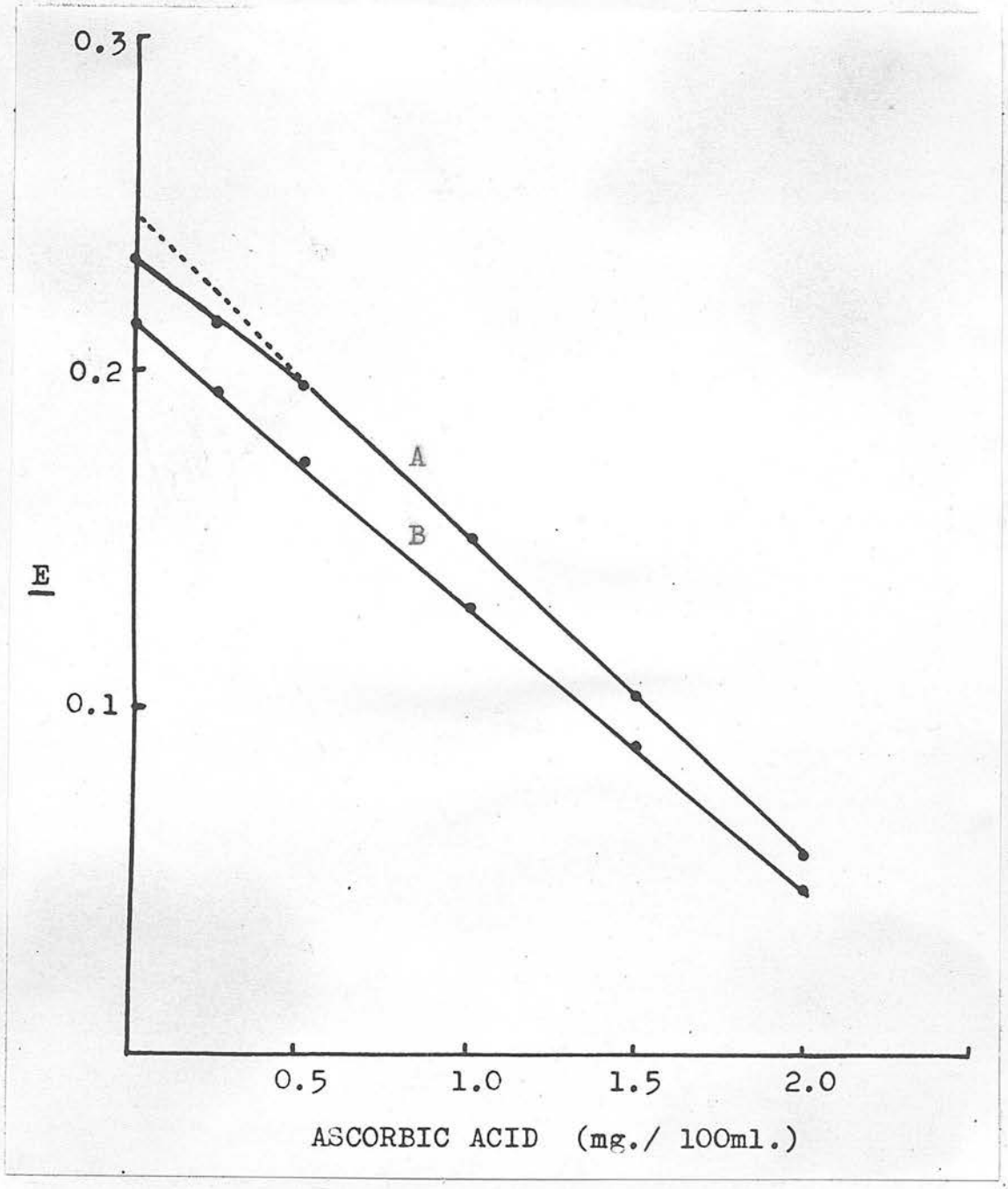


Fig.4. Calibration curves for the estimation of AA. A, by the 'unbuffered indophenol' method; B, by the 'buffered indophenol' method. Dotted line represents linearity in A.

TABLE 1. Indophenol reducing activity of various sulphur compounds

Results in terms of equivalent ascorbic acid concentration (10^{-5}M)

Compound	Conc. (10^{-5}M)	No addition						With CMB
		30 sec.		1 min.		5 min.		
		pH 4.5	pH 3.5	pH 4.5	pH 3.5	pH 4.5	pH 3.5	pH 3.5
$Na_2S_2O_3$	6	3.6	4.1	3.6	4.0	4.0	3.6	0.0
Na_2SO_3	25	5.0	4.8	5.5	5.5	8.4	7.7	2.7
Cysteine	50	7.1	2.6	10.3	6.8	20	20	0.0
2:3-dimercaptopropanol	50	6.8	5.1	9.8	7.5	13.5	11.5	0.1
Ergothioneine	50	0.0	2.7	0.0	4.2	0.0	9.6	0.0
Glutathione	50	3.3	0.7	5.8	1.3	11.7	6.3	0.0
H_2S	50	3.1	2.6	4.4	3.2	6.1	4.8	0.3
Homocysteine	50	0.7	0.3	1.3	0.4	6.4	1.6	0.1
Thiourea	150	2.0	3.2	2.5	3.6	2.5	7.0	0.0

metaphosphoric acid solutions of the following substances was measured at pH 4.6; methionine 5×10^{-4} M, glutathione 5×10^{-4}M, cysteine 5×10^{-4}M, thioglycolic acid 2×10^{-4}M, H_2S 5×10^{-4}M, thiosulphate 0.60×10^{-4} thiourea 1.5×10^{-4}M, and sulphite 2.5×10^{-4}. Indophenol at a concentration of 4mg./100ml. was used and the optical density of the solution was measured at intervals for five minutes after adding the dye (Fig.5). Thiosulphate and thiourea reacted rapidly with indophenol; by 60 sec. most of the fading had occurred and the dye was stable after 2 min. By contrast a solution containing 1.0×10^{-4}M AA was stable 30 sec. after adding the dye. The other compounds reacted more slowly and the fading of indophenol was incomplete at 5 min. At pH 3.5 (Fig.6) all the solutions except ergothioneine and thiourea, which are shown in Fig.7, reacted more slowly with indophenol than at pH 4.6. The indophenol reducing activity of these solutions, expressed as mg./100ml. "apparent ascorbic acid", is given in Table 1 and it can be seen that the presence of these substances could seriously interfere with the estimation of AA by the indophenol method. Other groups of substances such as reductones, pheonols, dihydroxymalic acid and hydroxytetronic acid will also reduce indophenol but were not examined.

<u>The use of the indophenol method to estimate AA in mammalian tissues. Protein precipitation.</u>

Metaphosphoric acid is the most suitable reagent for precipitating tissue proteins before applying the indophenol reaction. Protein determinations were done on plasma and erythrocyte filtrates after treatment with 3%, 6%, 10%, 15% and 20% (w/v) metaphosphoric

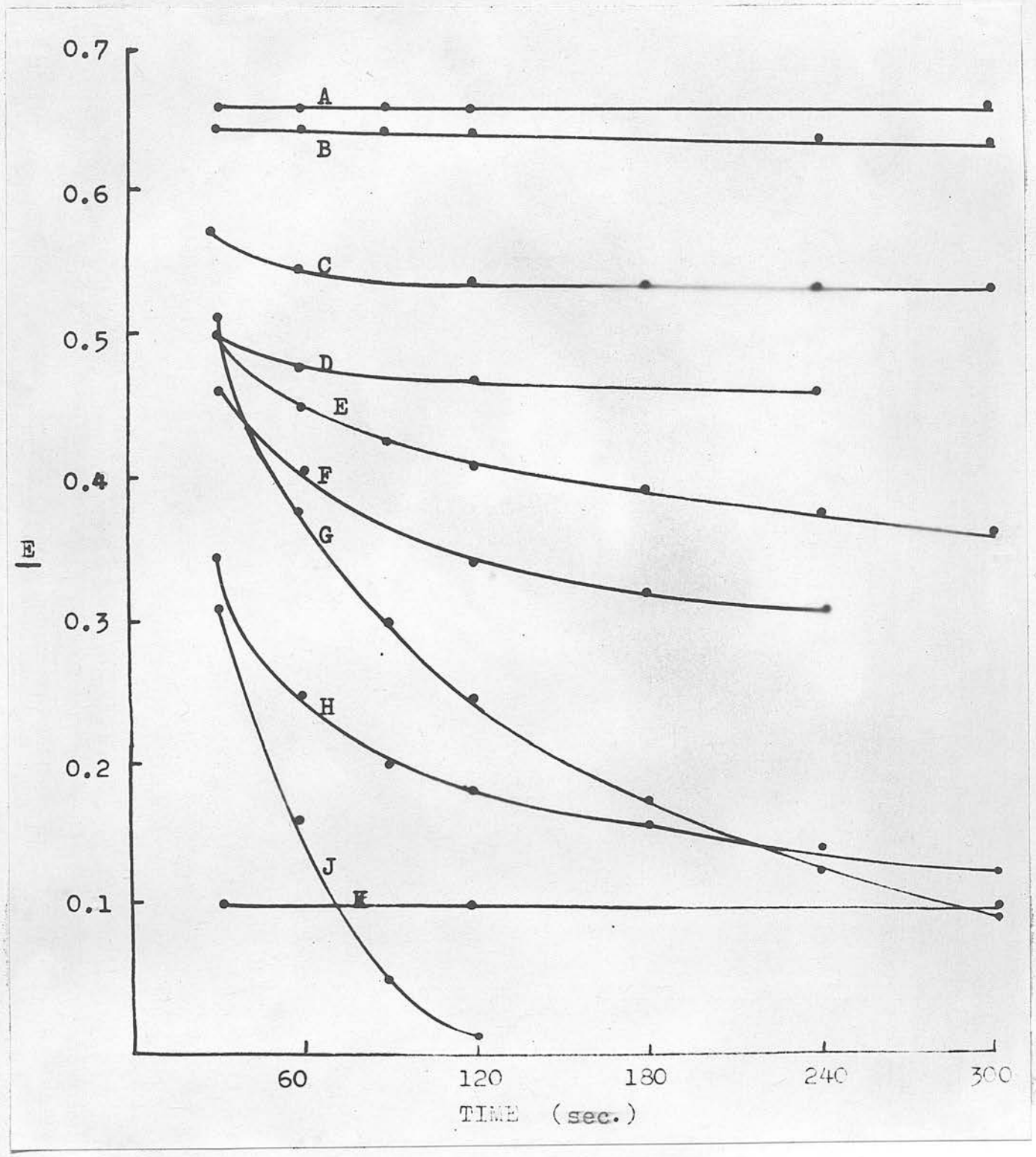


Fig. 5. The rates of reaction of S compounds with indophenol at pH 4.6. A, control; B, methionine, 5×10^{-4}M; C, thiourea, 15×10^{-4}M; D, $Na_2S_2O_3$, 0.6×10^{-4}M; E, H_2S, 5×10^{-4}M; F, Na_2SO_3, 2.5×10^{-4}M; G, glutathione, 5×10^{-4}M; H, thioglycollic acid, 2×10^{-4}M; J, cysteine, 5×10^{-4}M; K, AA, 1×10^{-4}M.

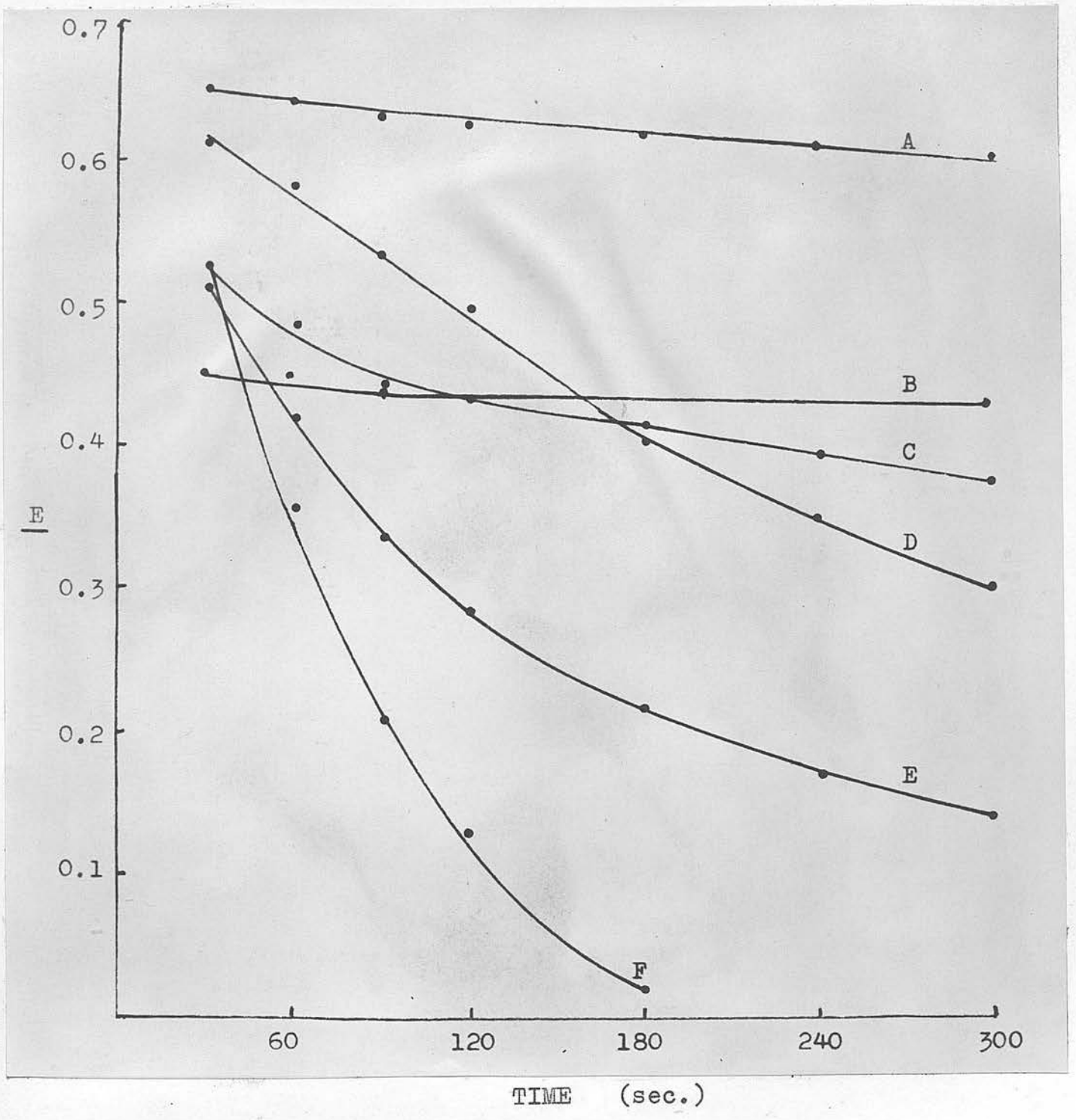


Fig.6. The rates of reaction of S compounds with indophenol at pH 3.5. A, control; B, $Na_2S_2O_3$, 0.6×10^{-4}M; C, H_2S, 5×10^{-4}M; D, glutathione, 5×10^{-4}M; E, thioglycollic acid, 2×10^{-4}M.; F, cysteine, 5×10^{-4}M.

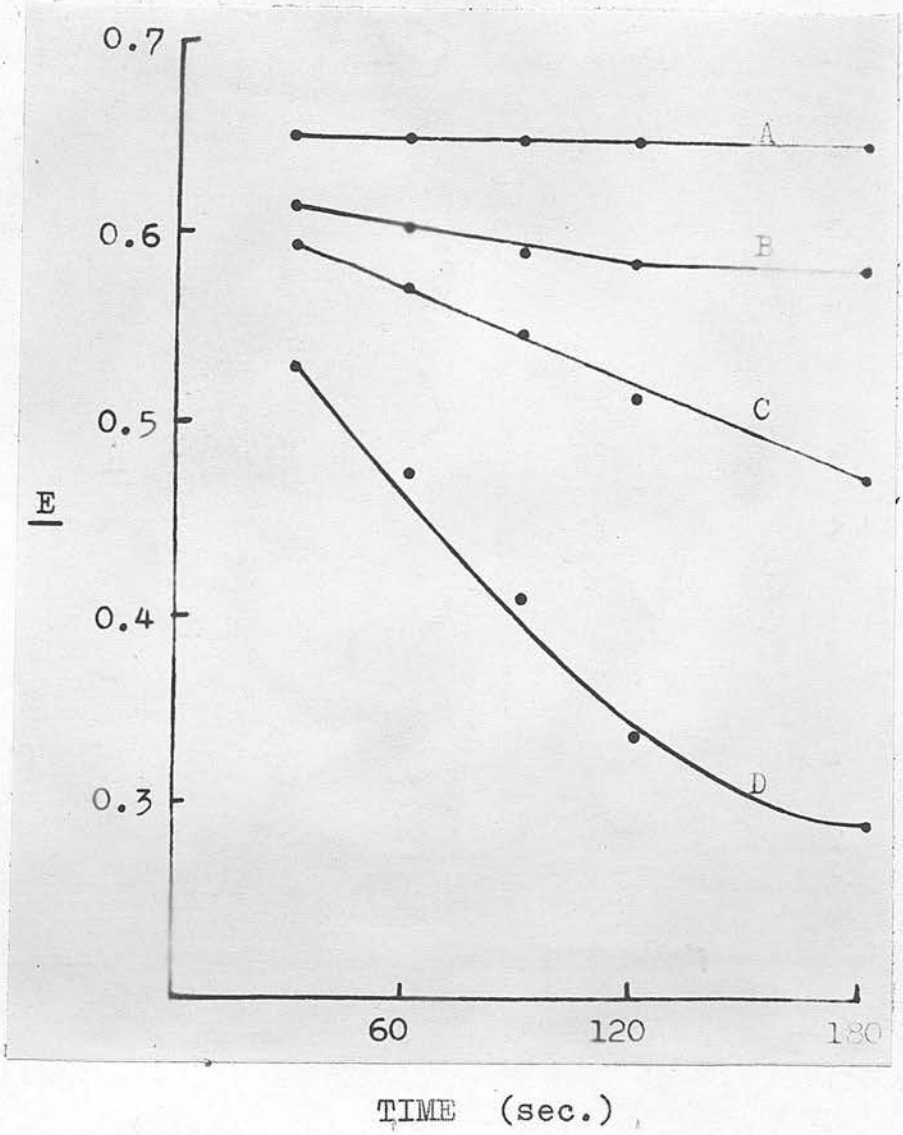


Fig.7. The rates of reaction of ergothioneine and thiourea with indophenol at pH 4.6 and 3.5. A, ergothioneine (5×10^{-4}M) at pH 4.6; B, thiourea (15×10^{-4}M) at pH 4.6; C, ergothioneine at pH 3.5; D, thiourea at pH 3.5.

TABLE 2. The effect of metaphosphoric acid concentration on protein precipitation

Material	HPO_3 (g/100ml)	AA: Without CMB (mg/100ml)	AA: With CMB (mg/100ml)	Blank E	Total AA (mg/100ml)	N.P.N. (mg/100ml)
Plasma A	3	0.13	0.13	0.045		29.5
	6	0.12	0.14	0.049	0.16	30.5
	10	0.14	0.11	0.050		35.5
	15	0.13	0.13	0.049		43.0
Plasma B	3	0.09	0.11	0.041		32.0
	6	0.09	0.13	0.049	0.10	31.5
	10	0.11	0.11	0.047		35.0
	20	0.09	0.14	0.047		54.0
Cells A (no CO)	3	insufficient to precipitate the protein				
	6	0.16	0.07	0.049	0.16	42.5
	10	0.16	0.12	0.057		45.0
	15	0.25	0.01	0.059		47.5
Cells B (no CO)	10	0.47	0.02	0.057	0.11	45.0
	20	0.29	0.05	0.059		45.0

acid. The total N content of plasma filtrates increased as the metaphosphoric acid concentration was raised from 3% to 20% (Table 2). The filtrates prepared with 3% were quite clear but above this concentration they were slightly cloudy; this is reflected in the higher blank values. When 3% metaphosphoric acid was used to precipitate protein from erythrocytes a brownish filtrate containing protein was obtained. A clear filtrate was obtained when 6% metaphosphoric acid was used but at higher concentrations the precipitation of protein was incomplete.

In all subsequent experiments 3% and 6% metaphosphoric acid was used for the precipitation of protein in plasma and erythrocytes respectively.

Fading of indophenol in protein-free filtrates of plasma.

Ascorbic acid was added to plasma in amounts sufficient to give final concentrations of 0.25, 0.5, 1.0 mg./100ml. The indophenol reducing activity of protein-free filtrates of these solutions was measured at intervals for 4 min. (Fig.8). Continuous fading of indophenol occurred with all the solutions but it was less rapid at the higher concentrations of AA. The AA solutions added were the same as those used in Fig.2 but the rate of fading was less rapid, probably because the pH of the final solution is slightly higher when the same solution of metaphosphoric acid is added to pure solutions of AA and to plasma solutions of AA. When the pH was raised to 4 by the addition of sodium citrate no fading of the solutions occurred during 30 min. The apparent increase in AA is plotted in Fig. 9.

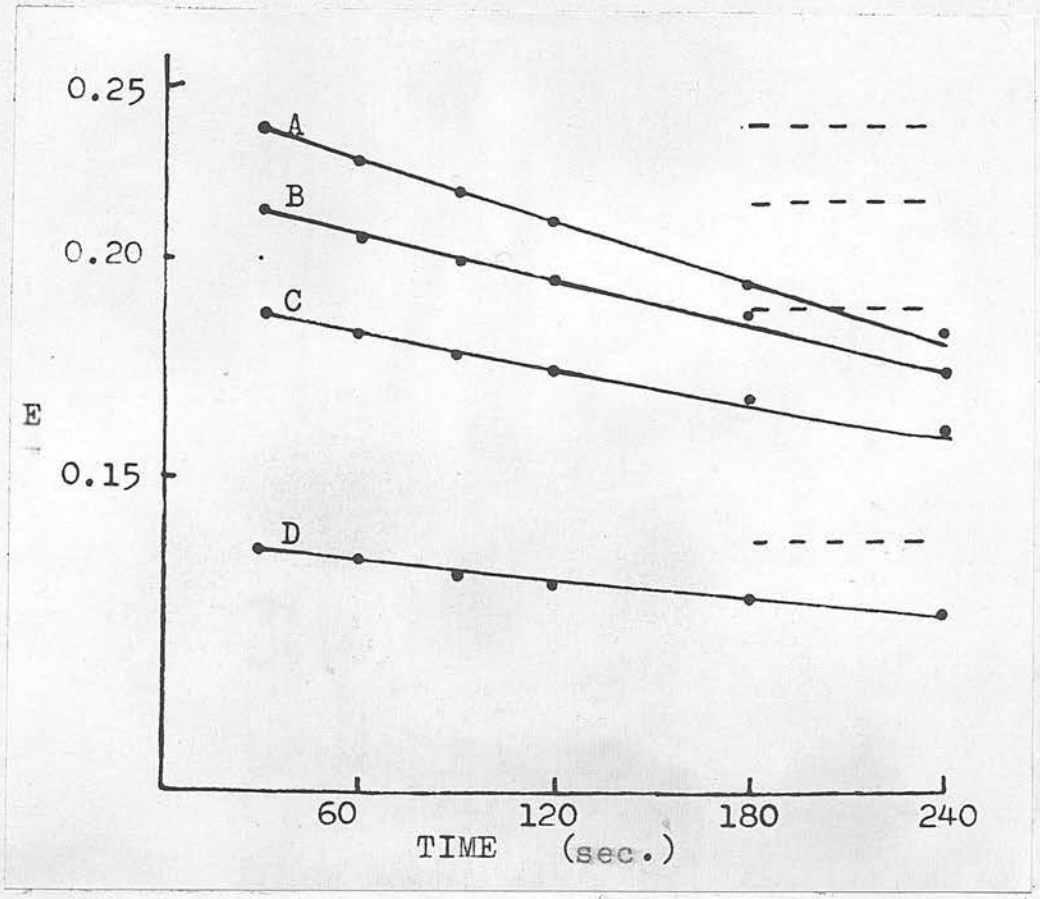


Fig.8. The effect of AA on the stability of indophenol with plasma filtrates at pH 1.5. Concentration of AA (mg./100ml.) :- A, no AA; B, 0.25; C, 0.5; D, 1.0. Broken lines show that no fading of indophenol occurred in these filtrates during 4 min. at pH 4.0.

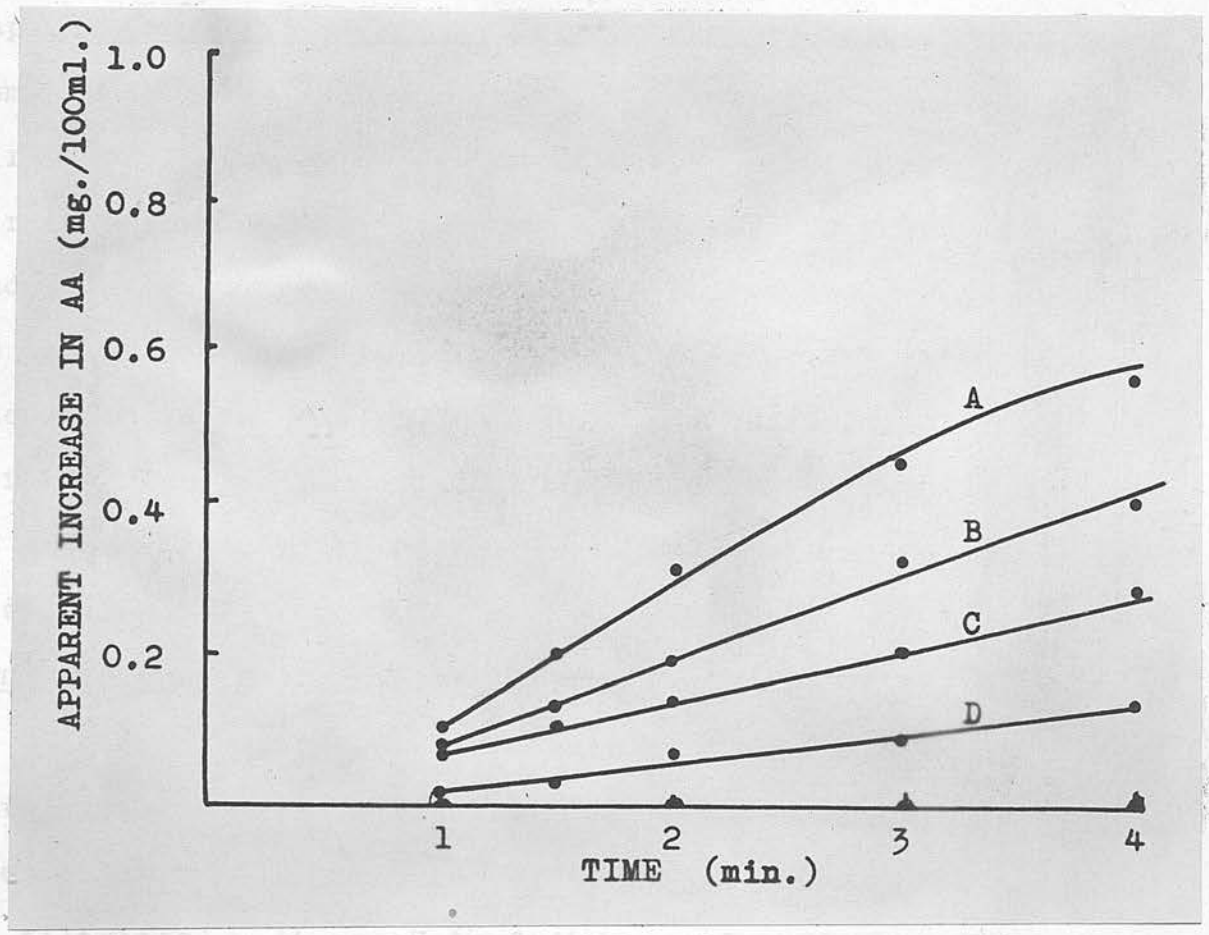


Fig.9. The apparent increase in AA content of standard solutions of AA added to plasma filtrates of pH 1.5. Concentration of AA (mg./100ml.):- A, no AA; B, 0.25; C, 0.5; D, 1.0. The base line shows no increase in AA in all the filtrates at pH 4.0.

If the same pH could be reached in filtrates of plasma and standard solutions of AA an accurate estimate of the plasma AA could be obtained using the indophenol method. However some metaphosphoric acid is always used in the precipitation of the plasma proteins so that the final pH is never the same. Since the rate of fading is so dependent on pH, particularly below pH 2, the rate of fading of indophenol will always be faster in the standard solutions than in the plasma filtrates. Because of this the AA content will be underestimated. For this reason an accurate measure of plasma AA will be obtained only when the pH of the reaction mixture is about pH 4, at which pH the spontaneous fading of both standard solutions and plasma filtrates is arrested.

Fading of indophenol in protein-free filtrates of erythrocytes.

Fading of indophenol in CO-saturated erythrocyte filtrates is much greater than in filtrates of plasma. The apparent increase in AA at high pH is shown in Fig.10 for three different cell filtrates. At no pH is fading completely arrested and in fact it is greater at the higher pH values. Evidence will be presented later to show that the presence of interfering substances is chiefly responsible for this increase. When the pH was raised from 2.4 to 5.1 there was a 10-fold increase in the apparent AA content of filtrate A and a 4-fold increase in filtrate B.

Specificity of the indophenol method.

The fading of indophenol in the presence of thiol and other S-containing compounds has already been discussed. These substances are present in mammalian tissues. The use of a known

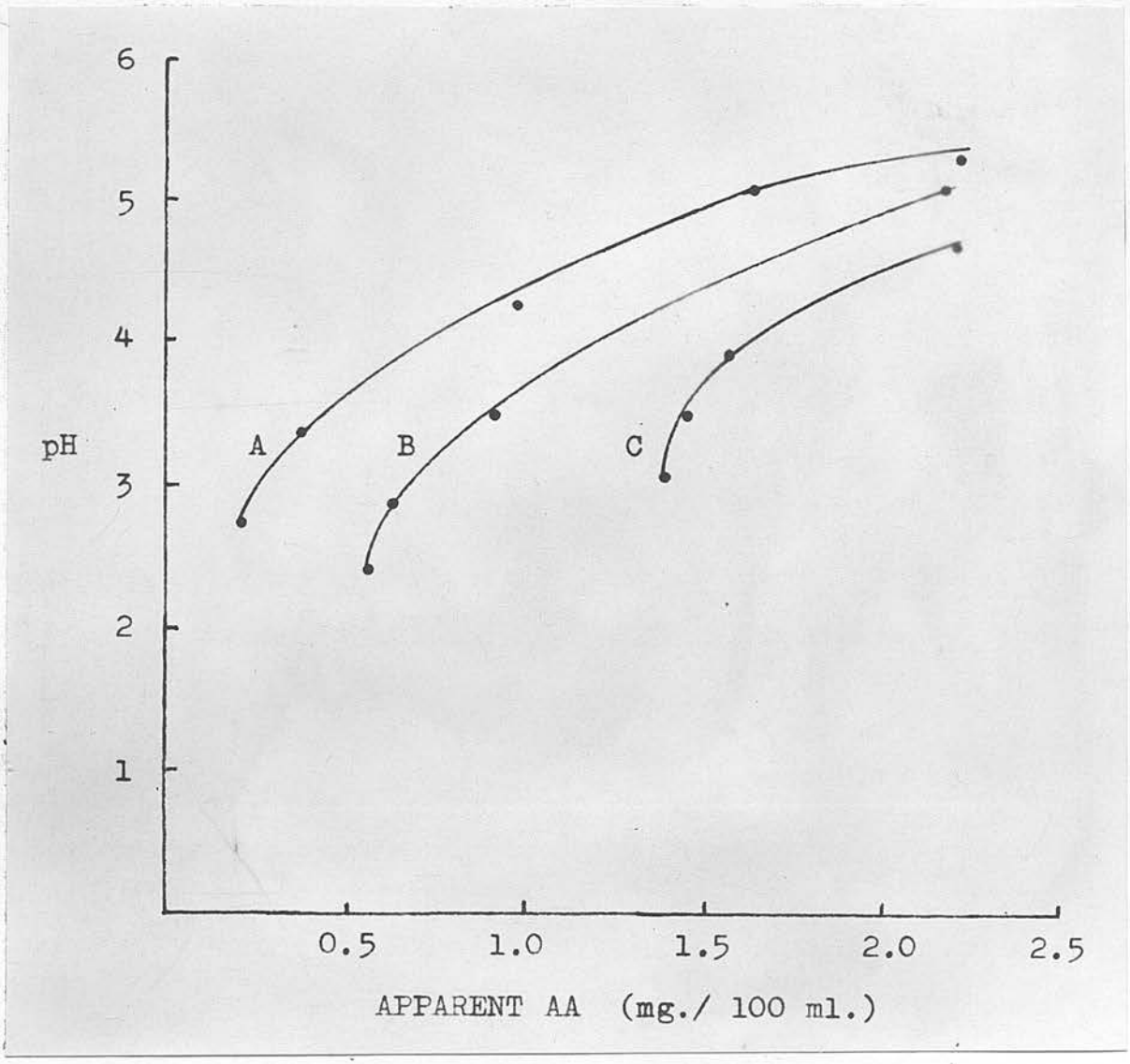


Fig.10. The effect of pH on the apparent AA content of 3 filtrates of erythrocytes.

TABLE 3. The effect of CMB on the recovery of AA

Material	Original AA content (mg/100ml)	AA added (mg/100ml)	AA found (mg/100ml)	Recovery (%)
Plasma	0.12	1.0	1.16	104
Erythrocytes	0.18	0.33	0.50	96
		1.0	1.17	99
Urine	0.98	1.0	2.00	102
Plasma filtrate	1.06	1.0	2.10	104
Erythrocyte filtrate	0.74	1.0	1.71	97
Urine filtrate	1.00	1.0	1.99	99
Liver filtrate	1.13	1.0	2.18	105

TABLE 4. The effect of CMB on the apparent AA content of plasma, erythrocytes and urine

(Results as means and range)

Material	n	AA Without CMB (mg/100ml)	AA With CMB (mg/100ml)	Difference (mg/100ml)
Plasma	41	0.56 (0.09 - 1.40)	0.55 (0.10 - 1.40)	0.01 (-0.09 - +0.07)
Erythrocytes	12	0.47 (0.21 - 1.39)	0.25 (0.10 - 0.94)	0.22 (+0.01 - +0.45)
Urine	12	0.77 (0.19 - 1.92)	0.65 (0.02 - 1.79)	0.12 (0.00 - +0.26)

SH-inhibitor, p-chloromercuribenzoic acid (CMB), in an attempt to increase the specificity of the indophenol reaction was therefore investigated. The standard curves obtained by the "buffered indophenol" method before and after the addition of CMB are shown in Fig.11 for solutions containing 0.5, 1.0, 1.5, 2.0 and 3.0 mg./100ml. AA. Good recoveries of AA added to plasma, erythrocytes (after haemolysis with saponin and saturation with CO) and diluted urine were obtained with the CMB method. AA added to metaphosphoric acid filtrates of plasma, erythrocytes, urine and liver was also satisfactorily recovered when estimated by the CMB method (Table 3). These results indicate that CMB does not interfere with the reaction of indophenol with AA. The effectiveness of CMB in removing S-containing compounds from 2% metaphosphoric acid solutions was then tested. CMB effectively suppressed the indophenol reducing activity at pH 4.5 of all the compounds tested (Table 1). The effect of CMB on protein free filtrates of plasma, erythrocytes and urine was therefore tested. The "apparent AA content" measured by the "buffered indophenol" method with and without the addition of CMB for 41 plasma, 12 erythrocyte and 12 urine filtrates are given in Table 4. No difference was observed after the addition of CMB to plasma filtrates. However the "apparent AA content" of the erythrocyte and urine filtrates was reduced by 50 and 15% respectively. The absence of slowly reacting substances from plasma is shown in Fig.12. There was no slow fading during 4 min. at pH 4.5 (curve D) and the results with and without CMB are identical at this pH. Erythrocytes do contain slowly

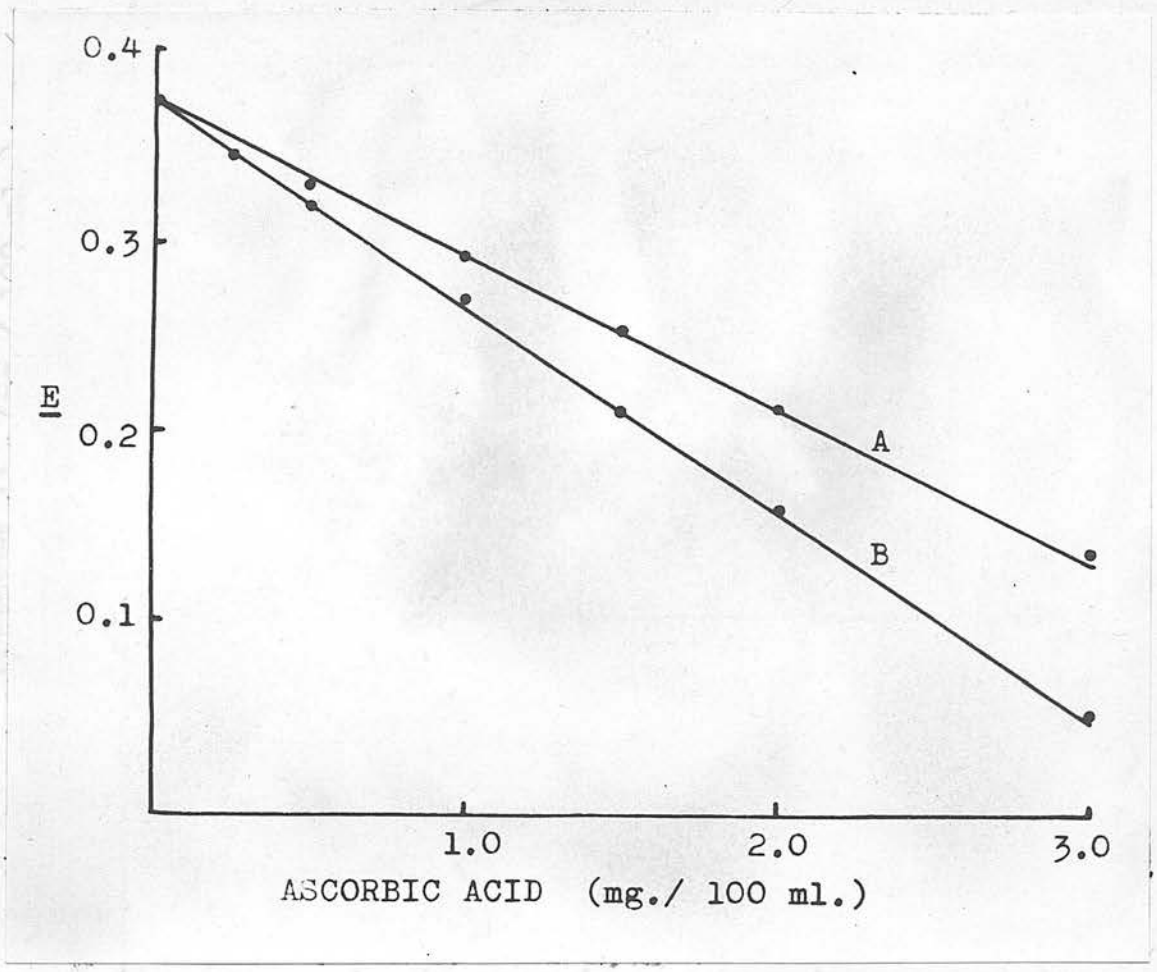


Fig. 11. Calibration curves for the estimation of AA. A, by the CMB method; B, by the 'buffered indophenol' method.

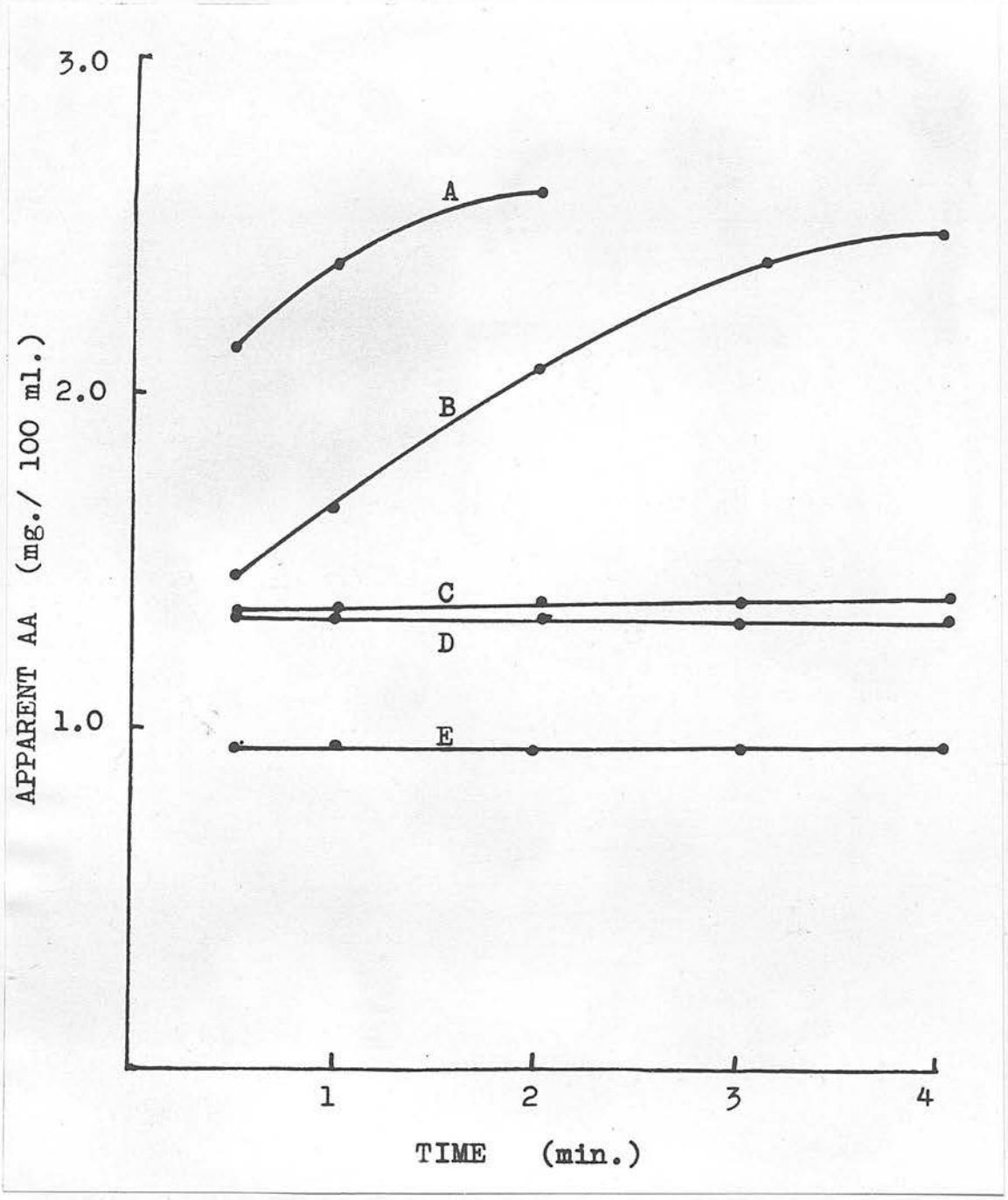


Fig. 12. The effect of CMB on the indophenol reducing activity of filtrates of plasma and erythrocytes at pH 3.5 and 4.0. A, erythrocyte filtrate at pH 4.5; B, erythrocyte filtrate at pH 3.5, C, plasma filtrate at pH 3.5; D, plasma filtrate at pH 4.5 and with CMB at pH 3.5 and 4.5; E, erythrocyte filtrate with CMB at pH 4.5.

TABLE 5. The effects of pH and CMB on the indophenol reducing activity of erythrocytes

Material	pH	Indophenol reducing activity 'buffered method' (mgAA/100ml)	CMB method (mgAA/100ml)	Difference
Blood A	2.4	0.20		0
	2.7	0.21		0.01
	3.4	0.38		0.18
	4.3	0.98	0.20	0.78
	4.8	1.65		1.45
	5.1	1.98		1.79
	5.3	2.12		1.92
Blood B	2.4	0.56		0.31
	2.9	0.62		0.37
	3.5	0.92	0.25	0.67
	4.6	1.81		1.56
	5.1	2.17		1.92
Blood C	3.1	1.39		0.34
	3.4	1.44		0.39
	3.9	1.55	1.05	0.50
	4.7	2.10		1.05
Blood D (no CO)	2.0	0.29		0.26
	4.5	0.47	0.03	0.44
	4.9	1.34		1.31

reacting substances (Curve A & B Fig.12) as is shown by the increase in "apparent AA". This increase is largely due to substances which react more rapidly at the higher pH values (Curve A & B). The addition of CMB almost completely removes these interfering substances since there was no apparent increase in AA when CMB was added (Curve E Fig.12). The striking effect which pH has on the AA values of erythrocytes is shown in Table 5. This effect of pH was absent after the addition of CMB. Above pH 4.5 the difference between the two methods was greater than 1 mg./100ml. AA. Substances such as glutathione and cysteine which a re known to occur in erythrocytes behave in this manner and may be responsible for the increase. When added to plasma they cause slow continuous fading of indophenol which is completely arrested by the addition of CMB (Fig. 13).

The effect of adding CMB to 2 diluted urine filtrates is shown in Fig.14. Urine contains substances which react slowly with indophenol at high pH values (Curve A & D) but their effect is only partly removed by treatment with CMB (Curves C & E). Some of the interfering substances in urine are therefore probably S-containing compounds. Urine also contains other compounds, possibly phenols, which are not affected by CMB. When GSH and cysteine were added to urine filtrates they caused slow continuous fading of indophenol (Fig.15).

In a few experiments it was shown that extracts of rat liver and kidney also contain considerable amounts of substances which react slowly with indophenol and this effect was removed by CMB. (Fig.16).

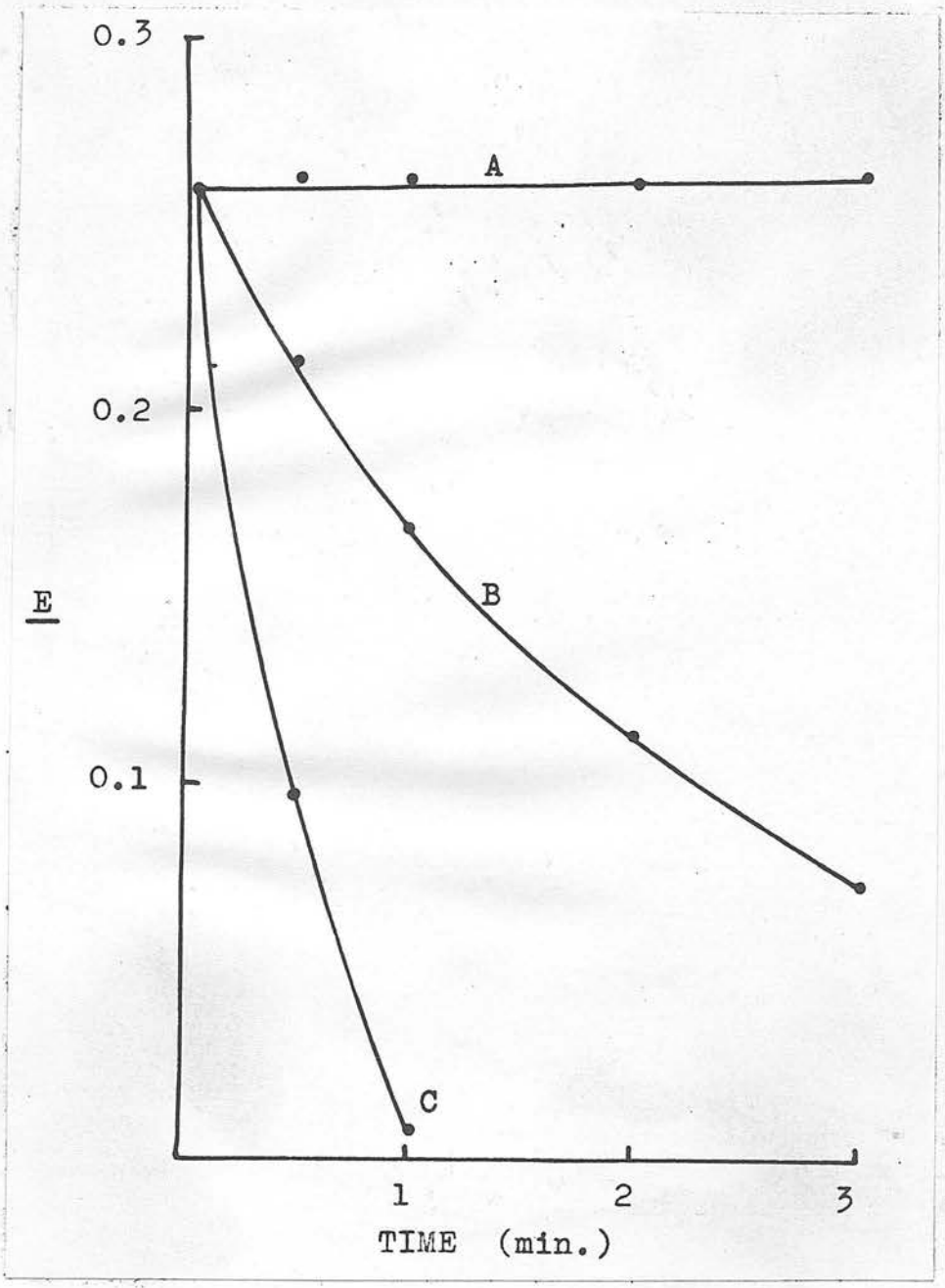


Fig. 13. The effect of glutathione and cysteine on the indophenol reducing activity of plasma filtrates at pH 4.2. Glutathione concentration, 20mg./100ml; cysteine concentration, 10mg./100ml. A, plasma filtrate; plasma filtrate + glutathione or cysteine + CMB; B, plasma filtrate + glutathione; C, plasma filtrate + cysteine.

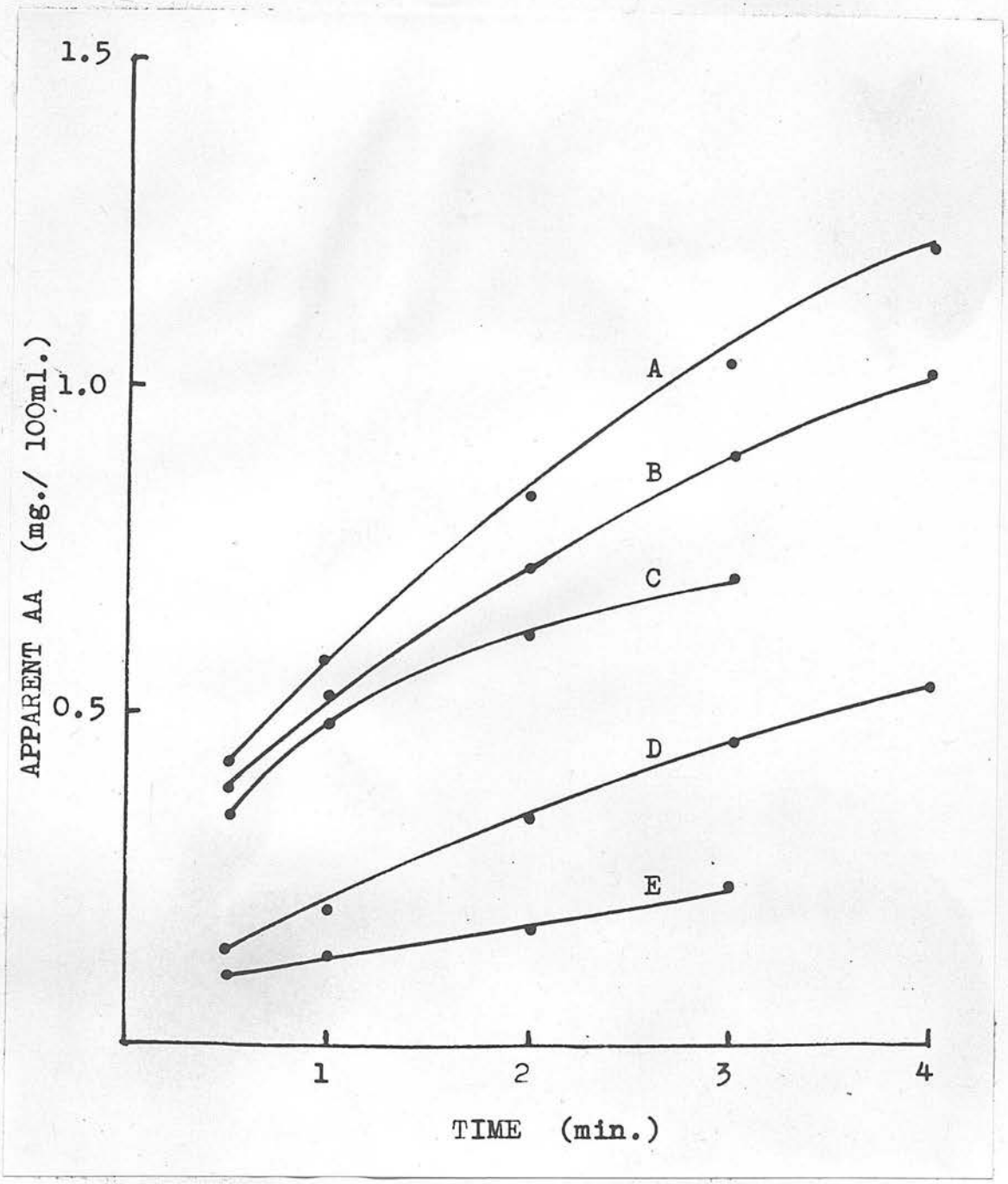


Fig.14. The effect of pH and CMB on the indophenol reducing activity of 2 diluted urines. Urine 1 :- A, pH 4.2; B, pH 3.5; C, + CMB, pH 4.2. Urine 2 :- D, pH 4.2; E, + CMB, pH 4.2.

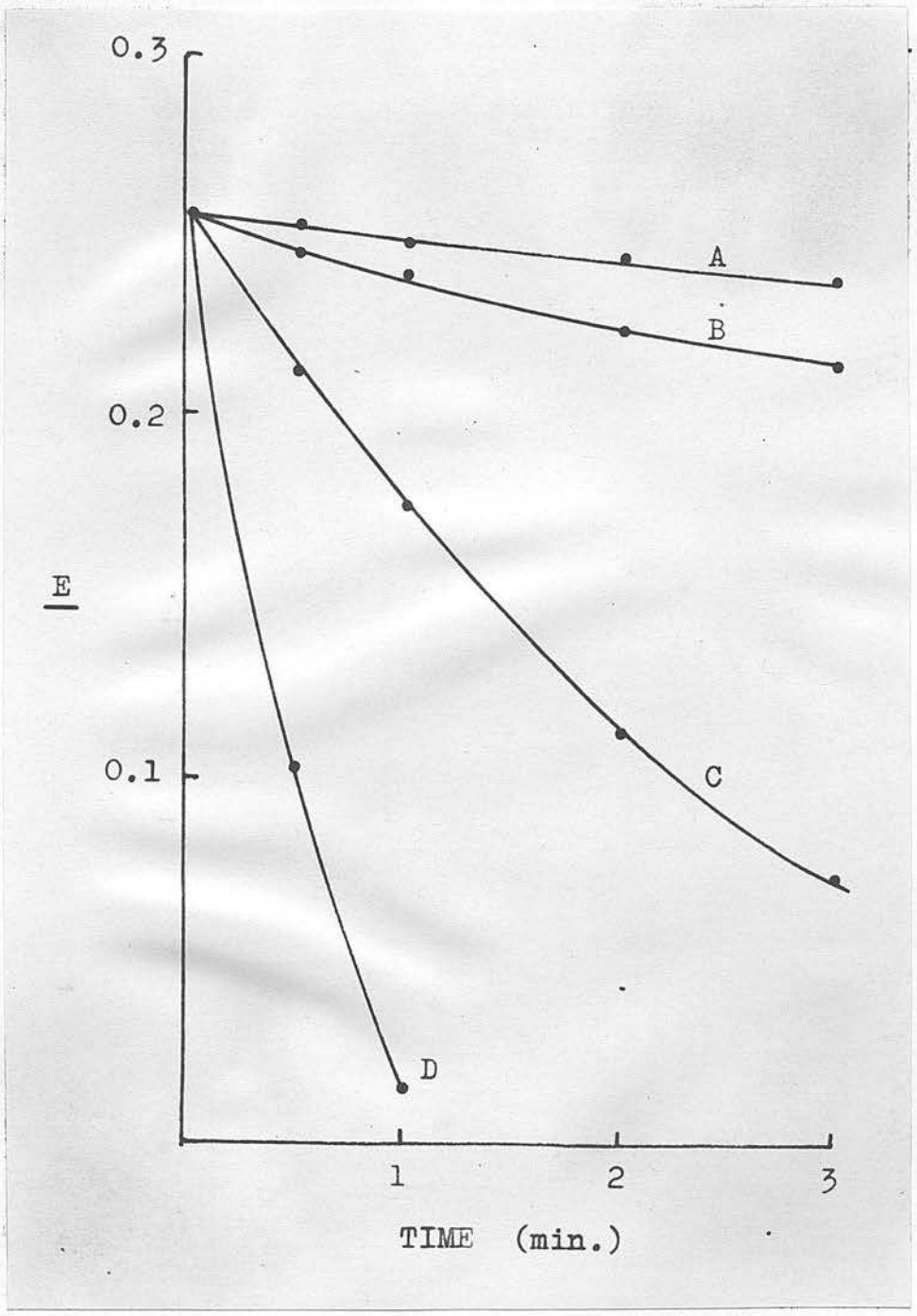


Fig.15. The effect of glutathione and cysteine on the indophenol reducing activity of diluted urine at pH 4.2. Concentration of glutathione, 20mg./100ml; concentration of cysteine 10mg./100ml. A, diluted urine + CMB; B, diluted urine; C, diluted urine + glutathione; D, diluted urine + cysteine.

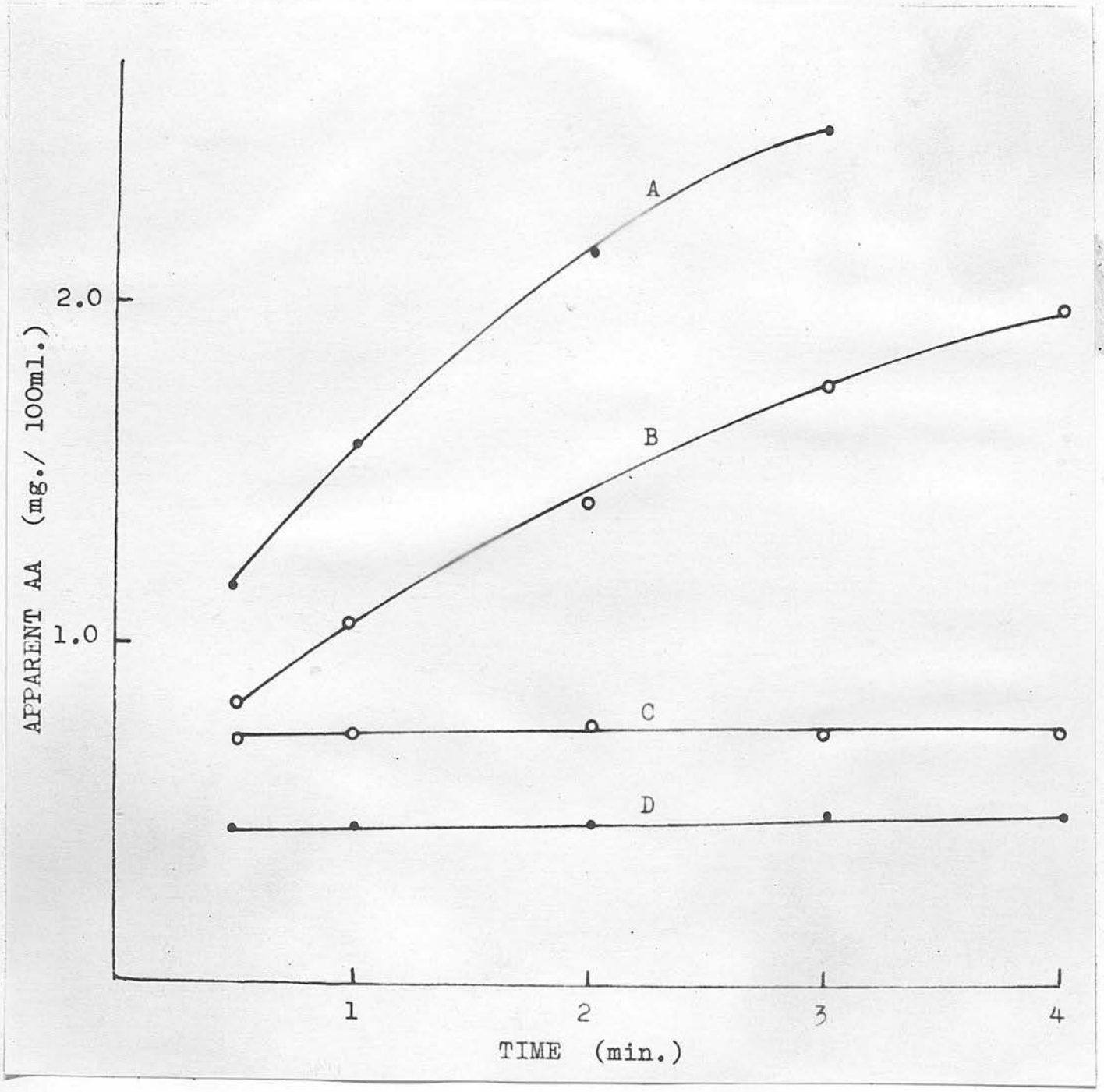


Fig.16. The effect of CMB on the indophenol reducing activity of rat liver and kidney filtrates. A, kidney filtrate; B, liver filtrate; C, liver filtrate + CMB; D, kidney filtrate + CMB.

TABLE 7. The effect of CMB on the indophenol reducing activity of erythrocytes

Apparent AA		Total AA
'Buffered method' (mg/100ml)	CMB method (mg/100ml)	Roe method (mg/100ml)
0.22	0	0.11
0.29	0.02	0.11
0.17	0.07	0.16
0.16	0.07	0.16
0.58	0.04	0.47

TABLE 6. The effect of CMB on the indophenol reducing activity of sulphur compounds added to biological materials

The compounds were added in amounts calculated to give an indophenol reducing activity approximately equal to 1mg AA/100ml. Results in terms of AA (mg/100ml).

Material	Additions				
	None	Glutathione (12mg/100ml)	Cysteine (5mg/100ml)	H_2S (1.4mg/100ml)	$Na_2S_2O_3$ (0.4mg/100ml)
Plasma filtrates	1.11	1.12	1.12	1.11	1.12
	1.01	1.01	1.06	1.02	1.04
	1.06	1.12	1.12	1.06	1.04
Erythrocyte filtrates	0.58	0.60	0.61	0.63	0.58
	0.53	0.58	0.49	0.50	0.54
	0.78	0.77	-	0.72	0.75
Diluted urine	0.79	0.77	0.79	0.75	0.77
	1.00	1.00	1.00	1.00	1.08
	0.68	0.66	0.58	0.64	0.64
Diluted liver filtrate	1.13	1.13	1.13	1.13	1.14

When glutathione, cysteine, H_2S and thiosulphate, in amounts calculated to give a reducing activity equivalent to approximately 1 mg./100ml. AA, were added to filtrates of plasma, erythrocytes, urine and rat liver, their reducing effect was completely inhibited by the addition of sufficient CMB. (Table 6).

Oxidation of ascorbic acid by oxyhaemoglobin.

It is well known that precipitation of erythrocyte protein not previously saturated with CO leads to the loss of AA due to its oxidation by the haemoglobin released. The usefulness of CMB as a means of removing interfering substances is well illustrated by a comparison of AA values of erythrocytes not treated with CO measured by the "buffered indophenol" method with and without CMB (Table 7). Practically no AA was detected in the protein-free filtrates after CMB treatment whereas a large amount of "apparent AA" was measured in the absence of CMB. Comparison of the values obtained by the CMB method and by the Roe method for total AA shows that practically all the AA had been oxidised to DHA by the precipitation procedure. The values obtained by the "buffered indophenol" method without CMB could not represent the true AA content. Oxidation of AA also occurs in plasma after slight haemolysis. The AA content of 1 plasma measured by the "buffered indophenol" method was reduced from 0.88 mg./100ml. to 0.62 mg./100ml. after slight intentional haemolysis.

TABLE 8. Comparison of different methods for the determination of of total AA in plasma Results as mg/100ml.

Roe method (a)	Roe method HPO_3 (b)	Roe method modified (c)	Bolin & Book method (d)	Bolin & Book modified method (e)
0.04	-	0.02	-	-
0.09	0.09	0.09	0.07	0.09
0.13	0.11	0.11	0.13	0.10
0.20	0.25	0.20	0.18	0.20
0.22	0.21	0.26	0.23	0.24
0.27	0.23	0.27	0.29	0.27
0.29	0.24	0.27	0.29	0.28
0.36	0.35	0.34	0.36	0.40
0.41	0.50	0.44	0.46	0.46
0.55	0.46	0.53	0.54	-
0.61	0.65	0.62	0.71	0.65
0.65	0.61	0.64	0.66	0.66
0.79	0.85	0.80	0.87	-
0.81	-	0.83	-	-
0.84	0.91	0.91	0.95	0.86
1.01	1.06	1.01	1.07	1.05
1.08	0.89	1.09	1.08	1.13
1.13	1.02	1.10	1.12	1.15
1.26	1.13	1.31	1.26	1.30
Mean difference from (a)	-0.02	0.00	+0.02	+0.02

Estimation of total AA using 2:4-dinitrophenylhydrazine.

The determination of total AA in biological materials by the method of Roe & Kuether involves the following procedures; (a) preparation of extracts, (b) oxidation of AA, (c) incubation with 2:4-dinitrophenylhydrazine to form the ozazone, (d) colour development with H_2SO_4 and (e) colorimetry of the resulting red brown material. Each of these procedures has been examined critically using human plasma and in some instances urine.

(a) Preparation of the extract.

Trichloroacetic acid, 6% and metaphosphoric acid, 3% were tested for their suitability as media for carrying out the dinitrophenylhydrazine procedure. To 1 vol. of plasma was added 3 vol. of 6% trichloroacetic acid, mixed thoroughly, allowed to stand for 10 min. and then filtered. Metaphosphoric acid filtrates were prepared by adding 2 vol. of plasma to 3 vol. of 3% metaphosphoric acid, mixed, stood for 10 min. and filtered. To 4 ml. of the filtrate was added 1 ml. of 30% trichloroacetic acid so that the final concentration of trichloroacetic acid was the same as in the trichloroacetic acid filtrates. Both filtrates were then treated with charcoal, dinitrophenylhydrazine and H_2SO_4 as in the original Roe & Kuether method. The mean difference between the total AA estimated in 17 plasma precipitated with trichloroacetic acid and those precipitated with metaphosphoric acid was 0.02mg/100ml. (range -0.21 to +0.09) (Table 8 column a & b). Thus precipitation of the plasma proteins with metaphosphoric acid instead of with trichloroacetic acid

TABLE 9. The effect of charcoal on the recovery of AA added to plasma filtrates

Material	Percentage recovery		
	0.25g.charcoal	0.45g.charcoal	0.9g.charcoal
Plasma filtrate + 1.0mg AA/100ml	103	102	100
Plasma filtrate + 2.0mg AA/100ml	106	101	98

caused a slight lowering of the AA content. It was necessary to add trichloroacetic acid to the metaphosphoric acid filtrate to ensure the oxidation of AA.

Oxidation of AA.

The effect on the recovery of AA added to trichloroacetic acid filtrates of plasma of adding different amounts of charcoal was examined. The amounts added were 0.45 g. as originally suggested by Roe & Kuether and quantities twice and one-half this amount. (Table 9). The recoveries of 1 mg. and 2 mg./100ml. AA using 0.25 g. charcoal were respectively 1% and 5% higher than when 0.45g. charcoal was used. The recoveries were 2% and 2.5% lower when 0.9 g. was used. It seems likely therefore that a small quantity of AA may be oxidised beyond DKG or that it may be adsorbed onto the charcoal if too much is used. Either alternative would lead to an under estimation of AA. On the other hand, charcoal may remove a small amount of interfering substance which would also account for the higher recoveries of AA when the smaller quantities of charcoal were added. Adding the charcoal to the trichloroacetic acid before adding the plasma did not interfere with the estimation of total AA. (Table 8 columns a & c). On the basis of these results it was decided to omit filtration before adding the charcoal.

Oxidation of AA by indophenol in metaphosphoric acid extracts of plasma was compared with charcoal oxidation of AA in trichloroacetic acid filtrates, using the Bolin & Book method of indophenol oxidation. A slight modification, in which one drop of conc. thiourea replaced the 2 ml. of metaphosphoric

acid - thiourea reagent was used to reduce the final volume by one-half, did not alter the recovery of added AA. The total AA in 16 plasmas measured by the Bolin & Book method and the modification of it are compared with the values obtained by the Roe & Kuether method in Table 8. The mean difference from the values obtained by the Roe & Kuether method was ±0.02mg./100ml. for both the Bolin & Book method and its modification, (range -0.02 to +0.10 and -0.03 to +0.05 respectively). Indophenol oxidation of AA in metaphosphoric acid filtrates may therefore slightly overestimate the total AA content.

Incubation procedure.

The rate of coupling of AA derivatives with dinitrophenylhydrazine was tested by incubating trichloroacetic acid filtrates of plasma and urine after charcoal incubation for times up to 6 hr. The rates were compared with the coupling rate of a standard solution containing 1 mg./100ml. AA which had been treated in exactly the same way. (Fig.17). Six replicates of the charcoal treated filtrates of plasma, urine and the standard treated with thiourea and dinitrophenylhydrazine were placed in the incubator at 37°C. One replicate of each was withdrawn after 0.5, 1, 2, 3, 4, and 6 hr., cooled immediately in ice and H_2SO_4 was added. After 0.5 hr. the optical density of the resultant red brown colour was read at 540 mμ and the results are plotted against time in Fig.17. In the first half hour of incubation the rates of coupling with dinitrophenylhydrazine were the same in urine and plasma, but thereafter the rate of coupling was slower in the urine than in plasma. After 2 hr.

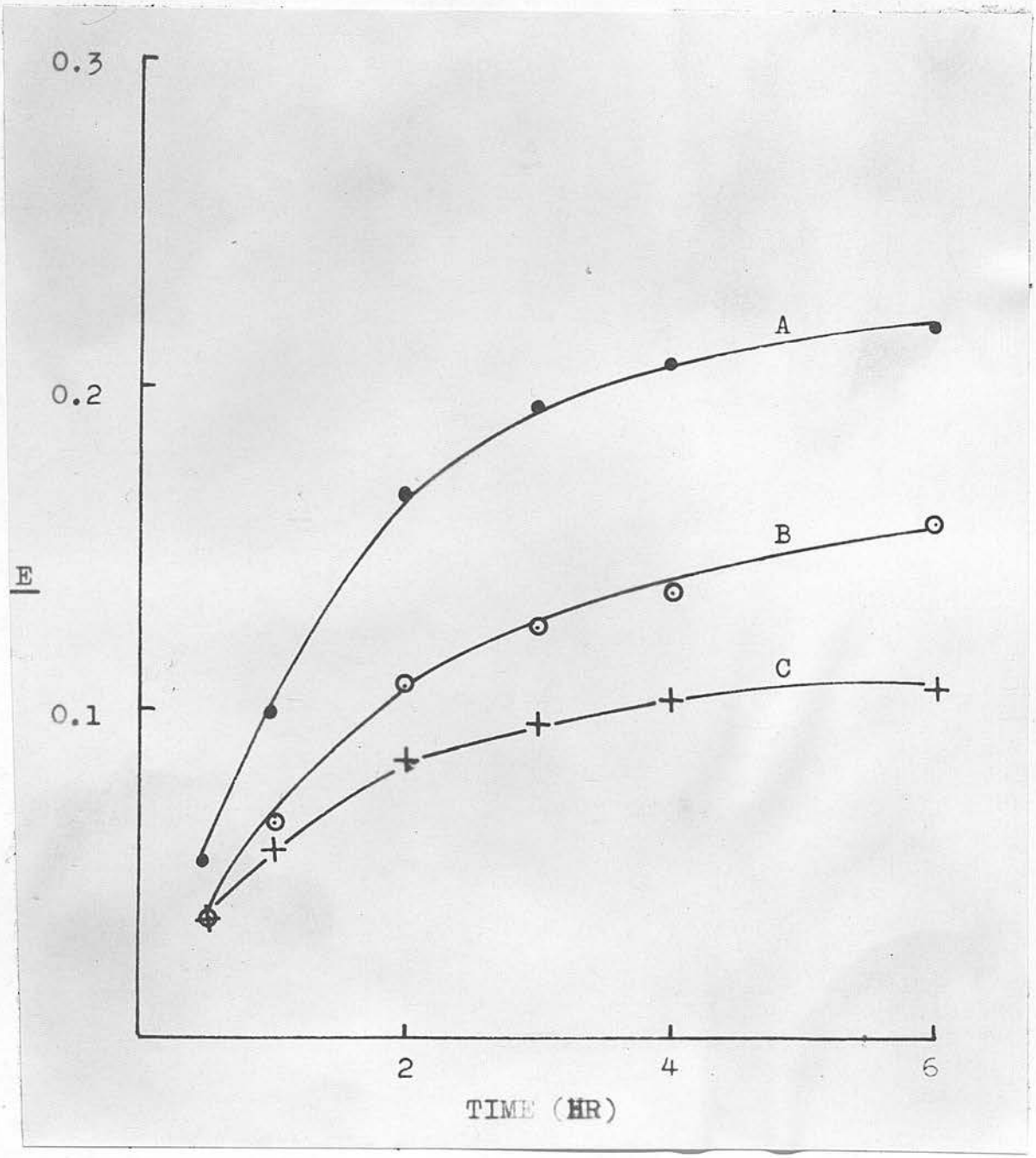


Fig.17. The rate of formation of the dinitrophenylhydrazine derivatives in an AA solution, in a plasma filtrate and in diluted urine. A, AA (1mg./100ml.); B, plasma filtrate; C, diluted urine.

TABLE 10. The effect of duration of incubation on the total AA content of plasma and urine Results as mg./100ml.

Hours of incubation		0.5	1.0	2.0	3.0	4.0	6.0
Plasma	total AA	0.63	0.62	0.62	0.64	0.64	0.64
Urine	total AA	0.63	0.55	0.49	0.49	0.49	0.49

TABLE 11. The effect of temperature of incubation on the total AA content of plasma Results as mg./100ml.

Incubation temp. 37°	Incubation temp. 60°	Difference
0.13	0.10	-0.03
0.19	0.20	+0.01
0.21	0.15	-0.06
0.25	0.27	+0.02
0.28	0.32	+0.04
0.30	0.32	+0.02
0.35	0.35	0.0
0.37	0.37	0.0
0.38	0.38	0.0
0.40	0.40	0.0
0.51	0.49	-0.02
0.55	0.60	+0.05
0.72	0.77	+0.05
0.75	0.86	+0.11
0.86	0.87	+0.01
0.87	0.95	+0.08
0.92	0.89	-0.03
0.95	0.98	+0.03
1.10	1.16	+0.06
1.25	1.33	+0.08
1.60	1.69	+0.09
Range 0.13 - 1.60	Range 0.10 - 1.69	Mean = +0.025

the rates of coupling in plasma and the standard were the same. The final concentration of AA in plasma and urine calculated from the corresponding standard at the appropriate times of incubation are given in Table 10. An incubation time of 3 to 4 hr. was selected as the most satisfactory time for plasma and urine. Increasing the temperature of incubation to 60°C and reducing the time from 3 hr. to 40 min. as suggested by Geschwind has been examined. The total AA content of 21 plasmas after incubation of the filtrates at 37°C for 3 hr. and at 60°C for 45 min. are given in Table 11. After incubation at 60°C the range of values is wider and the mean slightly higher. It was decided that slower incubation at 37°C gives more consistent results and this procedure was adopted.

Colorimetry

Roe & Kuether suggested that after the addition of H_2SO_4 to the incubated samples development of the brownish red colour should be allowed to continue for one-half hour before reading the optical density. The absorption spectrum of this coloured material in plasma, erythrocytes and urine extracts has been examined one-half hr., 18 and 120 hr. after adding the H_2SO_4. The absorption spectra of the resultant colour in a standard solution containing 0.8mg./100ml. AA and in a plasma filtrate are given in curves A & B respectively of Fig.18; the corresponding blanks are shown in curves D & C. The peak absorption of the AA derivative, reported to be at 518 mμ, is very flat in curves A & B. However, subtraction of the blank curves D & C gives curve E for the material in plasma and curve F for the

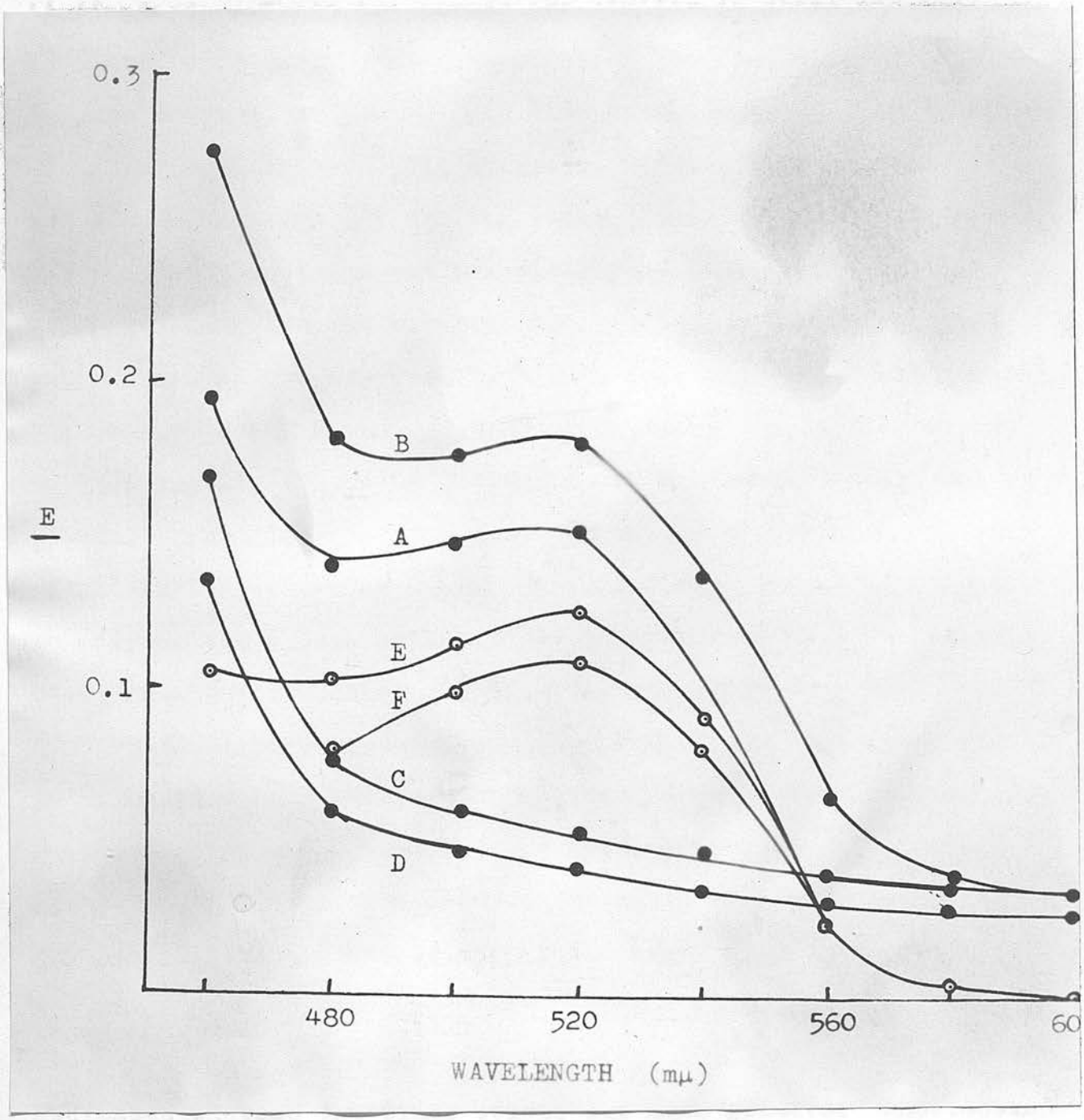


Fig.18. Absorption spectra of dinitrophenylhydrazine derivatives. A, AA (0.8mg./100ml.); B, plasma filtrate; C, plasma blank; D, control blank; E, B- C; F, A - D.

AA standard. These two curves are similar in shape and are characteristic for the AA derivative. The stability of the colour, measured at 540 mμ, was then examined at 18 hr. and 40 hr. after the addition of H_2SO_4 (Fig.19). The optical density of the plasma material (A) and its blank (E) increased slowly and steadily whereas there was practically no increase in the standard blank (F) and the standard itself (B) slowly decreased. Subtraction of the appropriate blanks from the plasma and standard values i.e. C and D, showed that there was a slight fall in the optical density of the AA derivative in both the standard and the plasma. The nature of the material in plasma which causes the blank reading and which slowly increases with time is not known. In general the plasma blank is little different from the standard blank in the first half hour after adding the H_2SO_4.

The absorption spectra and the stability of the derivatives in urine were then examined. The optical density at 540 mμ of a concentrated urine (total AA equivalent to 12 mg./100ml.) and of a blank prepared from it, were measured at intervals up to 120 hr. (Fig.20). The colour of the urine blank read at 0.5 hr. after the addition of H_2SO_4 was equivalent to 2 mg./100ml. AA. After 120 hr. the optical density of the urine had scarcely decreased whereas the blank colour was much greater. Subtraction of the optical density of the blank from that of the urine after 18 hr. showed that a considerable fading of the urinary AA had occurred. Similar curves were obtained for 2 other diluted urines (Fig.21) and again the urine blank samples contained a greater quantity of colour material than did the standard blank.

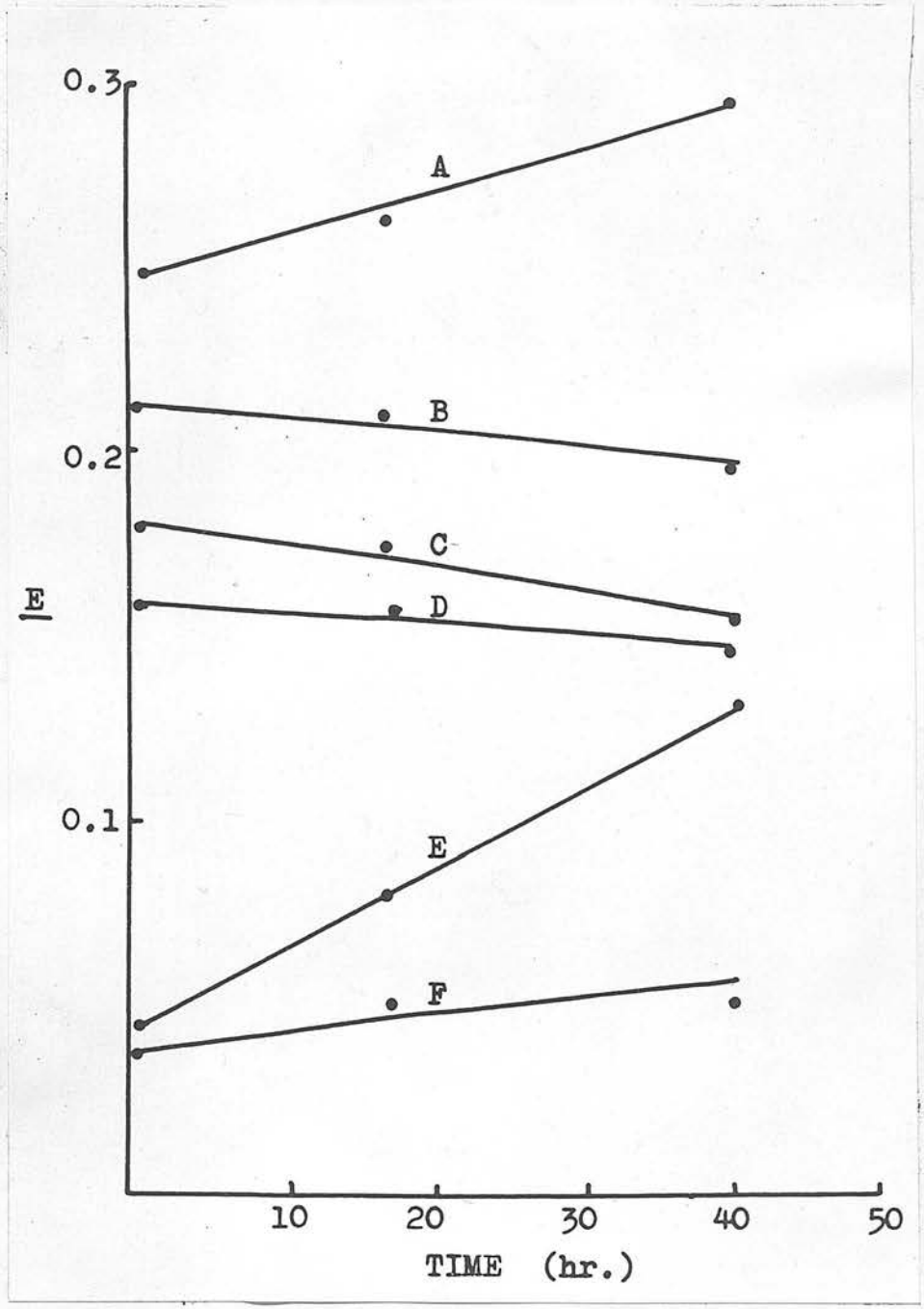


Fig.19. The stability of the dinitrophenylhydrazine derivatives of AA and plasma in 85% (w/v) H_2SO_4. A, plasma; B, AA (0.8mg./100ml.); C, A - E; D, B - F; E, plasma blank; F, control blank.

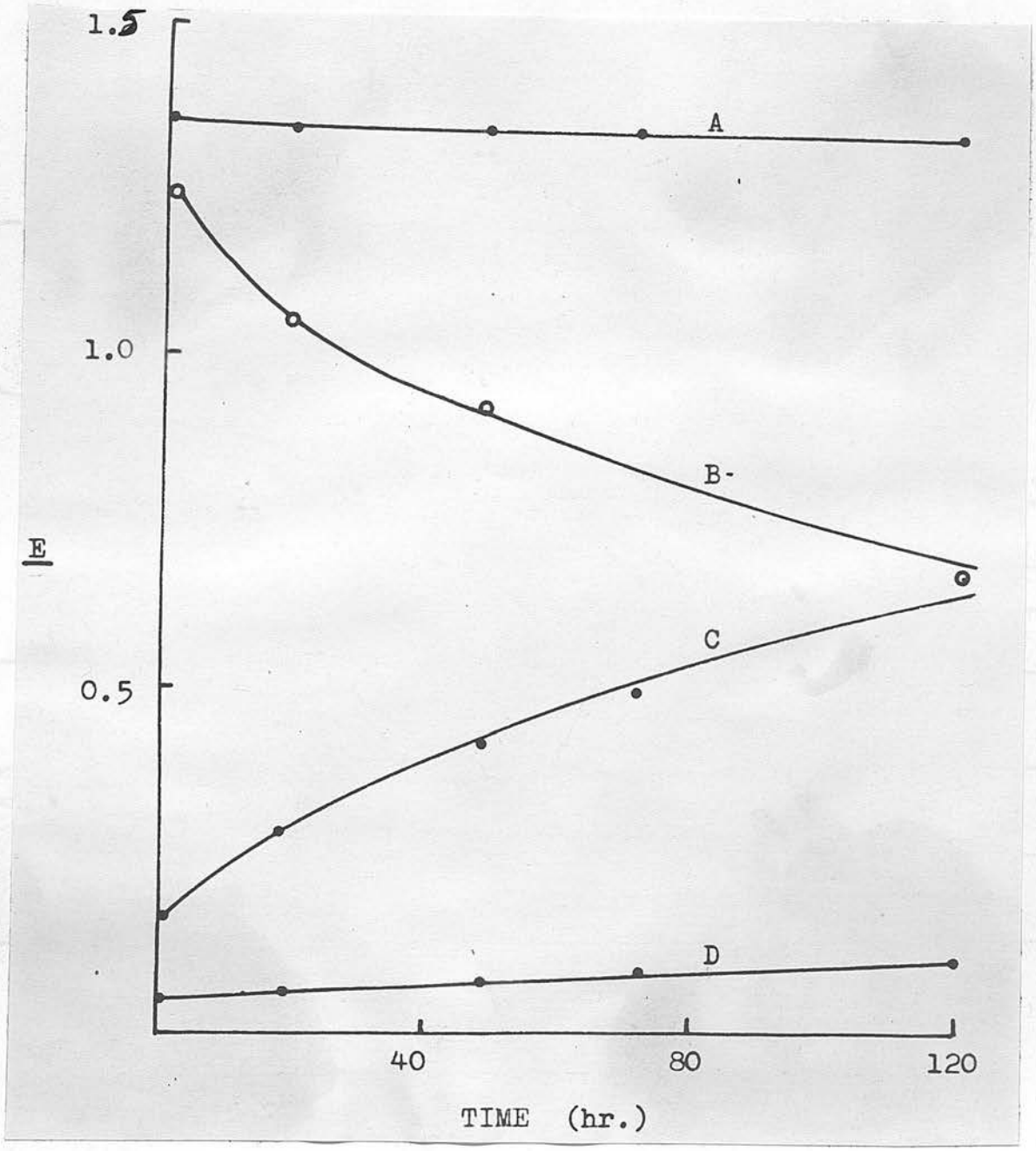


Fig.20. The stability of dinitrophenylhydrazine derivatives in urine. A, urine; B, urine - urine blank; C, urine blank; D, control blank from an AA standard (2mg./100ml.).

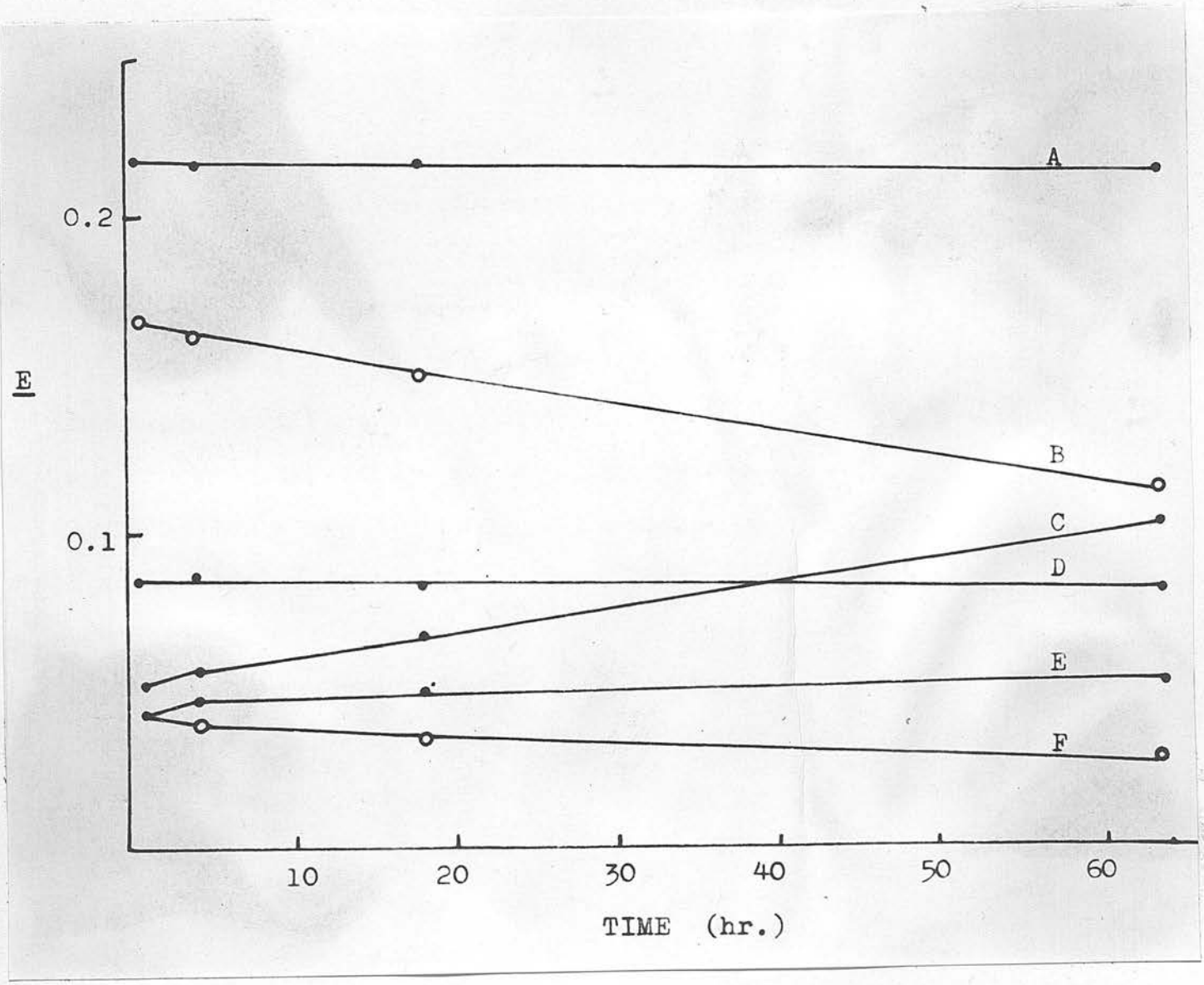


Fig.21. The stability of dinitrophenylhydrazine derivatives in 2 diluted urines. A, urine 1; B, A - C; C, urine blank; D, urine 2; E, D - F; F, urine 2 blank.

That the increase in optical density of the blank is probably not due to a derivative formed by slow coupling of AA with phenylhydrazine is shown in Fig.22. The absorption spectrum of the blank colour 18 hr. after the addition of H_2SO_4 lacked the peak at 518 mμ characteristic of the AA derivative, whereas the absorption spectrum of the urine derivative after subtraction of its blank is typical of AA even after 18 hr. These experiments clearly show that there is some material other than AA in urine which gives a slowly developing colour with phenylhydrazine. In the urine examined, this material was equivalent to as much as 10% of the total colour and after 120 hr. it accounted for one half the total. Although the colour of the AA derivative is stable for 4 hr. (Roe & Kuether) the most accurate estimate of total urinary AA is obtained only if the colour in the urine and the blank are measured one-half hour after the addition of H_2SO_4. In plasma, the blank colour is the same one-half hour after the addition of acid as that obtained from a tichloroacetic acid solution of AA treated in the same manner. However the plasma blank slowly increases with time and it is therefore advisable to read all the colours after they have developed for one-half hour. Examination of the absorption spectra of erythrocyte filtrates and blanks prepared from them showed that these solutions behaved in the same manner as did the plasma solutions.

Measurement of total AA using H_2S

Many workers have suggested the use of H_2S for the estimation of total AA in biological extracts. Measurement of the indophenol

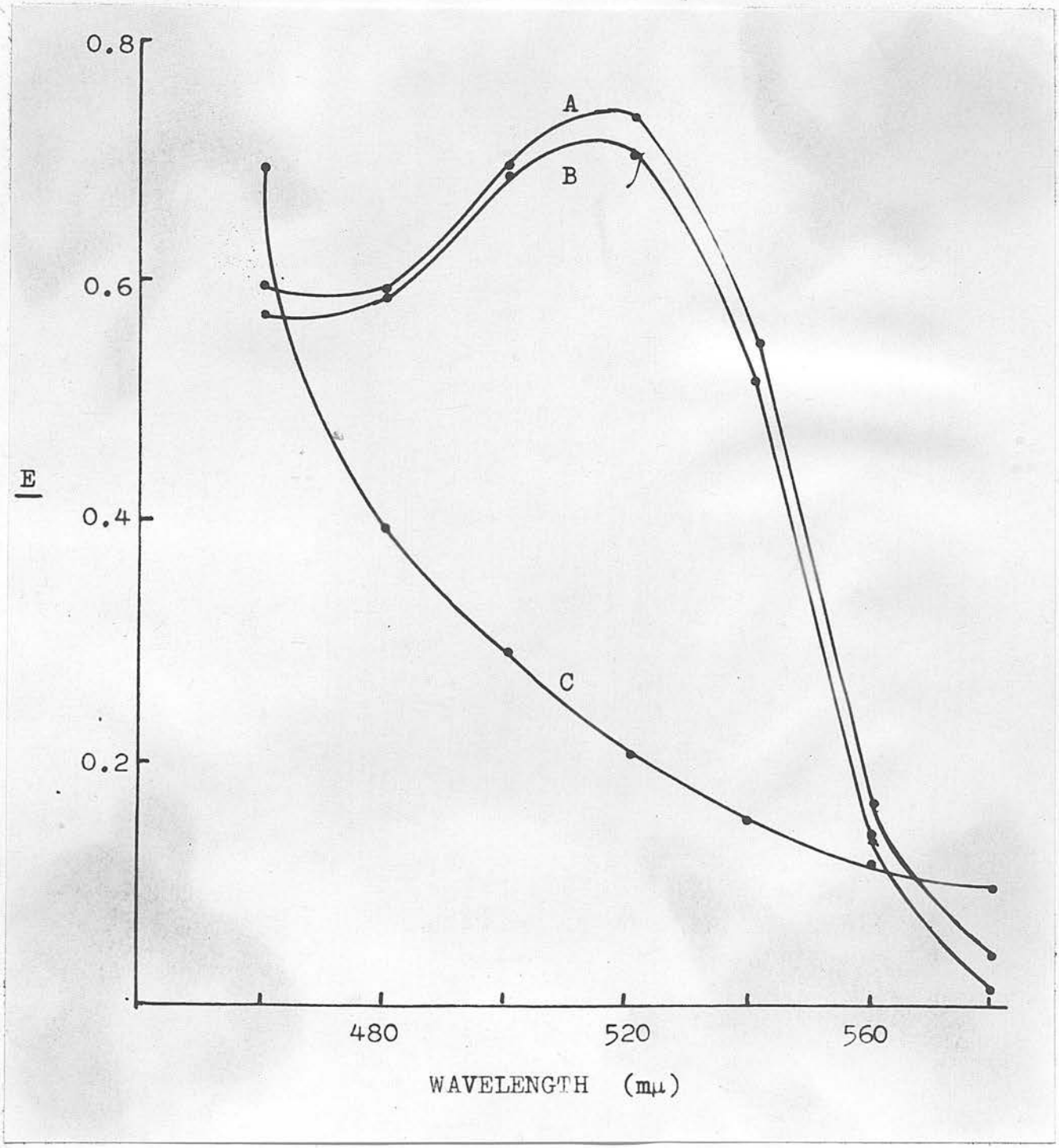


Fig.22. Absorption spectra of urinary dinitrophenylhydrazine derivatives after 18 hr. A, urine - urine blank after 1 hr.; B, urine - urine blank after 18 hr.; C, urine blank after 18 hr.

TABLE 12. The recovery of DHA added to plasma

Results as mg./100ml.

Material	DHA added	Buffered indophenol method: Before H_2S	Buffered indophenol method: After H_2S pH 1.8	Buffered indophenol method: After H_2S pH 3.5	Roe method
Plasma A	None	0.82	1.06	1.10	0.88
	0.5	0.82	1.12	1.74	1.38
Plasma B	None	1.12	1.26	1.45	1.11
	0.5	1.12	1.39	2.21	1.60
	1.0	1.12	1.47	3.11	2.60

TABLE 13. Comparison of the H_2S method with the indophenol and Roe methods. Results as mg. of AA/100ml.

Material	Buffered indophenol method	Buffered indophenol method after H_2S at pH 1.8	pH 3.5	+CMB	Roe method
Plasma	0.63	1.01	1.01	-	0.58
filtrates	0.88	1.00	-	-	0.85
	0.59	0.70	-	-	0.49
	1.02	1.22	-	-	1.01
	0.99	1.15	-	-	0.92
	0.16	-	0.34	-	0.14
	0.26	-	0.46	-	0.31
	0.19	-	0.59	-	0.09
	0.17	-	0.37	-	0.15
Erythrocyte	0.45	-	0.72	0.30	0.28
filtrates	0.39	-	0.42	0.13	0.08
	0.50	-	0.64	0.19	0.14

reducing activity before and after treatment with H_2S allows both reduced and total AA to be estimated in a sample. The difference between these two values is also used to calculate the quantity of DHA. The interpretation of results obtained by this method is made difficult, however, by the fact that H_2S reduces other materials in addition to DHA.

In several experiments the AA content of plasma and erythrocyte extracts was estimated with this method. Hydrogensulphide treatment of metaphosphoric acid solutions of DHA and AA buffered to pH 3.5 showed that 95 to 105% of these substances could be recovered. Very low recoveries of DHA were obtained when the solution was not buffered (pH approx. 1.8). The results obtained when known amounts of DHA were added to plasma are given in Table 12. Ascorbic acid was meaured by the "buffered indophenol" method before H_2S treatment, after H_2S treatment at pH 1.8 and 3.5 and by the Roe & Kuether method. Treatment with H_2S increased the indophenol value and at pH 3.5 the indophenol value was much more than the total AA value. This increase was considerably more than the theoretical increase due to the addition of DHA. The added DHA was not recovered after H_2S treatment at pH 1.8. None of the solutions gave a positive nitroprusside or lead acetate test so that no detectable H_2S remained in the solutions, which had had wet N_2 bubbled through them for $3\frac{1}{2}$ hr.

Table 13 shows the AA content of 9 plasma filtrates and 3 erythrocyte filtrates measured by the "buffered indophenol" method before H_2S treatment and after H_2S treatment at pH 1.8 or 3.5. The total AA measured by the Roe & Kuether method is

also given. For all the filtrates an increase in the indophenol value was observed after treatment with H_2S. It is unlikely that this increase was entirely due to DHA because the values were all considerably higher than the Roe & Kuether values. A faint positive nitroprusside test was observed in the cell filtrates even after they had been treated with N_2 for 3 hr. The last traces of H_2S are probably more difficult to remove from cell filtrates than from plasma or standard solutions. It has already been shown that erythrocytes contain substances that react slowly with indophenol. Most probably treatment with H_2S increases the amount of interfering materials with indophenol reducing activity.

The results of these experiments were so forbidding that it was thought unprofitable to investigate further the H_2S method. More suitable conditions for the procedure may exist but the conditions used in the above experiments are those reported in the literature as optimal. Application of the CMB reagent to H_2S treated filtrates would eliminate interference due to traces of H_2S. The results of its use with 3 erythrocyte filtrates after H_2S treatment are given in Table 13. Most of the interference due to S compounds, either traces of H_2S or thiols formed by H_2S reduction, has been removed. However the AA values are still slightly higher than those obtained by the Roe & Kuether method.

<u>The estimation of the vitamin C content of plasma erythrocytes and urine by different methods</u>.

In the past, opinions have differed on whether any of the

TABLE 14. The DHA content of plasma determined by the difference between the Roe method and the 'buffered' indophenol method

Results as mg./100ml.

Roe method	Buffered method	DHA	Roe method	Buffered method	DHA
0.03	0.09	-0.06	0.54	0.59	-0.05
0.04	0.02	+0.02	0.55	0.49	+0.06
0.06	0.21	-0.15	0.55	0.49	+0.06
0.09	0.05	+0.04	0.58	0.63	-0.05
0.09	0.19	-0.10	0.61	0.63	-0.02
0.10	0.09	+0.01	0.64	0.55	+0.09
0.13	0.05	+0.08	0.65	0.64	+0.01
0.13	0.16	-0.03	0.70	0.69	+0.01
0.14	0.16	-0.02	0.75	0.75	0.0
0.15	0.17	-0.02	0.79	0.80	-0.01
0.16	0.13	+0.03	0.81	0.91	-0.10
0.19	0.14	+0.05	0.82	0.91	-0.09
0.20	0.08	+0.12	0.84	0.89	-0.05
0.22	0.21	+0.01	0.85	0.88	-0.03
0.27	0.22	+0.05	0.86	0.80	+0.06
0.28	0.23	+0.05	0.87	0.87	0.0
0.29	0.27	+0.02	0.92	0.91	+0.01
0.29	0.19	+0.10	0.95	0.95	0.0
0.30	0.40	-0.10	0.95	0.99	-0.04
0.31	0.26	+0.05	1.01	1.08	-0.07
0.35	0.32	+0.03	1.01	1.02	-0.01
0.36	0.36	0.0	1.08	1.07	+0.01
0.37	0.40	-0.03	1.08	1.14	-0.06
0.38	0.31	+0.07	1.10	1.04	+0.06
0.40	0.40	0.0	1.10	1.09	+0.01
0.41	0.45	-0.04	1.13	1.16	-0.03
0.44	0.45	-0.01	1.20	1.13	+0.07
0.44	0.44	0.0	1.26	1.30	-0.04
0.49	0.59	-0.10	1.37	1.40	-0.03
0.51	0.50	+0.01	1.57	1.33	+0.24
0.51	0.35	+0.16	1.60	1.75	-0.15

Mean = +0.002

vitamin C in blood exists in the oxidised state i.e. as dehydro-ascorbic acid. Until 1943 when Roe & Kuether suggested their phenylhydrazine method, the vitamin had been estimated as total AA by the H_2S method. This method, as has just been shown, cannot be considered sufficiently reliable as a means for calculating the DHA content of tissue extracts; more so because the analysis necessarily depends on a determination by difference. It is at present generally agreed that a small fraction of blood vitamin C is present as DHA. However in the absence of a specific chemical method for the estimation of DHA any statement of the actual amount of DHA present in blood or its constituents must be regarded with much caution.

(a) Plasma

In the course of the present investigation the AA content of a number of plasmas (heparinised or oxalated) from normal subjects was determined by several methods. In Table 14 are presented the analyses of 62 plasmas by the "buffered indophenol" method and by the Roe & Kuether method. DHA is calculated by difference The mean DHA found in this series was 0.002 mg./100ml. (S.D.= 0.07, range -0.15 to +0.24.) The total AA content ranged from 0.03 to 1.60 mg./100ml. with a mean of 0.59. The mean difference between the AA content measured by the buffered and the Roe & Kuether method was not significant, ($t=0.05, n=62, p=0$). The correlation coefficient ($r=0.046$) was not significant, so that there was no measurable DHA at any plasma level of total AA.

The same statistical tests were applied to a series of 49 plasmas (Table 15) in which the total AA was measured by the

TABLE 15. The DHA content of plasma determined by the difference between the Roe method and the 'unbuffered' indophenol method

Results as mg./100ml.

Roe method	Unbuffered method	DHA	Roe method	Unbuffered method	DHA
0.04	-0.05	+0.09	0.61	0.56	+0.05
0.09	-0.14	+0.23	0.65	0.58	+0.07
0.13	-0.01	+0.14	0.70	0.70	0.0
0.19	0.15	+0.04	0.75	0.75	0.0
0.20	-0.02	+0.22	0.79	0.67	+0.12
0.22	0.16	+0.06	0.81	0.85	-0.04
0.27	0.16	+0.11	0.82	0.85	-0.03
0.29	0.11	+0.18	0.84	0.80	+0.04
0.30	0.20	+0.10	0.85	0.75	+0.10
0.35	0.36	-0.01	0.87	0.87	0.0
0.36	0.24	+0.12	0.92	0.88	+0.04
0.37	0.27	+0.10	0.95	0.85	+0.10
0.38	0.46	-0.08	0.95	0.88	+0.07
0.40	0.33	+0.07	1.01	0.91	+0.10
0.41	0.29	+0.12	1.01	1.06	-0.05
0.44	0.44	0.0	1.08	1.08	0.0
0.44	0.35	+0.09	1.08	1.10	-0.02
0.44	0.33	+0.11	1.10	1.03	+0.07
0.49	0.43	+0.06	1.10	1.09	+0.01
0.51	0.35	+0.16	1.13	1.12	+0.01
0.51	0.10	+0.41	1.20	1.10	+0.10
0.54	0.63	-0.09	1.26	1.29	-0.03
0.55	0.32	+0.23	1.37	1.35	+0.02
0.55	0.50	+0.05	1.60	1.75	-0.15
0.58	0.50	+0.08			

Mean = +0.06

Roe & Kuether method and the reduced AA was measured by the "unbuffered indophenol" method. In this series the mean DHA was 0.065 mg./100ml. (S.D.=0.09, range -0.15 to +0.41). The estimation of AA by the unbuffered method gave values which were significantly lower ($t=5$, $n=49$, $p=<.001$) than those obtained by the Roe & Kuether method. There was a significant negative correlation ($r=-0.36$) between the total AA values (mean 0.59, range 0.04 to 1.60), and the calculated DHA values.

When the concentration of total AA in plasma was 0.2mg./100ml. or less, the optical density measured by the "unbuffered indophenol" method of the filtrates was greater than the optical density of the blank solution and extrapolation of the standard curve beyond zero gave the negative values for unbuffered indophenol analyses shown in Table 15. These negative values are undoubtedly due to the difference in the rates of fading of indophenol at low pH in the plasma solution and in the standard solutions, as shown earlier in Figs. 2 & 8. Because the pH of metaphosphoric acid extracts of plasma is probably slightly higher than that of metaphosphoric acid solutions of the standards, the rate of fading of indophenol added to the solutions is less in the former than in the latter. This effect is more obvious when the AA concentration is very low since there is insufficient AA present to cause a partial stabilisation of the dye. The difference between the Roe & Kuether and the "unbuffered" values is therefore unlikely to be a true measure of the DHA content, particularly when the AA concentration is very low.

TABLE 16. Comparison of methods for the determination of vitamin C in erythrocytes

Results as mg./100ml.

Apparent AA			Total AA	DHA
'Buffered' method	CMB method	Difference	Roe method	Roe - CMB
0.39	0.11	0.28	0.08	-0.03
0.30	0.17	0.13	0.08	-0.09
0.27	0.12	0.15	0.10	-0.02
0.50	0.10	0.40	0.14	0.04
0.21	0.14	0.07	0.18	0.04
0.36	0.18	0.18	0.20	0.02
0.27	0.18	0.09	0.23	0.05
0.45	0.21	0.24	0.28	0.07
0.21	0.20	0.01	0.29	0.09
0.79	0.34	0.45	0.44	0.10
0.62	0.25	0.37	0.47	0.22
1.39	0.94	0.45	1.19	0.25
Mean 0.47	0.25	0.22	0.31	0.06

(b) Erythrocytes

In a smaller series of experiments the vitamin C content of human erythrocytes (saturated with CO) was determined using the Roe & Kuether "buffered indophenol" and the CMB methods (Table 16.) In every instance, except one, the "buffered indophenol" method gave values which were considerably higher than those obtained by either the Roe & Kuether or the CMB methods. These higher values are undoubtedly due to the presence in erythrocytes of interfering materials which react with indophenol. When these substances were removed by treatment with CMB more valid AA values were obtained. The mean DHA (calculated by subtracting the CMB value from the Roe & Kuether value) for this series was 0.063mg./100ml. (S.D. = 0.09, range -0.02 to +0.27.) A significant positive correlation, r= +0.903, of the DHA with the total AA (mean 3.68mg./100ml., range 0.08 to 1.19) was found. The estimation of AA by the CMB method gave values which were significantly lower than those given by the Roe & Kuether method ($t = 2.431$, $n = 12$) indicating that by the use of these two methods a significant amount of the total AA in erythrocytes may be DHA. The use of the indophenol method without treatment with CMB is of no value as a method for determining the AA content of erythrocytes.

(c) Urine.

It was considered of interest to examine the vitamin C content of a small number of urines by some of the different methods in order to see if any of the total AA was excreted as DHA. The AA content of diluted urines determined by 5 different methods is given in Table 17 but because so few urines were

TABLE 17. Comparison of the methods for the determination of vitamin C in diluted urine

Results as mg./100ml.

Apparent AA				Total AA
'Buffered method'	'Unbuffered method'	CMB method	HCHO method	Roe method
0.31	-	0.13	-	0.36
0.47	-	0.36	-	0.43
0.53	-	0.42	-	0.42
0.28	-	0.25	-	0.32
0.19	-	0.02	-	0.14
0.48	0.67	-	-	0.26
1.57	2.1	-	-	1.39
0.45	0.52	0.60	0.05	0.50
1.12	1.28	1.55	0.97	1.31
0.37	0.69	0.58	0.97	0.51
0.98	1.32	0.82	0.42	0.46
0.69	0.94	0.68	0.04	0.60
0.22	0.30	0.24	0.30	0.59
1.95	-	-	-	1.49

examined only tentative conclusions can be drawn. Comparing the results obtained by using the "buffered indophenol" the CMB and the formaldehyde methods with those using the Roe & Kuether method it was found that they did not differ significantly ($t = 0.33$, 0, and 0.58 respectively). The "unbuffered indophenol" method however gave values which were significantly higher than the Roe & Kuether values ($t = 5.1$). It is known that both the "unbuffered" and the "buffered indophenol" methods over-estimate the AA content of urine because urine contains interfering materials which react with indophenol. Some but not all the interfering material can be suppressed by the use of CMB. In a larger series in which only CMB and "buffered indophenol" methods were compared it was found that the former method gave values which were 15% lower. The formaldehyde method is reported to remove most of the interfering materials and it was thought that this method might give lower values than either the buffered or unbuffered methods. Significantly lower values were not however obtained probably because of the greater technical difficulties of the method. Probably very little DHA was excreted in the urines examined.

DISCUSSION

Reduction of indophenol is the most widely used method for the measurement of the AA content of mammalian tissues. The inherent difficulties of the method due to the fact that indophenol is reduced by substances other than AA have not always been realised. Van Eekelen & Emmerie (1936) suggested the removal of interfering substances by precipitation with mercuric acetate. Their subsequent removal of excess mercuric ions with H_2S, however, introduces a further source of error. King (1936) has stated that interference caused by glutathione and some other substances can be eliminated if the reaction is carried out in a strongly acidic medium. On the other hand it has been shown by Mindlin & Butler (1937-38) and confirmed in the course of this investigation that indophenol itself fades considerably at low pH. Only when the pH is above 4 does the spontaneous fading of the dye become inappreciable. That AA contributes some stabilising effect to the fading of indophenol has been shown by Mindlin & Butler (1937-38), Lugg (1942) and Stewart, Horn & Robson (1953). The stabilisation is however only temporary and at concentrations less than 1 mg./100ml. it is not completely effective even for a very short time (5 min.) Since the AA content of human plasma and erythrocytes is usually less than this it would seem more advantageous to raise the pH to a value where fading of indophenol does not occur i.e. pH 4, than to rely on the small amounts of AA present for stabilisation. Furthermore the addition of plasma or a solution of AA to 3% (w/v)

metaphosphoric acid does not result in filtrates of identical low pH so that the fading of indophenol with test solutions prepared in this way does not proceed at the same rate as in corresponding standard solutions. By raising the pH to 4 this source of error can be eliminated. The only advantage to be gained by measuring the decolorisation of indophenol by AA at low pH is the suppression of fading due to interfering substances such as glutathione. However, the interference due to some other substances e.g. ergothioneine and thiosulphate is greater at low pH. Evelyn et al. (1938) suggested that a correction for the fading at pH 4 due to interfering substances in urine could be made by taking successive measurements of indophenol reducing activity and extrapolating to zero time. Since this procedure involves a curvilinear and not a linear extrapolation very considerable errors may be introduced.

A better approach to this problem is therefore to measure the decolorisation of indophenol at a pH greater than 3.5 and devise a procedure for the removal of the interfering substances. For this purpose Lugg (1942) proposed the use of formaldehyde which will condense with both AA and many interfering substances to an extent which depends on the pH. AA condenses slowly with formaldehyde at pH 1.5 and rapidly at pH 3.5. Most interfering substances except reductones and hydroxytetronic acid condense rapidly at pH 1.5. After condensation at pH 1.5 and 3.5 the difference in indophenol reducing activity gives a measure of the AA content in the absence of reductones and hydroxytetronic acid. The latter substances are probably not present to any extent in

plasma and erythrocytes. Modifications of this procedure have been used for the determination of AA in urine by Mapson (1953) and by McSwiney, Clayton & Prunty (1954). However Snow & Zilva (1944) have pointed out that careful control of the conditions for the condensation is needed and the procedure itself is extremely laborious (Mapson & Harris, 1947).

The recoveries of added AA are lower when estimated by the formaldehyde method than when using the standard indophenol procedures. The formaldehyde method can therefore be used successfully only when the AA content is relatively high e.g. in urine, (McSwiney et al., 1954) whereas in blood the AA concentration is too low for the method to be used with advantage.

Many of the substances which cause slow fading of indophenol contain sulphur. The presence of such substances e.g. glutathione, cysteine and ergothioneine has been demonstrated in blood and other mammalian tissues and these substances are usually blamed for the slow fading of indophenol in mammalian tissue extracts (Bessey, 1938; King, 1941). It is well known that CMB forms complexes with thiols (Barron & Singer, 1945) and this substance is frequently used as a specific -SH inhibitor. It was thought therefore that the use of CMB might be a satisfactory means of increasing the specificity of the indophenol method for AA. Preliminary experiments showed that CMB did not interfere with the indophenol method when it was applied to aqueous solutions of AA. A linear standard curve was obtained with AA solutions containing 50 mg./100ml. of CMB. When CMB was added to solutions of glutathione, cysteine, ergothioneine, homocysteine, 2:3-dimercaptopropanol, hydrogen

sulphide, thiouea and thiosulphate it completely inhibited the indophenol reducing activity of the compounds. This inhibition persisted for at least 5 min. Although the indophenol reducing activity of sodium sulphite was not completely inhibited it was considerably reduced by CMB. The effect of CMB on the indophenol reducing activity of metaphosphoric acid extracts of plasma, erythrocytes, urine and rat liver to which glutathione, cysteine, H_2S, and thiosulphate had been added, was then examined. CMB again completely inhibited the indophenol reducing activity of these compounds but did not affect the recovery of AA added to the extracts.

The effect of CMB on the indophenol reducing activity of protein-free filtrates of human plasma, erythrocytes and urine and of rat liver and kidney was next examined. CMB had a negligible effect on the activity of plasma filtrates. However slow decolorisation of indophenol was absent in these filtrates which confirms the previously reported absence from plasma of substances which interfere with the indophenol reaction. Metaphosphoric acid extracts of erythrocytes, urine, liver and kidney all contain appreciable amounts of substances which react slowly with indophenol, Treatment with CMB completely inhibited the slow decolorisation of indophenol by the filtrates of erythrocytes, liver and kidney. Presumably the interfering substances in these tissues consist chiefly of S-compounds e.g. glutathione and ergothioneine. The fact that no progressive decolorisation of indophenol occurred after the addition of CMB to these extracts

suggests that the results obtained by this procedure represent the actual AA content. The possibility must be considered that interfering materials which react rapidly with indophenol and are not inhibited by CMB are also present. Mapson & Harris (1947) using a "continuous flow" method for the estimation of AA reported that of a wide range of substances tested only hydroxy-tetronic acid reacted with indophenol at the same rate as AA under similar conditions of pH.

Slow decolorisation of indophenol also occurred with filtrates of urine; this was only partly inhibited by CMB. Mapson (1953) has shown that two groups of interfering substances are present in urine. One group, the S-compounds, rapidly forms complexes with HCHO at pH 0.6. This is the group inhibited by CMB. The second group reacts slowly with HCHO at pH 3.5 but not at all at pH 0.6; the nature of these substances is not known. It is assumed that the second group of substances corresponds to the material not inhibited by CMB and to the material which Evelyn et al. (1938) found to be unaffected by mercuric acetate. Since CMB does not remove all the interfering material present in urine, the formaldehyde method may provide a more accurate method for the estimation of AA in urine.

It is concluded that preliminary treatment of tissue extracts with CMB greatly improves the specificity of the indophenol reaction for the estimation of AA in some biological tissues. The procedure suggested provides a simple, specific method for the measurement of AA in erythrocytes liver and kidney but when applied to urine includes a small amount of interfering material.

The formation of a dinitrophenylhydrazine derivative as suggested by Roe & Kuether (1943) provides a method which is specific for AA and its derivatives. The manipulations required for its use in the separate determination of AA, DHA and DKG (Roe et al., 1948) are, however, cumbersome. For the measurement of reduced and total AA therefore the simplest procedure to use is a reliable indophenol method and the Roe & Kuether (1943) method.

None of the modifications of the original Roe & Kuether method offer any advantages in the determination of total AA in plasma and erythrocytes. When metaphosphoric acid was used in place of trichloroacetic acid for the precipitation of protein slightly lower values were obtained, indicating that oxidation was not complete in these extracts. On the other hand, when indophenol was used in place of charcoal as an oxidising agent in metaphosphoric acid extracts, as suggested by Bolin & Book (1947), slightly higher values were obtained. The quantity of charcoal used for the oxidation has a slight effect on the recovery of AA added to plasma filtrates. If too little charcoal is used slightly higher results are obtained. In contrast when larger quantities of charcoal were used the percentage recovery of added AA was less. These results may be explained either by the assuption that a little AA is absorbed onto the charcoal or that some oxidation beyond DHA occurred. The charcoal itself may remove a small amount of interfering material.

Increasing the temperature of the coupling to about 60°C as suggested by Geschwind et al. (1951) gave somewhat less consistent results and its only advantage is the saving of time. Further

increase of the temperature to 100°C for 5 min. (Meyer, Haselbach & Boissonnas, 1952; Shaffert & Kingsley, 1955) could result in poor reproducibility since small errors in the time would be important. This has been demonstrated by Glick, Alpert & Stecklein (1953).

The omission of thiourea and the use of glacial acetic acid in place of 85% H_2SO_4 (Bolomey & Kemmerer, 1946, 1947) was soon refuted by Mills & Roe (1947) who pointed out the necessity for a reducing substance to prevent interference in the final colour development by oxidants such as Fe^{+++} and H_2O_2. Interference by sugars such as glucose and fructose is diminished by the use of 85% H_2SO_4, in which medium, the derivatives of the sugars fade in half an hour to such a low level that they cause practically no interference at a wavelength of 540 mμ.

It has been confirmed that the peak absorption of the ascorbic acid derivative is at 518 mμ and that in plasma, erythrocytes and urine any absorption due to interfering substances occurs at lower wavelengths. The optical density of the interfering substances increases slowly with time whereas that of the ascorbic acid derivative decreases. This phenomenon is most evident in urine extracts where the colour of the interfering materials can account for as much as 10% of the total colour developed after half an hour and after 120 hr. it may represent 50% of the total colour. That this increase in colour is not due to slow coupling with AA in the blank solution was shown by the absence of the characteristic AA peak at 518 mμ even after 120 hr. Furthermore, standard AA solutions to which dinitrophenylhydrazine was added after incubation did not increase at

this rate. Although the AA derivative is reported by Roe & Kuether (1943) to be stable for 4 hr. it seems advisable to read the optical density of the urine solutions not later than half an hour after the addition of the acid.

The only other widely used method for the determination of total AA depends on the reduction of DHA by H_2S and the subsequent measurement of the total indophenol reducing capacity. Serious doubt is cast on the validity of this method since H_2S itself reduces indophenol and although the last traces of H_2S can be removed from metaphosphoric acid solutions of pure DHA by treatment with wet N_2 for 3 hr., even after a much longer time some H_2S may remain in tissue extracts. Furthermore many workers have reported that treatment of tissue extracts with H_2S may produce interfering material not present originally (Johnson, 1933; Mack & Tressler, 1933; King, 1936; Kellie & Zilva, 1936; Bessey, 1938b). In the present investigation it was found that treatment of filtrates of plasma and erythrocytes resulted in AA values considerably higher than those obtained by the Roe & Kuether method. These values therefore could not represent the true AA content of the extracts. Similar findings on the presence of increased amounts of interfering substances in whole blood (Butler & Cushman, 1940) and in urine (Evelyn et al. 1938) after treatment with H_2S have been reported.

The validity of the H_2S method can be greatly improved by the use of CMB which completely inhibits interference due to traces of H_2S and thiol compounds. However since the Roe & Kuether method already provides a specific method for the determination of total AA (i.e. AA + DHA + DKG) the only useful application of the H_2S

method combined with the CMB method would seem to be for the estimation of the AA + DHA as distinct from the DKG which is not reduced by H_2S and is reported to have no anti-scorbutic activity.

Widely divergent views have been held on the amount and even the existence of DHA in blood or its components. It is almost certain that these differences rest on the technical difficulties of the methods used in its determination.

Most workers agree that some AA in plasma becomes oxidised if the sample is allowed to stand for any length of time. The analyses must be carried out as soon as possible after withdrawal of the blood and if protein precipitation is not achieved within 0.5 hr., the results must be viewed with suspicion. Practically all the methods which have been used for the estimation of AA give good recoveries of added AA. However recovery tests, while valuable as a check, do not provide information as to whether interfering materials are included but only show that the method will estimate the added AA. Evidence on the suitability of methods needs therefore to be checked rigorously.

Since no simple, specific, chemical method is available for the determination of DHA in biological tissues its estimation depends upon the difference between the values obtained for reduced and total AA. These differences are vanishingly small. Furthermore DHA is unstable at the pH of blood so that any figure for the quantity of DHA in blood must be stated with caution, particularly if the total AA is low.

Some early workers believed that most of the vitamin C in blood serum or plasma was exclusively DHA (Plaut & Bülow, 1935;

TABLE 18. The DHA content of blood as reported by other authors.

Authors	Material	AA method	Total AA method	DHA values
Van Eekelen & Emmerie (1936)	serum	titration	H_2S	None
Kellie & Zilva (1936)	g.pig plasma	titration	H_2S	None
Borsook et al. (1937)	plasma	titration	H_2S	None
Farmer & Abt (1938)	plasma	titration	H_2S	None
Lowry et al. (1945)	serum	spectrophotometric	Roe	0 to 0.09
Lloyd et al. (1945)	plasma	spectrophotometric	Roe	0.1 to 0.3
Daubenmerkl (1949)	serum	spectrophotometric	Roe	0.15
Todhunter et al. (1950)	serum	spectrophotometric	Roe	None
Chen & Schuck (1950)	blood	not stated	not stated	Some DHA
Davey et al. (1952)	serum plasma	titration	Roe	0 to 0.15
Damron et al. (1952)	g.pig blood	Roe et al.(1948)	Roe et al. (1948)	0.03
M.R.C. Report No 280 (1953)	plasma	spectrophotometric	Roe	0 to 0.18
Stewart et al. (1953)	plasma	spectrophotometric	Roe H_2S	0.07 to 0.34
Banerjee & Belavady (1953)	whole blood	micro-titration	H_2S	0.09
McSwiney et al. (1954)	plasma	titration	Geschwind et al.	0 to 1.0
Harkness & Donovan (1955)	plasma	not stated	not stated	Some DHA
Shaffert & Kingsley (1955)	whole blood	modifications of Roe		0.9 to 1.0
	Serum			0.4

Fujita & Ebihara 1937). The conclusions and the chemical methods which have been used by different workers to derive DHA values are set out in Table 18. Comparison of the results is difficult since different methods of analysis have been employed. That all the AA in plasma is in the reduced form was reported by Kellie & Zilva, (1936), Borsook, Davenport, Jeffreys & Warner (1939) and Farmer & Abt (1936). However these studies were carried out before the Roe & Kuether method was developed so that total AA was measured by the H_2S method and the reduced AA was measured by the titration procedures. Both the latter methods may be subject to serious errors. Todhunter et al. (1950) found no significant difference between values for serum obtained by a photoelectric indophenol method and the dinitrophenylhydrazine method of Lowry, Lopez & Bessey (1945). The latter group also found that these methods gave similar results.

More recently up to 0.34 mg./100ml. or 28% of the total AA has been reported to be present in plasma or serum as DHA. Several investigators (Davey et al. 1952; McSwiney et al. 1954; Banerjee & Belvady, 1953) have used a titration procedure for estimating reduced AA. Sulpho-salicylic acid was used as the protein precipitant by the latter group and this reagent is not regarded as satisfactory for AA analyses. That the differences observed between the total and reduced AA may result from the time delays before analysis of the specimens and are not due to the preformed DHA has already been suggested by Lloyd et al. (1945) and Daubenmerkl (1949). It is unfortunate that an "unbuffered indophenol" method was used in the careful study of

Stewart et al. (1953). The only investigations in which the dinitrophenylhydrazine method was used for both total and reduced AA have given widely differing results. Roe and his collaborators found only 0.03 mg./100ml. DHA and no DKG in guinea pig blood, whereas Schaffert & Kingsley (1955) using a modification of the Roe procedure found the highest values yet reported for DHA. However they used a termperature of 100°C for 5 min. for the coupling procedure, a practice which has been severely criticised by Glick, Alpert & Stecklein (1953). Chen & Schuck (1950) reported that they found DHA in blood but give no details of their methods or other values they obtained.

In the present investigation it has been found that no significant difference exist between AA analyses of plasma carried out by the "buffered indophenol" method and by the Roe & Kuether procedure. It is therefore concluded that no measurable DHA exists in human plasma when these methods are employed. However when the AA was measured by the "unbuffered indophenol" method, the mean DHA was 0.065 mg./100ml. Furthermore, a significant negative correlation was found between the total AA and the DHA. This is attributed to the known inaccuracy of the "unbuffered indophenol" method when the total AA content is low. It is considered that the "buffered indophenol" method gives a more reliable indication of the AA content and by derivation, therefore of the DHA.

Indophenol methods were quite unreliable when used on erythrocyte filtrates unless interfering materials were removed by CMB. The mean DHA calculated by the difference between the

CMB and Roe & Kuether values was 0.063mg./100ml. A significant positive correlation was found between the DHA and the total AA content. The lack of specificity of the indophenol method when used to estimate the AA content of erythrocytes, in which there are large amounts of interfering material, probably accounts for the absence in the literature of any estimate for the DHA content of erythrocytes.

Both DHA and DKG have been reported in urine (Penney & Zilva, 1943). In normal urine the presence of large amounts of interfering materials makes the use of the indophenol method for estimating reduced AA impracticale unless special procedures are used to remove them. Mapson (1953) examined the AA content of urine by indophenol titration, by the Roe & Kuether method and by the formaldehyde method. He obtained reasonable agreement between the dinitrophenylhydrazine and formaldehyde results though the latter usually yielded slightly lower values. The dye titration method gave results approx. 10 times greater. In the present investigation it was found that urine sometimes contains considerable amounts of interfering materials which react with dinitrophenylhydrazine. In those urines in which the vitamin C was estimated by the Roe & Kuether method, the "unbuffered indophenol", the CMB and theHCHO methods, the "unbuffered" results were much higher than the Roe & Kuether figures. In a larger series of experiments the "buffered" method was compared with CMB method. CMB reduced the values by 15% and even so all the interfering material was not removed. On the basis of these experiments it was decided that there was insufficient data to make any estimate of the amounts of DHA and DKG in urine.

SECTION II.

The reduction of dehydroascorbic acid by erythrocytes

A. The reduction mechanism

B. The reduction mechanism in various diseases

C. The effect of certain drugs on the reduction mechanism

INTRODUCTION

DHA is an extremely unstable substance in aqueous solution, particularly at pH above 7. Nevertheless it is clear from the results of the numerous studies on the role of AA and DHA in a wide variety of biological processes that DHA is of importance in metabolic reactions. Even before the structure of AA was completely elucidated it was recognised that the reversibly oxidised form of vitamin C was an anti-scorbutic substance (Zilva, 1928; Tillmans, Hirsch & Siebert, 1932; Hirst & Zilva, 1933). More recent work on the bioassay of DHA using guinea pigs (Fox & Levy, 1936; Gould & Shwachman, 1943), on the excretion of DHA in man (Johnson & Zilva, 1934; De Ritter, Cohen & Rubin 1951) and on the effect of DHA dosage on plasma levels and urinary excretion of AA (Todhunter, et al., 1950; Clayton, McSwiney & Prunty, 1954) confirms its anti-scorbutic properties. On the other hand, DKG is not anti-scorbutic (Hirst & Zilva, 1933; Borsook et al., 1937; Penney & Zilva, 1943,b). This fact may explain why Roe & Barnum (1936) found that DHA had only 25% of the activity of AA whereas other workers (Borsook et al., 1937; Gould, & Shwachman; 1943; Todhunter et al., 1950) consider that DHA is utilised almost as efficiently as AA. The preparations of the former workers may have contained considerable amounts of DKG.

The administration of DHA does not raise the level of tissue AA as efficiently as does a comparable dose of AA (Fox & Levy, 1936; Bülow, 1936; Damron et al., 1952). Penney & Zilva (1943b) suggested that the amount of DHA given was important and that small

doses were quantitatively converted to AA whereas with large doses there was a decrease in its utilisation. They suggested that delays during absorption provided greater opportunities for its conversion to DKG.

Work on isolated mammalian tissues confirms the existence of biological mechanisms which can reduce DHA. Preparations of liver, kidney, adrenal and intestine readily reduce DHA (Borsook et al., 1937; Schultze, Stotz & King, 1937-38). Plasma however lacks this property (Plaut & Bülow, 1935; Schultze et al., 1937-38; Borsook et al., 1937). These last workers also reported that in whole blood no reduction occurred but since they examined only the plasma fraction of the blood their results are not unexpected. Schultze and his coworkers found that both whole blood and suspensions of erythrocytes reduce DHA. Lloyd (1951) and Panteleva (1950) have confirmed that the erythrocytes are the DHA-reducing components of blood.

Ascorbic acid is also very unstable in aqueous solution particularly at pH above 7.0. Early workers observed that AA was less readily oxidised when incubated with tissue extracts than it was in pure solution (Quastel & Wheatley, 1934; Mawson, 1935; Kellie & Zilva, 1935). The existence of some stabilizing mechanism was postulated by Green (1933). The suggestion that glutathione (GSH) might be involved in the protection of AA was put forward by De Caro & Giani (1934) and was further investigated by Barron, Barron & Klemperer, (1936). Mawson (1935) considered that cystine and cysteine might also be involved. Substances such as purines and creatinine (Giri & Krishnamurthy, 1941) have

also been suggested as "protective" agents. It is now generally agreed that GSH is the principle stabilising substance in biological materials.

Oxidation of AA certainly occurs in biological preparations. Kellie & Zilva (1935) noted that, of the tissues they examined, washed leucocytes were outstanding in that they did not protect AA from oxidation. Haemochromogens also readily oxidise AA (Barron, De Meio & Klemperer, 1936; Lemberg, Legge & Lockwood, 1939). This reaction depends on the presence of copper. Stotz, Harrer, Schultze & King in 1937 observed a slow aerobic oxidation of AA, added to guinea pig liver brei, which did not involve copper. They suggested that a cytochrome system was responsible for the reaction. However, no specific enzyme comparable with the AA oxidase which is found in plants and which catalyses the direct oxidation of AA by atmospheric oxygen, has been identified in animal tissues. Enzyme systems which indirectly catalyse the oxidation of AA exist in both plant and animal tissues. Little is known of the biological systems(if any) which convert the primary oxidation product DHA to DKG by opening the lactone ring. That the reaction is dependent on the pH has been shown by Penney & Zilva (1943,b). Information on the further breakdown of AA to oxalic acid and water is also scanty.

There is strong evidence for an association between AA and GSH in biological systems. Tissues which have a high content of AA usually contain a reasonable amount of GSH and furthermore, are metabolicly active. The action of GSH in protecting AA from oxidation has already been cited. GSH will reduce solutions of

DHA without the addition of catalysts (Hopkins & Morgan, 1936). At physiological pH and temperature the straightforward reaction

$$DHA + 2GSH \rightleftharpoons AA + GSSG$$

is too slow and would require an enzyme system for it to be of importance in cellular respiration. Hopkins and his collaborators demonstrated the presence of a DHA-reductase in plants which catalysed this reaction. That such a system might provide a hydrogen transfer system analagous to the cytochrome system was suggested by Crook, (1941). No DHA reductase has been isolated from animal tissues. Schultze et al. (1937-38) concluded that the reduction of DHA depended on a reversible reaction with thiol compounds. Mawson (1935) observed that the quantity of GSH required for the reduction of DHA was considerably less than the equation required. In addition to GSH and fixed -SH groups, Kinkawa (1944) postulated that a reducing mechanism, which was not enzymic but was heat stable, existed in animal tissues. Rall & Lehninger (1952) demonstrated the presence in rat liver of a GSH reductase which involved triphosphopyridine nucleotide (TPN). However they could not demonstrate the reversibility of the reaction

$$GSSG + TPNH_2 \longrightarrow 2GSH + TPN$$

In pea seedlings this reaction has been coupled with the DHA reductase system. The GSH reductase of plants is specific and does not catalyse the reduction of other disulphides such as cystine, nor does it involve diphosphopyridine nucleotide (DPN) (Mapson & Goddard, 1951).

Indirect evidence that DPN may be involved in the reduction

of DHA in plant tissues has been put forward by James & Cragg (1943,1944). The route of hydrogen transfer may be by triosephosphate, DPN and AA. The hypothesis that systems producing reduced DPN may reduce DHA is supported by the work of Waygood (1950). Matthews (1951) has described a preparation which, in the presence of methylene blue or AA, catalysed the oxidation of DPNH by oxygen. Although the hydrogen acceptor was not DHA it was suggested that an intermediate such as monodehydroascorbic acid might function in this way. A system for the reduction of DHA which involves the participation of a dehydrogenase of α-hydroxy acids has also been postulated (James and Cragg, 1943).

Our knowledge of the role of AA and DHA as an intermediate in cellular respiration has been more closely examined in plants than animals. No evidence exists that AA is a general respiratory catalyst in mammalian tissues though its reversible oxidation makes this a tempting hypothesis. Special oxidation processes in which AA plays an essential part e.g. the metabolism of tyrosine, have been investigated by numerous workers. Knox and le May-Knox (1951) have demonstrated that AA is a major cofactor in the oxidation of tyrosine in rat liver. It is known that AA has some function in the formation of intercellular materials but so far the exact role of AA in these processes has not been established. That AA enhances the enzymic conversion of folic acid to folinic acid has been reported by Nichol & Welch (1950) and Shive, Bardos, Bond & Rogers (1950). Work on the degradation of haemoglobin indicates that AA and oxyhaemoglobin can react directly (Lemberg, Legge, & Lockwood, 1941) and this reaction may be concerned with bile

pigment formation.

Many workers have commented on the possibility of a relationship between AA and carbohydrate metabolism. Patterson (1949,1950) made the important contribution that DHA has a diabetogenic effect on rats. Recently Bhattacharya (1955) has studied the GSH content of rat tissues after the administration of diabetogenic doses of DHA. Despite the vast literature on the relationship of AA to the adrenal hormones no clear picture of the function of AA in this field has emerged.

The metabolism of AA in blood has not been studied intensively. Practically all the AA in blood plasma and erythrocytes is in the reduced form. It is reported however that AA does not enter the erythrocytes readily (Golden & Sargent, 1952). A slow transfer of AA into erythrocytes had earlier been reported by Heinemann (1938) but in 1941 the same worker showed that this was due to the presence of leucocytes. Panteleva (1950) observed a very slow movement of AA into the erythrocytes of man, horse, cat and rabbit. In the animal, AA must enter the cell by some process since the concentration of AA there can be raised by oral administration of AA. In contrast, it has been found that DHA readily enters the erythrocytes of human blood (Panteleva, 1950; Lloyd, 1951; Lloyd & Parry 1954;) and is there reduced. The final concentration in the cell is higher than in the plasma. Erythrocytes from other species e.g. horse, pig, dog, cat and rabbit, do not seem to be readily permeable to DHA.

Schultze *et al.* (1937-38) reported that GSH and fixed -SH groups were responsible for the reduction of DHA by animal tissues

including the erythrocytes. They did not consider that there was any evidence for the participation of an enzyme system. More recent work by Thomson (1955) has shown that an enzyme system must be involved directly or indirectly. The system was sensitive to temperature, pH and heavy metals. Dialysis destroyed the capacity for reducing DHA and since the addition of GSH to the dialysate did not completely restore the reducing activity, GSH or fixed -SH groups cannot be entirely responsible for the reduction of DHA in erythrocytes. Lloyd (1954) and Thomson (1955) have shown that when successive amounts of DHA are added to a suspension of erythrocytes the total quantity of DHA which the cells can reduce is very large and that it is difficult to exhaust the reducing capacity. These findings give further support to the view that enzymic processes are involved.

Little is known of the part played by the leucocytes in AA metabolism, except that the total AA concentration is much higher than that of either the erythrocytes or plasma. Lloyd (1951) has established that AA is taken up by the leucocytes. Both Kellie and Zilva (1935) and Lloyd (1951) report that leucocytes readily oxidise AA.

The only report on changes in the erythrocyte reducing mechanism in disease is that of Thomson (1955) who showed that blood from patients with pernicious anaemia reduced DHA more rapidly than did normal blood. Factors such as diet, exercise and disease have been reported to affect the tissue reduction of DHA. Parrot and Gazave (1951) have claimed that liver from guinea pigs fed on a synthetic diet with no vitamin C had a low

DHA reducing activity which was restored by the addition of catechol. Training on a treadmill also increased the degree of tissue reduction (Schroll, 1938). Vinokurov & Silakova, (1944) have reported a much lower than normal reducing activity of liver from patients with tuberculosis and pneumonia. A similar result has been reported for the kidney and duodenum of patients who had died from tuberculosis or cancer (Matusis, 1951). However, this author found no abnormality in the reducing power of the pancreas and adrenals. During scurvy and experimental diabetes guinea pig organs appeared to reduce DHA more slowly.

An enormous amount of study has been directed towards the elucidation of the relation of AA to the metabolism of the adrenal cortex. In a comprehensive review Pirani (1952) suggests that the function of AA is probably non-specific and related to cellular activity, although many workers, on the basis of indirect evidence, consider that it has a specific function. A comprehensive review of this aspect of AA metabolism is beyond the scope of this thesis but some points are pertinent. Booker, Dent, Hayes, Harris & Green (1951) have found that both plasma and erythrocyte levels of AA increased when the following materials were administered to rats and dogs; an adrenocortical extract, cortisone and desoxycortisone. Stewart, Horn & Robson (1953) found that in man the parenteral administration of ACTH caused an increase in the plasma AA and the disappearance of any oxidised fraction of the plasma AA. Oral administration of cortisone similarly resulted in a decrease of plasma DHA but left the level of total AA unchanged.

Vogt (1948) found that ACTH does not increase the total AA

content of adrenal venous blood. Lloyd & Sinclair (1953) consider, on the basis of Vogt's work, that ACTH could cause the oxidation of AA to DHA and its susequent degradation to oxalic acid. That cortisone causes tissues to become more reducing has been shown by Loxton & Le Vay (1953) who observed a fall of 25 mV in tissue potential 2 hours after its administration.

Many workers have been interested in possible changes in ascorbic acid metabolism during disease. Studies on plasma levels of AA and on saturation tests have indicated that during some diseases, particularly tuberculosis and the collagen diseases, the reserves of AA in the body become depleted (Andreae & Brown, 1946; Rhinehart, Greenberg & Baker (1936); Rhinehart, Greenberg & Christie (1936); Hall, Darling & Taylor, 1939; Abbasy, Gray Hill, & Harris (1936); Abbasy, Harris, Ellman & Gray Hill, 1937).

A similarity between the pathological changes in chronic scurvy and in collagen diseases has been suggested by Rhinehart, Connor & Metteir (1934). Subsequent work however has failed to substantiate an aetiological relation between AA and the rheumatoid diseases (Waksman, 1949). Adrenocortical hormones are reported to be beneficial in the treatment of these diseases (Hensch, Kendall, Slocumb & Polley, 1950). Other workers have suggested that large doses of AA have beneficial effects in rheumatic diseases (Massell, Warren, Patterson & Lehmus, 1950) and in experimentally induced arthritis (Bacchus, 1951). The simultaneous administration of AA and DOCA to patients with rheumatoid arthritis was claimed by Lewin & Wassén (1949) to have favourable effects. This claim has not been substantiated by later work for either rheumatoid

arthritis (Spies, Stone de Maeyer & Niedermeier, 1949; Bywaters, Dixon & Wild, 1950; Margolis & Caplan, 1951) or for experimentally induced arthritis (Maclean, 1951). Little information is available on the effects of the simultaneous administration of AA and ACTH or cortisone. Hensch et al. (1950) did not find that AA enhanced the effect of the latter compound when given to patients with rheumatoid arthritis.

The purpose of the experiments described in this section was three-fold:-

A) To investigate the system which brings about the reduction of DHA in normal erythrocytes.

B) To examine the effect of disease, particularly rheumatoid arthritis, iron-deficiency anaemia, tuberculosis and scurvy on the erythrocyte reducing system.

C) To examine the effect of the administration of AA, salicylate, cortisone, ACTH, and nicotinic acid on the erythrocyte reducing mechanism.

METHODS

The estimation of AA.

The "buffered indophenol" method as described in Section 1 was found to be satisfactory for the determination of the AA content of metaphosphoric acid extracts of plasma and of intact and haemolysed erythrocytes. Since the estimations were carried out after the addition of large amounts of DHA (usually 10mg./100ml.) considerable dilution of the erythrocyte extracts was needed and therefore interference due to indophenol-reducing substances other than AA was minimal. Dilution to bring the final concentration of AA to approximately 1 mg./100ml. was carried out during the protein precipitation procedure and further dilution if necessary was done in the spectrophotometer cuvette.

The estimation of AA and DHA.

The CMB adaptation of the "buffered indophenol" method was used on filtrates of haemolysed erythrocytes (prepared with 3% MPA) which had been treated with H_2S to reduce any DHA to AA. The DHA content was estimated by subtraction of the indophenol value obtained before treatment with H_2S from that obtained after H_2S treatment.

The estimation of total AA.

The method used for the estimation of total AA (AA + DHA + DKG) in plasma, haemolysed and intact erythrocytes after the addition of DHA was that of Roe & Kuether (1943) with the minor modification described in Section 1, in which filtration of the precipitated proteins before the addition of charcoal was omitted. Dilution

to bring the final AA concentration into the range of the standard curve was carried out during protein precipitation and again when the filtrates were prepared for incubation with phenylhydrazine.

The estimation of total -SH groups.

The estimation of total -SH groups in extracts of erythrocytes which had been haemolysed with water and to which 10 mg./100ml. of DHA had been added was carried out with the help of Dr.Bhattacharya. The amperometric titration method of Kolthoff & Harris (1946) was used. No interference with the method was caused by the presence of AA or DHA (Bhattacharya, 1955, unpublished).

The estimation of reduced pyridine nucleotides.

The fluorimetric method of Levitas, Robinson, Rosen, Huff & Perlsweig (1947) for the determination of total pyridine nucleotides in erythrocytes was used for the determination of reduced DPN and TPN in haemolysed erythrocytes to which 10 mg./100ml. of DHA had been added. Pyridine nucleotides condense with acetone in alkaline medium to form a highly fluorescent, stable compound. Of the substances tested Huff and his associates found that only pyridine nucleotides and N-methyl nicotinamide formed the fluorescent compound with acetone. Pure N-methyl nicotinamide was used as the internal standard.

The fluorescence was measured in a Farrand Model A fluorometer. The primary filter was a Corning No.5860 filter and the fluorescent light was filtered through a combination of Corning No.4308 and No.3389 filters with maximum transmission between 4500 and 5000 A°.

Preparation of DHA.

DHA was prepared from AA by oxidation with an ethereal

solution of quinone according to the method of Patterson (1950). The AA was dissolved in 0.9% (w/v) saline.

Procedure for the measurement of the rate of reduction of DHA by human erythrocytes.

Intact erythrocytes. Heparinised human blood, immediately after withdrawal (never longer than 20 min. after withdrawal) from the antecubital vein, was centrifuged for 20 min. The plasma was removed, and the leucocyte layer discarded. The erythrocytes were then re-suspended in sufficient plasma, or in a mixture of plasma and normal saline, so that the reconstituted "blood" contained 30% (v/v) erythrocytes. Leucocyte counts were done occasionally and showed that it is not possible to remove the whole of the leucocytes. The suspension of erythrocytes was saturated with CO as described in Section 1. An amount of a saline solution containing 100 mg./100ml. of DHA was added, sufficient to give a final concentration of 10 mg./100ml. of blood. The blood, in a stoppered tube filled with CO, was then incubated at 37°C for the required time, usually 15 min., and then centrifuged for 10 min. at a standard speed. The plasma and erythrocytes were separated and precipitated for vitamin C estimations by the "buffered indophenol" and Roe & Kuether methods. It was found convenient to precipitate the erythrocytes in a graduated centrifuge tube. One ml. of erythrocytes was measured into the tube and into it the precipitating acid was pipetted. Thorough mixing was achieved by stirring with a glass rod and then the tube was corked and inverted several times. Because of the manipulations required, it was difficult to measure the reduction rate at times

less than 30 min. after adding the DHA.

Haemolysed blood. Three volumes of RBC, separated so far as possible from the other blood components, were haemolysed with 7 vol. of water. The solution was saturated with CO and DHA added to give the required concentration. Aliquots of the haemolysed solution were withdrawn at specified times and precipitated with metaphosphoric acid for the "buffered indophenol" and H_2S methods, and with trichloroacetic acid for the Roe method. Using this procedure it was possible to measure the rate of reduction immediately and at intervals of 5 min. after the addition of DHA.

Procedure for obtaining blood from animals other than man

Blood was obtained from normal rats, rabbits, and guinea pigs. The animals were anaesthetised with pentobarbital and dissected to expose the abdominal aorta. Blood was withdrawn into a heparinised syringe. Professor D. Whitteridge kindly supplied a sample of blood from a rhesus monkey. The blood was withdrawn from the jugular vein of an animal anaesthetised with pentobarbital.

TABLE 19. The stability of DHA in 0.9 % (w/v) NaCl

Time after preparation (min)	AA (mg/100ml)	Total AA (mg/100ml)	DHA (mg/100ml)	Loss of DHA (%)
0	0	11.0	11.4	0
30	0	11.0	11.1	2
150	0	11.0	11.1	2

RESULTS.

A. The reduction mechanism in normal erythrocytes.

The stability of DHA in normal saline at pH 5.5.

The stability of a solution of DHA in normal saline (0.9% w/v sodium chloride) was tested immediately, 30 min. and 150 min. after its preparation by comparing its indophenol reducing activity before and after treatment with H_2S with the total AA content measured by the Roe & Kuether method. Aliquots of the solution containing approximately 10 mg./100ml. DHA were added to 3% metaphosphoric acid and 6% trichloroacetic acid immediately after its preparation and after 30 min. and 150 min. A portion of the metaphosphoric acid solution was immediately treated with H_2S at pH 3.5 and 37°C. The remainder was kept for the measurement of AA by the "buffered indophenol" method. A precipitate of sulphur, formed during treatment with H_2S, was removed by filtration. The extracts which had been treated with H_2S and the trichloroacetic acid extracts were diluted a thousand-fold for the measurement of AA. The undiluted DHA solution had no indophenol reducing activity even after standing 150 min. so that no reduction of DHA had occurred in this time at pH 5.5. The total AA content, as measured by the Roe method, was also unchanged. Immediately after preparation the reducible ascorbic acid i.e.DHA, was 103% of the total AA and 150 min. later it was 101%.

These results (Table 19) show that at pH 5.5. DHA is stable in saline solutions and it was concluded that the quinone oxidation method for preparing DHA was satisfactory. In the experiments

TABLE 20. The stability of DHA in plasma

Time after addition of DHA	AA	Increase in AA	Total AA	Decrease in Total AA
(min)	(mg/100ml)	(%)	(mg/100ml)	(%)
30	0.35	2	7.32	26
60	0.43	3	4.92	50
120	0.45	3	1.56	84

described in this section the DHA solutions were always used within 150 min. and usually within 30 min. of preparation.

The stability of DHA in normal saline at pH 7.1

The pH of a freshly prepared saline solution of DHA was increased to 7.1 by the addition of phosphate buffer. The total AA content and the indophenol reducing activity before and after treatment with H_2S was measured immediately and at 10, 20, 40, and 60 min. after the pH was raised. The total AA content remained unchanged and only 1% of this was detected in the solution as AA after 60 min., showing that no spontaneous reduction of DHA occurs at pH 7.1. By reduction with H_2S, progressive conversion of DHA to DKG at pH 7.1 was observed. (Fig.23). At a pH close to that of blood, DHA solutions are therefore unstable. In 60 min. one-half the DHA was converted to DKG. It was also noticed that after 24 hr. saline solutions of DHA began to turn yellow. This was accelerated by making the solution more alkaline. Furthermore, shortly after addition of the alkali, the DHA solutions became strongly acid (pH 2.0) suggesting the formation of DKG.

The stability of DHA in human plasma.

A saline solution of DHA was added to plasma, which had been equilibrated with CO, to give a final concentration of 10 mg./100ml. DHA. The plasma concentration of AA and the total AA was measured at 30, 60 and 120 min. after the addition of DHA. A slight increase of 2% in the AA content was observed after 120 min. but more striking was the rapid fall in total AA. (Table 20). In the table the quantity of AA initially present in the plasma has been subtracted

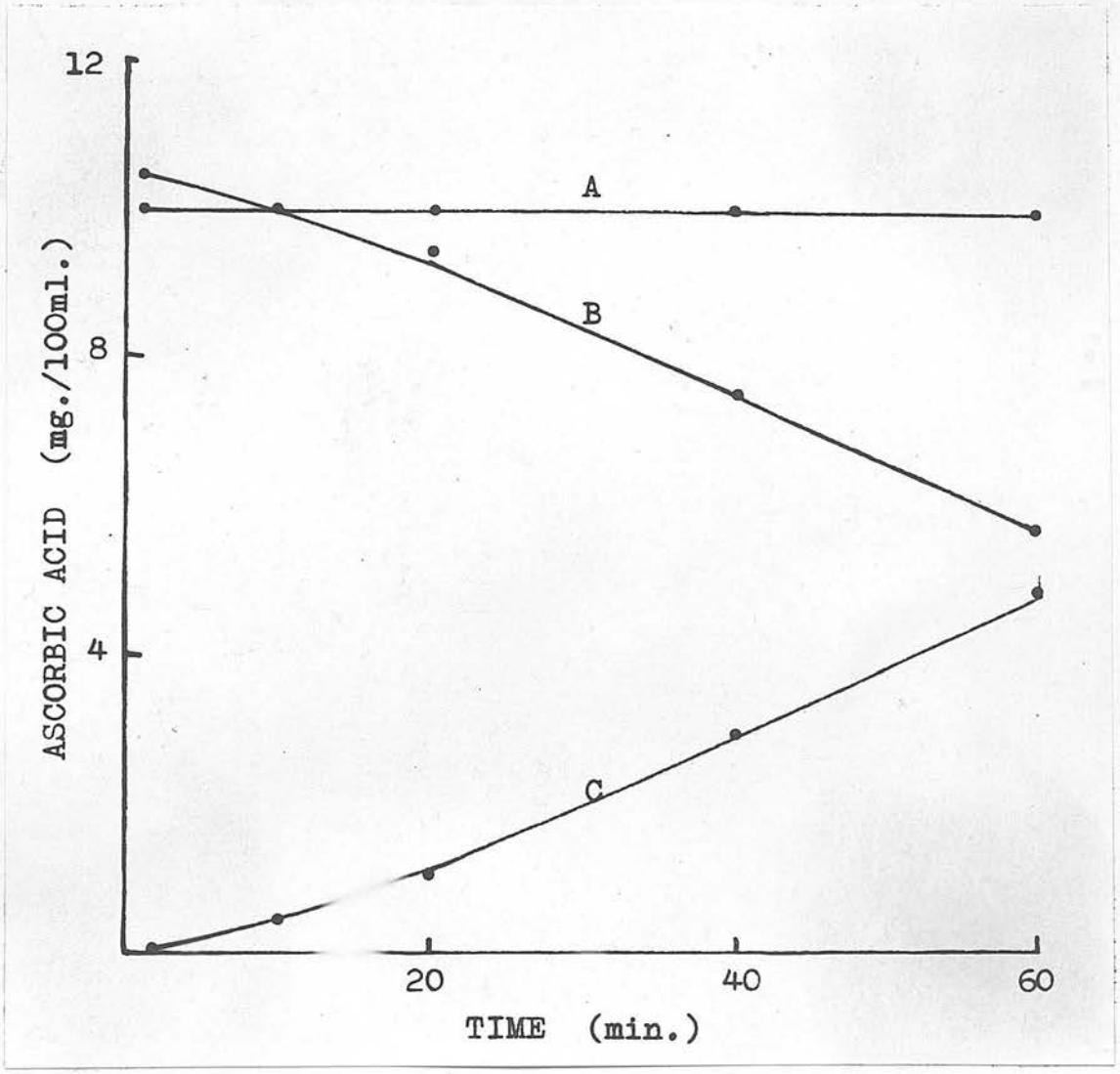


Fig.23. The stability of DHA at pH 7.1. A, total AA; B, total AA reduced by H_2S (DHA + AA); C, DKG.

TABLE 22. The reduction of DHA by intact erythrocytes

Time (min)	Plasma			Erythrocytes			Whole blood			Reduction of DHA (%)
	AA	Total AA	DHA+DKG	AA	Total AA	DHA+DKG	AA	Total AA	DHA+DKG	
	(mg/100ml)			(mg/100ml)			(mg/100ml)			
20	0.46	6.54	6.08	13.28	19.68	6.40	3.63	9.70	6.07	37
35	0.46	5.16	4.70	18.40	24.24	5.86	5.08	9.99	4.91	52
60	0.59	5.16	4.57	18.10	21.72	3.62	5.27	9.45	4.18	57
120	1.34	5.13	3.79	17.60	18.72	1.12	5.65	8.57	2.92	67

TABLE 21. The fate of AA added to leucocyte-poor blood

Time after addition of AA (min)	Plasma		Cells		Whole blood	
	AA	Total AA	AA	Total AA	AA	Total AA
	(mg/100ml)		(mg/100ml)		(mg/100ml)	
30	16.20	15.48	0.68	2.13	11.54	11.48
60	16.40	15.36	0.76	2.31	11.70	11.45
120	15.40	13.92	1.00	3.42	11.08	10.77

from the readings. After 120 min. only 16% of the added DHA could be detected by the Roe & Kuether method. It is therefore concluded that in plasma although a very slight reduction of DHA to AA may occur most of it is oxidised further than the DKG stage. Since no special precautions were taken to remove the leucocytes completely, e.g. by filtration through a Seitz filter, the destruction of DHA may have been due to leucocytes.

The fate of AA added to normal human blood.

To a CO-saturated sample of human blood from which the leucocytes had been removed AA was added to give a concentration of approximately 10 mg./100ml. Both the AA and total AA were determined in the plasma and erythrocytes after 30, 60 and 120 min. Even after 120 min. only a very small amount of the AA had diffused into the erythrocytes (Table 21). A small amount of the total AA (16%) was destroyed during this time.

The fate of DHA added to normal blood.

To a CO-saturated sample of human blood from which the leucocytes had been removed as completely as possible, and with the p.c.v. adjusted to 30, DHA was added to give a concentration of approximately 10mg/100ml.. The $\overset{\cdot\cdot}{AA}$ and the "total AA was determined in both the plasma and erythrocytes 20, 30, 60, and 120 min. after the DHA had been added. The DHA + DKG content of these fractions was calculated by difference. The AA, DHA + DKG and "total AA" in the whole sample was then calculated and from these values the percentage of the added DHA which had been reduced, was determined (Table 22). The course of the reaction is shown graphically in Fig.24. The time required for the separation of

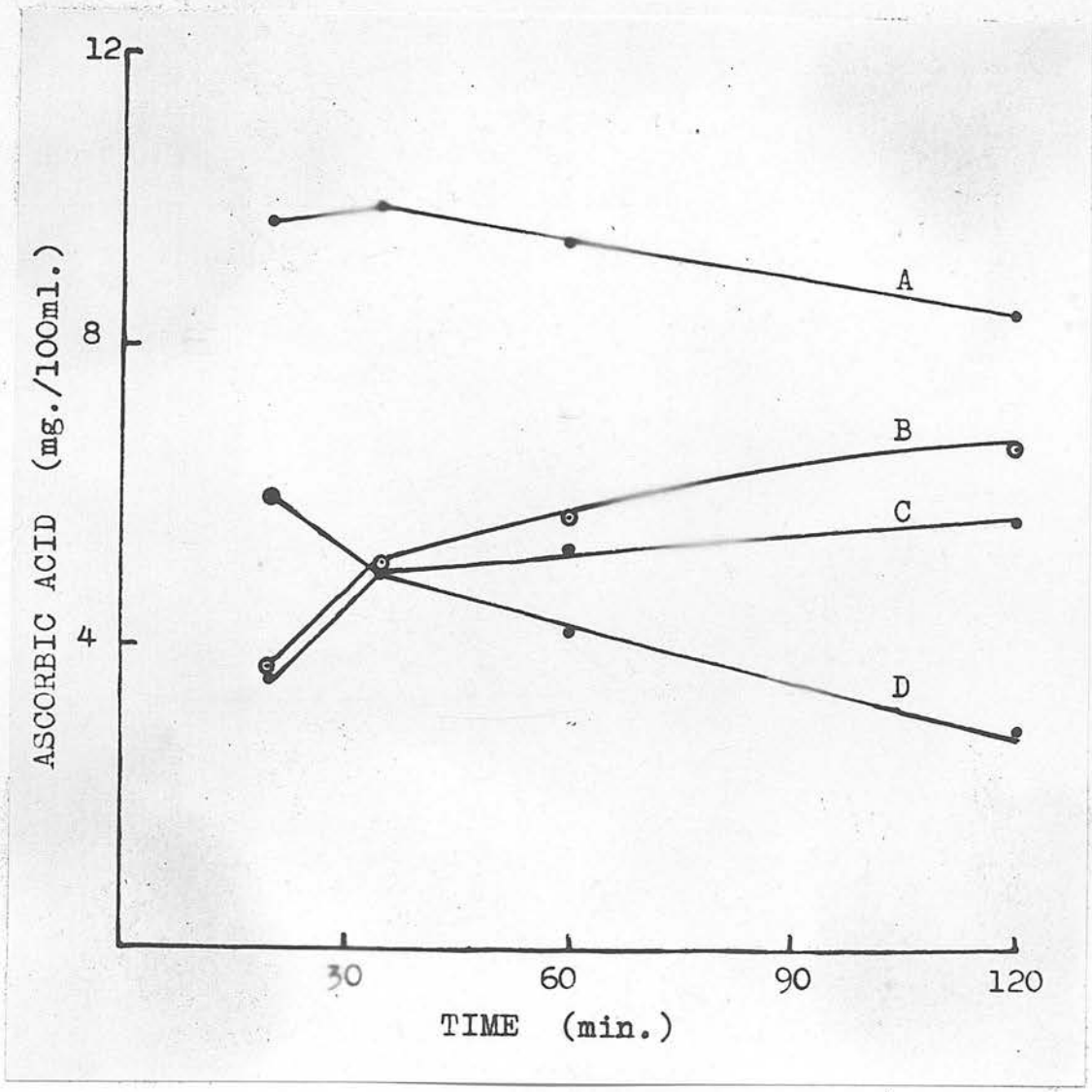


Fig. 24. The fate of DHA added to intact erythrocytes. A, total AA; B, percentage reduction of DHA; C, AA produced; D, DHA + DKG.

TABLE 23. The reduction of DHA by intact erythrocytes

Time (min)	Plasma			Cells			Whole blood					Reduction of DHA (%)
	AA	Total AA	DHA+DKG	AA	Total AA	DHA+DKG	AA	Total AA	DHA+DKG	DHA	DKG	
	(mg/100ml.)			(mg/100ml.)			(mg/100ml.)					
30	0.76	7.20	1.37	16.80	16.80	19.19	4.93	9.70	5.39	0.46	4.31	46
60	0.84	6.69	0.97	19.68	18.88	22.12	5.74	10.00	5.71	0	4.29	53

plasma and erythrocytes made it impossible to get values earlier than 20 min. after the addition of DHA. The concentration of AA rose sharply in the first 35 min. and almost all of it was located in the erythrocytes. After 35 min. the "total AA" was falling slowly, indicating that some destruction beyond DKG was taking place. Since the formation of AA after 35 min. proceeded slowly, the steady rise in the value for the "percentage" reduction after this time was largely due to the fall in the total AA. In blood therefore, the rate of reduction of DHA reached an optimal value between 20 and 35 min. after the addition of DHA. Within this time one-half of the added DHA had disappeared from the plasma and was found as AA in the erythrocytes. Since AA does not diffuse at all readily into the erythrocytes it was confirmed that reduction of DHA had taken place in the erythrocytes.

In another experiment DHA was added to normal blood and after 30 and 60 min. the plasma and erythrocytes were analysed for AA, total AA and total AA reducible by H_2S. Unfortunately the values obtained for the AA and DHA after H_2S reduction were higher than the corresponding total AA values, due to the non-specificity of the H_2S method. However the results (Table 23) show that even after 30 min. practically no DHA remained in either the plasma or erythrocytes and that the part of it which had not been reduced to AA had been converted to DKG. Furthermore, the DKG was nearly all in the plasma. It is probable that the conversion of DHA to DKG is brought about by the leucocytes since even after these had been removed as far as possible by different-

ial centrifugation the leucocyte counts of the reconstituted blood were between 1500 and 2000. Thomson (1955) has shown that when the leucocytes are completely removed by filtration through a Seitz filter DHA is not converted to DKG by plasma.

The reduction of DHA by haemolysed erythrocytes.

Erythrocytes from normal blood were haemolysed by adding 7 vol. of water to 3 vol. of erythrocytes. By this means it was possible to follow the reduction of DHA during the first 30 min. after its addition. DHA, to give a final concentration of 10mg./100ml. was added and the concentration of AA, total AA and total AA reducible by H_2S were determined at intervals of 3, 10, 20, 30, and 60 min. The results are shown in Fig.25. The curves for DHA and DKG are calculated by difference. There was no destruction of total AA in 60 min. and the concentration of AA increased steadily although the rate of formation was greater in the first 30 min. After 60 min. a little DHA remained. Approximately 16% of the DHA was converted to DKG at 60 min. and no more was formed even though the reaction has been followed for as long as 160 min. (Fig.26).

Comparison of the reduction of DHA by haemolysed and unhaemolysed erythrocytes.

The reactions which take place in a solution of haemolysed erythrocytes and in a suspension of intact erythrocytes differ in two respects. Firstly, in the haemolysed solutions, the total AA does not fall whereas in suspensions of intact erythrocytes it begins to fall after 60 min. Secondly, the amount of DKG

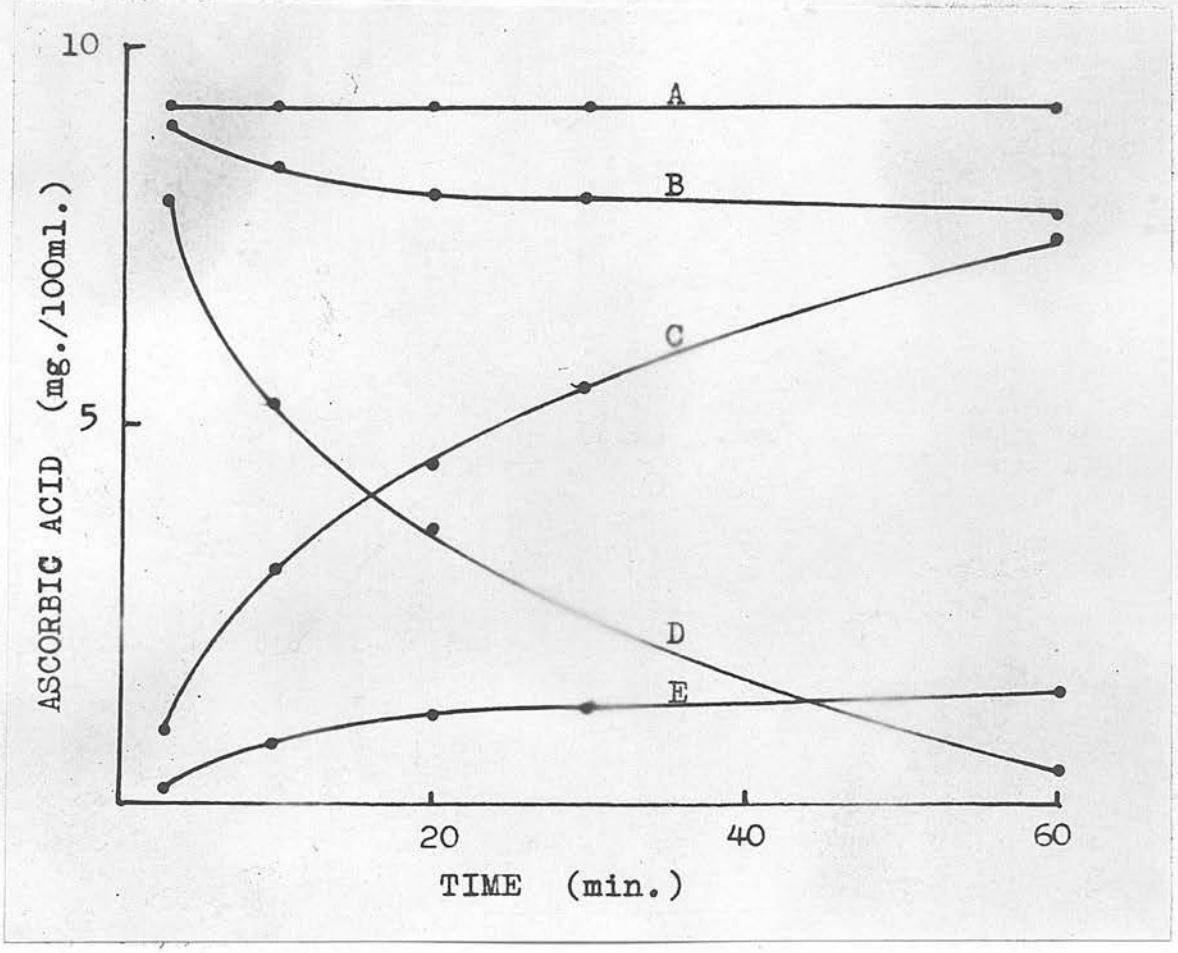


Fig.25. The reduction of DHA by haemolysed erythrocytes. A, total AA; B, total AA reduced by H_2S (DHA + AA); C, AA; D, DKA; E, DHA.

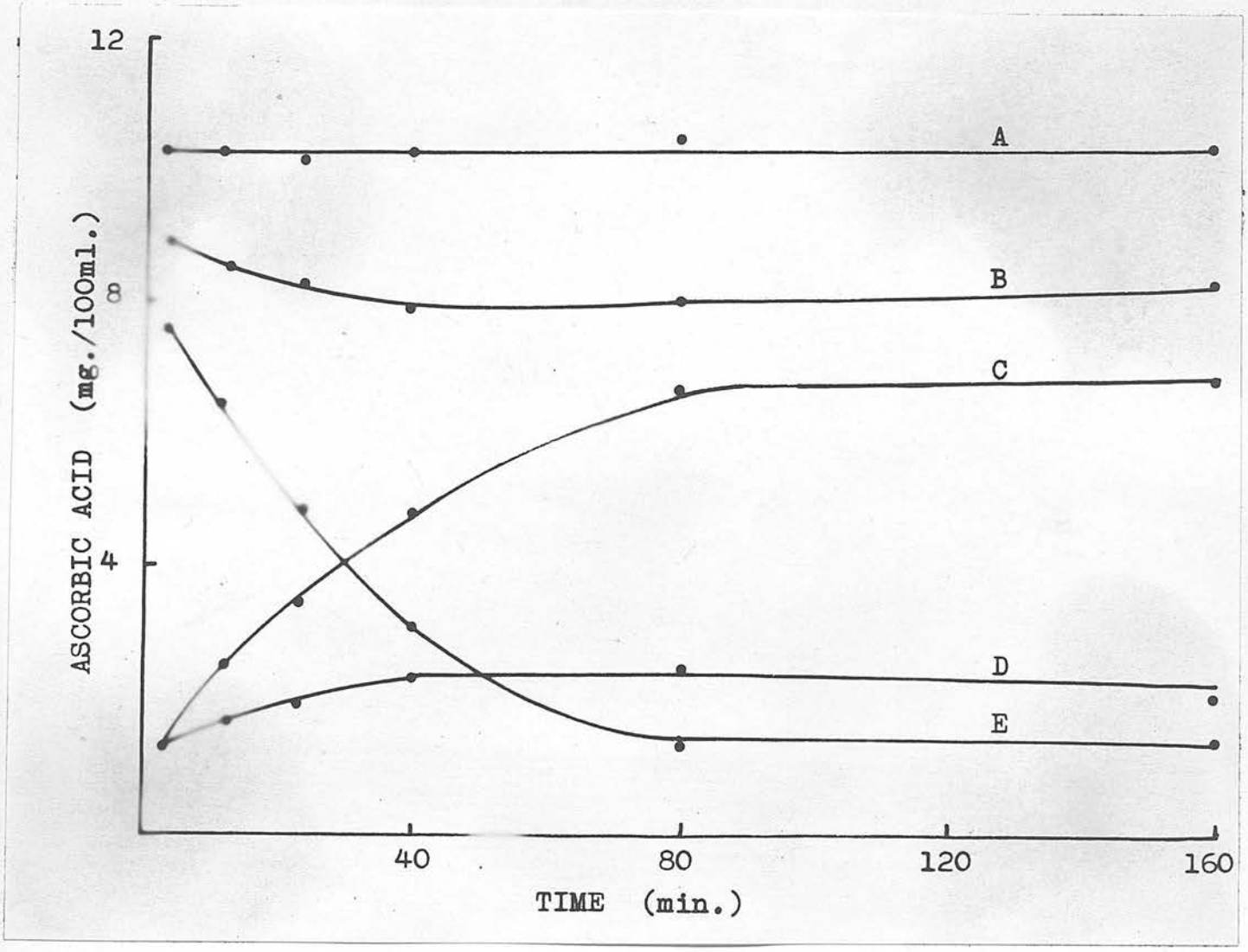


Fig.26. The reduction of DHA by haemolysed erythrocytes. A, total AA; B, total AA reduced by H_2S (DHA + AA); C, AA; D, DKG; E, DHA.

TABLE 25. The reduction of DHA by intact and haemolysed erythrocytes from different species

Animal	Weight (g)	Plasma AA (mg/100ml)	Reduction of DHA	
			Intact (%)	Haemolysed (%)
guinea pig	340	0.73	68	58
guinea pig	360	0.86	66	-
guinea pig	280	0.84	64	-
guinea pig	290	1.24	57	61
guinea pig	500	0.91	6	-
guinea pig	600	1.60	8	86
guinea pig	650	1.13	13	-
guinea pig	600	1.44	7	63
guinea pig (2)	280 600	0.90	52	70
rabbit	-	0.99	2	28
rabbit	-	0.35	1	39
rabbit	-	0.63	1	39
rat (4)	-	0.64	3	61
rat (3)	-	1.71	0	-
monkey	adult	0.68	80	77

TABLE 24. The reduction of DHA by intact and haemolysed erythrocytes

Subject	Condition	Reduction of DHA	
		Intact	Haemolysed
		(%)	(%)
T.B.	Normal	42	42
A.I.	Normal	46	41
J.O.	Normal	46	44
A.C.	Rheumatoid Arthritis	70	76
P.J.	Rheumatoid Arthritis	70	65

present after 30 min. in haemolysed solutions was never as great as that observed in the one suspension of intact erythrocytes in which the reducible total AA was measured. Consequently some AA continued to be formed after 30 min. in the haemolysed solutions whereas reduction was virtually complete in this time in the intact cells, probably because the supply of DHA was exhausted due to conversion of part of it to DKG. Comparing the values for the percentage reduction of DHA by intact and haemolysed erythrocytes (Table 24) it was found that after 30 min., the rate of reduction was the same.

The reduction of DHA by haemolysed and intact erythrocytes of other species

The ability of both haemolysed and intact erythrocytes from guinea pigs, rats, rabbits and a monkey to reduce added DHA was examined, and the results are given in Table 25. Intact erythrocytes from rats and rabbits did not reduce added DHA. However when the cells were laked reduction occurred readily. A very small amount of reduction occurred in intact erythrocytes from adult guinea pigs and this was greatly in increased when the cells were haemolysed. In contrast, intact cells from young guinea pigs very readily reduced added DHA. Both intact and haemolysed erythrocytes from an adult monkey readily reduced DHA. Erythrocytes of all these species reduce DHA to varying degrees but there seems to be species differences in the transfer of DHA across the cell membrane. In rabbits and rats DHA added to blood does not pass easily into the erythrocytes and remains in the plasma. In guinea pigs DHA appears to pass much more

TABLE 26. The effect of inhibitors on the reduction of DHA

Inhibitor	Concentration (M)	AA produced after 15 min. (mg/100ml)	AA produced after 40 min. (mg/100ml)
None	-	4.88	8.56
Potassium cyanide	0.01	4.16	4.48
Iodoacetic acid	0.01	2.88	3.20
Azide	0.05	5.64	9.60
Diethyldithio-carbamide	saturated solution	5.64	9.20
Urethane	0.35	5.00	7.76
Sodium arsenite	0.05	2.72	2.88
Sodium fluoride	0.05	5.00	7.04

readily into the cells of young animals. The adult monkey resembles man in that DHA readily enters from the plasma into the erythrocytes.

The effect of inhibitors on the reduction mechanism.

The effect of a number of known enzyme inhibitors was tested in the hope that the type of reactions involved in the reduction of DHA might be elucidated. DHA was added to haemolysed erythrocytes to give a final concentration of approximately 40 mg./100ml. Aliquots of 9.5ml. were immediately pipetted into tubes containing 0.5ml. of the following inhibitors; potassium cyanide, iodoacetic acid, sodium azide, sodium diethyldithiocarbamate, urethane, sodium arsenite and sodium fluoride. The final concentrations of the inhibitors and the amount of AA formed in 15 and 40 min.are given in Table 26. Only iodoacetic acid and sodium arsenite depressed the production of AA in 15 min. but after 40 min. the production of AA was reduced by one-half in the presence of potassium cyanide. Diethyldithiocarbamate, which is reported to be specific for copper-containing enzymes, was without effect. Sodium azide and urethane, which inhibit a number of dehydrogenases did not inhibit the production of AA. Cyanide, iodoacetic acid and arsenite inhibit -SH enzymes but by no means specifically.

The effect of different concentrations of a specific -SH inhibitor, CMB, has also been examined. Aliquots of a haemolysed solution of erythrocytes, to which 10 mg. of DHA had been added, were transferred to tubes containing different concentrations of CMB. CMB inhibited the reduction of DHA and the inhibition

TABLE 27. The inhibition of reduction by CMB

CMB (mg/100ml)	AA produced (mg/100ml)	Reduction of DHA (%)
0	3.92	58
22	3.20	48
44	2.88	43
66	2.72	41

TABLE 28. The concentration of total SH groups during reduction of DHA

Time after addition of DHA (min)	AA produced (mg/100ml)	Total SH (μg/0.1ml)
1	0.54	16.17
10	3.51	14.85
15	4.22	16.50
20	4.82	16.17
32	6.22	16.50

progressively increased with the higher concentrations of CMB (Table 27).

The concentrations of inhibitors are much larger than those usually required for enzyme inhibition. However since the DHA reducing system has not even been partially purified, large amounts of any inhibitor would probably be required to block the reduction completely. CMB for example will probably combine with all -SH groups on the cell proteins and with GSH. Nevertheless, since some degree of inhibition was obtained with all the -SH inhibitors, these experiments confirm the reports in the literature that GSH and fixed -SH groups are involved in the reduction mechanism.

The fate of -SH groups during the reduction of DHA

If -SH groups are involved in the reduction of DHA a fall in their concentration might be expected during the course of the reduction. Total -SH groups were measured by an amperometric titration procedure in haemolysed erythrocytes at intervals of 1, 10, 15, and 32 min. after 10mg./100ml. of DHA was added. The reduction of DHA at these times was also followed by estimating the amount of AA produced (Table 28). Although DHA reduction was normal no change in total -SH groups was found. Since both free and bound -SH groups are reported to bring about the reduction of DHA (Schultze et al., 1937, and Thomson, 1955) this experiment provides strong evidence that some mechanism for the rapid regeneration of the oxidised -SH groups exists in haemolysed erythrocytes.

The fate of reduced pyridine nucleotides (DPN and TPN) during the reduction of DHA.

TABLE 29. Reduced pyridine nucleotides during reduction of DHA

Sample	Time after addition of DHA (min)	Reduced pyridine nucleotides (mg/100ml RBC)	Reduction of DHA (%)
Blood A	2	8.87	
	5	7.17	
	10	6.07	
	20	6.07	
	25	6.67	
	30	-	50
Blood B	1	9.20	
	5	9.90	
	10	10.73	
	15	9.93	
	20	9.40	
	30	-	49

The possibility that either DPN or TPN or both these co-enzymes might be involved in the reduction of DHA or in the regeneration of -SH groups was examined. The total reduced pyridine nucleotides were measured in CO - saturated blood containing 10 mg.% (w/v) DHA. Analyses were carried out on two samples of blood immediately and at intervals of 5 min. after addition of DHA. After 30 min. the amount of DHA which had been reduced was determined. No change was observed in the concentration of the reduced co-enzymes during the first 25 min. after addition of DHA although approximately one-half of the DHA had been reduced to AA (Table 29). These experiments however do not exclude the possibility that reduced pyridine nucleotides are directly responsible either for the reduction of DHA or for the regeneration of GSH and fixed -SH groups, since the co-enzymes may be rapidly oxidised from other substrates.

The effect of substrates on the reduction of DHA.

It has been shown in a previous experiment that there is no fall in the concentration of total thiol groups during the reduction of DHA. If thiol groups are responsible for the reduction of DHA in erythrocytes some mechanism must be postulated for their regeneration. Regeneration of -SH groups could be coupled to the oxidation of some other substrate. Such an oxidation may be aerobic or anaerobic. Although the erythrocyte preparations were always saturated with CO, it is extremely difficult to exclude traces of oxygen completely. The possibility that oxidation of glucose, which is a normal metabolite of erythrocytes, might

TABLE 30. The concentration of glucose during reduction of DHA

Time after DHA addition (min)	No added DHA Glucose (mg/100ml)	+ DHA (50mg/100ml) Glucose (mg/100ml)	AA produced (mg/100ml)
1	148	157	1.29
5	152	152	3.96
10	150	158	5.42
15	-	154	6.38
20	152	153	7.68
30	154	161	9.28

TABLE 31. The effect of added glucose on the reduction of DHA

Time after DHA addition (min)	No added glucose			+ glucose (50mg/100ml)		
	AA (mg/100ml)	Total AA (mg/100ml)	Reduction (%)	AA (mg/100ml)	Total AA (mg/100ml)	Reduction (%)
15	3.88	10.56	37	3.92	10.56	37
30	6.20	10.44	59	5.68	9.72	58

be coupled with the regeneration of thiol groups was next examined.

The concentration of glucose was followed at intervals of 5 min. for one-half hour in a haemolysed solution of erythrocytes to which DHA, to give a concentration of 50 mg./100ml., was added. Glucose was also estimated by the method of Hagedorn & Jensen in solutions of the same erythrocytes to which no DHA had been added, in order to correct for any breakdown of glucose not concerned with the reduction of DHA. Table 30 shows that the concentration of glucose remained unaltered in both the solutions, although large amounts of AA were formed in the solution to which DHA had been added. The role of glucose was further tested by comparing the rate of reduction of DHA in haemolysed erythrocytes and in haemolysed erythrocytes enriched with 50 mg./100ml. glucose. To a solution of haemolysed erythrocytes DHA was added to give a final concentration of 10 mg./100ml. Immediately, 9.5ml. of the haemolysed erythrocytes were added to 0.5ml. of a solution containing 100 mg. glucose per 10 ml. A further 9.5 ml. were added to 0.5 ml. water as a control. AA and total AA were estimated after 15 and 30 min. and the amount of DHA reduction was compared (Table 31). Reduction proceeded at the same rate in both the solutions. Since glucose does not increase the rate of DHA reduction and the concentration of glucose does not fall during the reduction of DHA it is concluded that glucose is not concerned in the reduction mechanism.

<u>The effect of other substrates on the rate of reduction of DHA</u>.

To aliquots of haemolysed erythrocytes, containing 10 mg.

TABLE 32. The effect of added substrates on the reduction of DHA

Substrate (50mg/100ml)	AA produced (mg/100ml)	Reduction of DHA (%)	pH
None	3.00	39	7.0
Alcohol	3.04	40	7.0
Sodium pyruvate	3.32	43	7.0
Sodium tartrate	3.60	47	7.1
Sodium succinate	3.44	44	7.2
Sodium formate	3.56	46	7.2
Sodium lactate	3.64	47	7.3

of DHA /100ml. the following substrates were added to give a final concentration of 50 mg./100ml. sodium lactate, sodium succinate, sodium tartrate, sodium pyruvate, sodium formate and alcohol. The results are given in Table 32. Alcohol certainly was without effect on the reduction mechanism. Although the other substrates slightly increased the amount of DHA which was reduced it is probable that the increase was due to the slightly higher pH in the erythrocyte solutions and not due to any direct effect of the substrates. Under the conditions of the experiment no substrate was found which markedly increased the rate of reduction of DHA either directly or through the regeneration of thiol groups. However, the added substrates may have been used by other competitive systems.

B. The reduction of DHA by erythrocytes from normal subjects and from patients with rheumatoid arthritis, iron deficiency anaemia, tuberculosis and scurvy.

It has been reported (Vinokurov & Silakova, 1944; Matusis, 1951) that in some diseases tissue reduction of DHA is not normal. The capacity of erythrocytes to reduce DHA was therefore investigated in a group of individuals in normal good health, and in hospitalized patients with rheumatoid arthritis, tuberculosis and scurvy. Erythrocytes from hospital out-patients considered to have iron deficiency anaemia were also examined. Tuberculosis and rheumatoid arthritis were selected for this study because it has been suggested that some disturbance of vitamin C metabolism may occur in these diseases. Scurvy, on the other hand is the classical vitamin C deficiency disease. Iron deficiency anaemia was examined in order to determine whether the haemoglobin content of the erythrocytes was related to the DHA reducing mechanism. Furthermore, rheumatoid arthritis is usually accompanied by a normocytic hypochromic anaemia. The possibility that any change in reducing capacity might be associated with a chronic illness or infection was examined by comparing the tubercular and rheumatoid groups with the normal group.

The bloods which were examined were either fasting specimens or were obtained 2-3 hours after a light meal. The percentage reduction 30 min. after the addition of DHA to intact erythrocytes was determined under the standard conditions described on page 61. The haematocrit reading, after removal of the leucocytes, was always adjusted to 30($\pm$ 3). The calculation

TABLE 33. Data on the reduction of DHA and haematological values in normal subjects and in patients with various diseases. Values given are means.

Group	n	Total AA (mg/100ml)	Hb (%)	RBC Count mill/cmm	C.I.	MCHC	Reduction of DHA (%)	DHA + DKG Plasma Cells (mg/100ml)		AA Plasma Cells (mg/100ml)		Total AA Plasma Cells (mg/100ml)	
Normal	41	0.215	100	5	1.0	33	48	4.13	1.16	0.23	4.61	4.37	5.77
Rheumatoid Arthritis	31	0.231	80	4.79	0.83	28	62	3.22	0.52	0.35	5.59	3.57	6.10
Fe deficiency Anaemia	7	0.235	60	4.23	0.71	29	63	2.95	0.40	0.23	5.94	3.19	6.34
Tuberculosis	8	0.190	104	5.40	0.97	33	54	3.33	1.26	0.15	5.15	3.50	6.39
Scurvy	3	0.0	60	2.93	1.03	28	61	3.66	0.47	0.13	6.16	3.79	6.63
Fe deficiency +Scurvy	10	-	60	3.84	0.81	28	62	3.17	0.42	0.20	6.01	3.37	6.43

of the percentage reduction was based on the exact p.c.v. achieved. Data on the reducing capacity, on the total AA in plasma before addition of DHA and on the haematological values are summarised in Table 33.

In a group of 41 "normal" subjects the mean percentage reduction of added DHA was 48 (S.D. = 5.9, range 32-59). Haematological data on these individuals was not available but the normal values given by Discombe (1954) are included for comparison with those of the other groups. In blood from 31 cases of rheumatoid arthritis the mean percentage reduction was 62 (S.D. = 9, range 44-89). These patients were all receiving salicylate or codeine phosphate. In a few cases the reducing capacity was examined during salicylate therapy and later during codeine treatment. No difference in the percentage reduction of DHA was observed. In rheumatoid arthritis the range of the percentage reduction was wider than in the normal group and, as shown in Fig. 27, the distribution curves for the two groups overlap. The mean percentage reduction for the patients with rheumatoid arthritis was significantly higher than normal ($p < 0.01$). The concentration of total AA in the plasma before the addition of DHA was not different from normal. The haematological data show the hypochromic normocytic anaemia typical of the disease.

Comparison of the data on the reduction mechanism in the rheumatoid patients with that on normal subjects shows that the concentration of total AA in the cells was greater in the rheumatoid group. The concentration of AA in the cells was also greater. In the rheumatoid group 90% of the erythrocyte

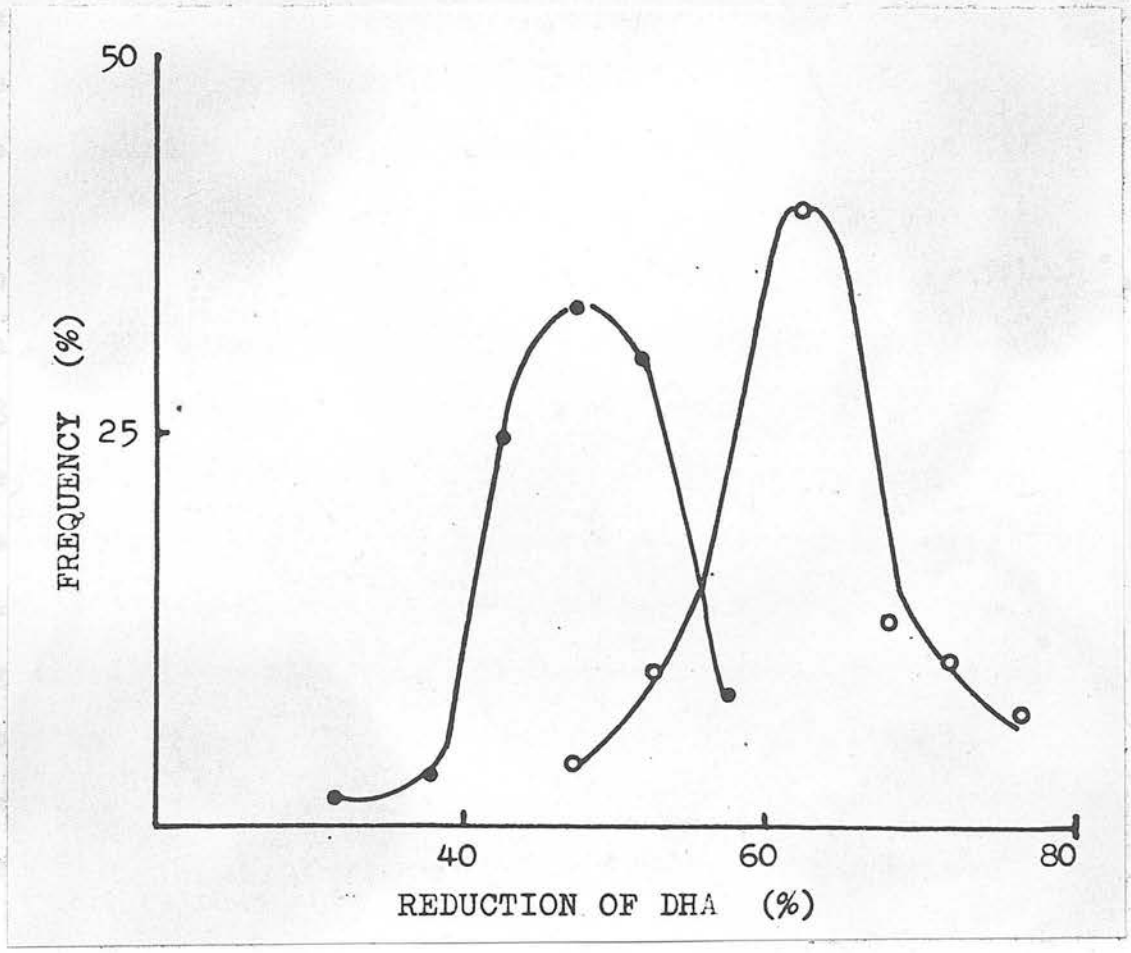


Fig. 27. Frequency distribution curves for the percentage reduction of DHA by erythrocytes. A, normal erythrocytes (n= 41); B, erythrocytes from patients with rheumatoid arthritis (n= 31).

vitamin C was present as AA whereas only 80% was in this form in the normal group.

Statistical analysis of the results obtained for the blood from patients with iron-deficiency anaemia, tuberculosis and scurvy was not carried out because the number of patients available was too small. The 3 cases of scurvy should be considered with the iron deficiency group since they were all anaemic. In this group the mean percentage reduction was 62 (range 54 to 73) and the individual figures for the reduction mechanism are comparable with those of the rheumatoid group. The concentration of AA in the plasma before the addition of DHA was also similar. In the 3 cases of scurvy this value was zero.

The mean percentage reduction of DHA in the tuberculosis group was a little higher than normal. Blood was obtained from these patients immediately on admission to hospital and before any drug therapy was started. The haematological pattern for this group was normal. The initial levels of total AA in the plasma were a little lower than in the other groups.

The most striking feature of this investigation is the inverse relation between the percentage reduction and the mean corpuscular haemoglobin content (MCHC). Since the reduction of DHA is a function of the erythrocytes, the MCHC should provide the best haematological index for correlation with the percentage reduction if it is a function of the haemoglobin content of the erythrocytes. MCHC values below 30 are considered to be good evidence of an iron deficiency anaemia. In all groups where this index is less than 30, the percentage reduction was consider-

ably greater than normal. It was unfortunately not possible to extend the investigation of the iron deficiency group and to measure the percentage reduction after the anaemia had been corrected. In another study, Thomson (1955) has shown that in 8 patients with pernicious anaemia, the mean percentage reduction of DHA by erythrocytes was 67. Thomson suggested that the percentage reduction was inversely related to the haemoglobin concentration of the blood. Unfortunately, values for the MCHC are not included, however this index is generally not low in pernicious anaemia. If the percentage reduction is related to the haemoglobin concentration in the erythrocyte it is difficult to understand why the percentage reduction of DHA should be high in both iron deficiency and in pernicious anaemia.

TABLE 34. Variation in the percentage reduction of DHA in the same individuals

Subject	Condition	Date	Reduction of DHA (%)
A.I.	Normal	4.10.54	39
		6.10.54	36
		6.7.55	39
B.I.	Normal	8.12.54	51
		6.5.55	55
		15.6.55	50
		14.9.55	54
J.O.	Normal	10.9.54	43
		12.9.55	45
		4.10.55	41
M.B.	Rheumatic Fever	15.10.54	53
		19.10.54	49
		26.10.54	51
W.M.	Rheumatoid Arthritis	16.10.54	63
		18.10.54	62
		24.11.54	63
		31.1.55	66

C. The effect of drugs on the reduction of DHA by intact erythrocytes

It has been shown that the capacity of erythrocytes to reduce DHA is considerably increased in some diseases. The effect on the reduction of DHA of several drugs which are commonly used in the treatment of these diseases and which produce a clinical improvement in the condition of the patients, was studied. The standard conditions of CO saturation, p.c.v., temperature and time under which the reduction of DHA by intact erythrocytes was measured have already been described. Blood was routinely obtained 14 hr. after the last meal.

The normal variation in the reduction of DHA by erythrocytes from the same individual was first examined .

Variation in the percentage reduction of DHA in the same individual

During the course of a year blood was obtained from the same individuals for different experiments which required the measurement of the percentage reduction of DHA under standard conditions. In no instance did the capacity to reduce DHA alter by more than 5% (Table 34). In the normal individuals the constancy of the reduction capacity is remarkable and, in fact, supplies a useful test of the accuracy of the experimental manipulations. Blood from hospital patients was not available over such long periods. One case of rheumatoid arthritis (W.M.) was followed for 4 months; the percentage reduction did not vary by more than 4% during this time. Since rheumatoid arthritis is a disease marked by periods of remission of the clinical symptoms it is possible that the percentage reduction of DHA may not always exhibit this stability. Unfortunately no reliable, objective index of the progress of the

disease is available. Throughout the period of investigation W.M. was considered to be an "active" case of rheumatoid arthritis. Although the short term effects of several drugs were tested during this time the values for the percentage reduction of DHA given in Table 34 were determined during control periods at a reasonable time after the administration of the drugs.

The effect of cortisone and salicylate on the reduction mechanism.

In view of the clinical improvement in rheumatoid disease which follows treatment with large doses of acetyl salicylate (salicylate) or of adrenocortical drugs, the effect of these drugs on the reduction capacity of erythrocytes was examined in normal subjects and in patients with rheumatic disease.

(a) The effect of salicylate ingestion on the percentage reduction of normal erythrocytes. Blood was obtained from a subject in normal health and the percentage reduction of DHA by the erythrocytes was determined. Two days later daily dosage with 80 grains of salicylate began. Blood samples were obtained 3 days and 5 days after the beginning of salicylate ingestion. Throughout the experiment 500 mg. of AA were taken daily. The subject had reached saturation with AA before the first blood sample was withdrawn. Since the concentration of AA in the blood does not affect the reduction mechanism (as found in the large series of normal individuals) and the high initial levels of AA are allowed for in the calculation of the percentage reduction, the daily ingestion of 500 mg. of AA probably does not influence the effect of the salicylate. The results are presented graphically in Fig. 28a. Even after 5 days of salicylate treatment no change

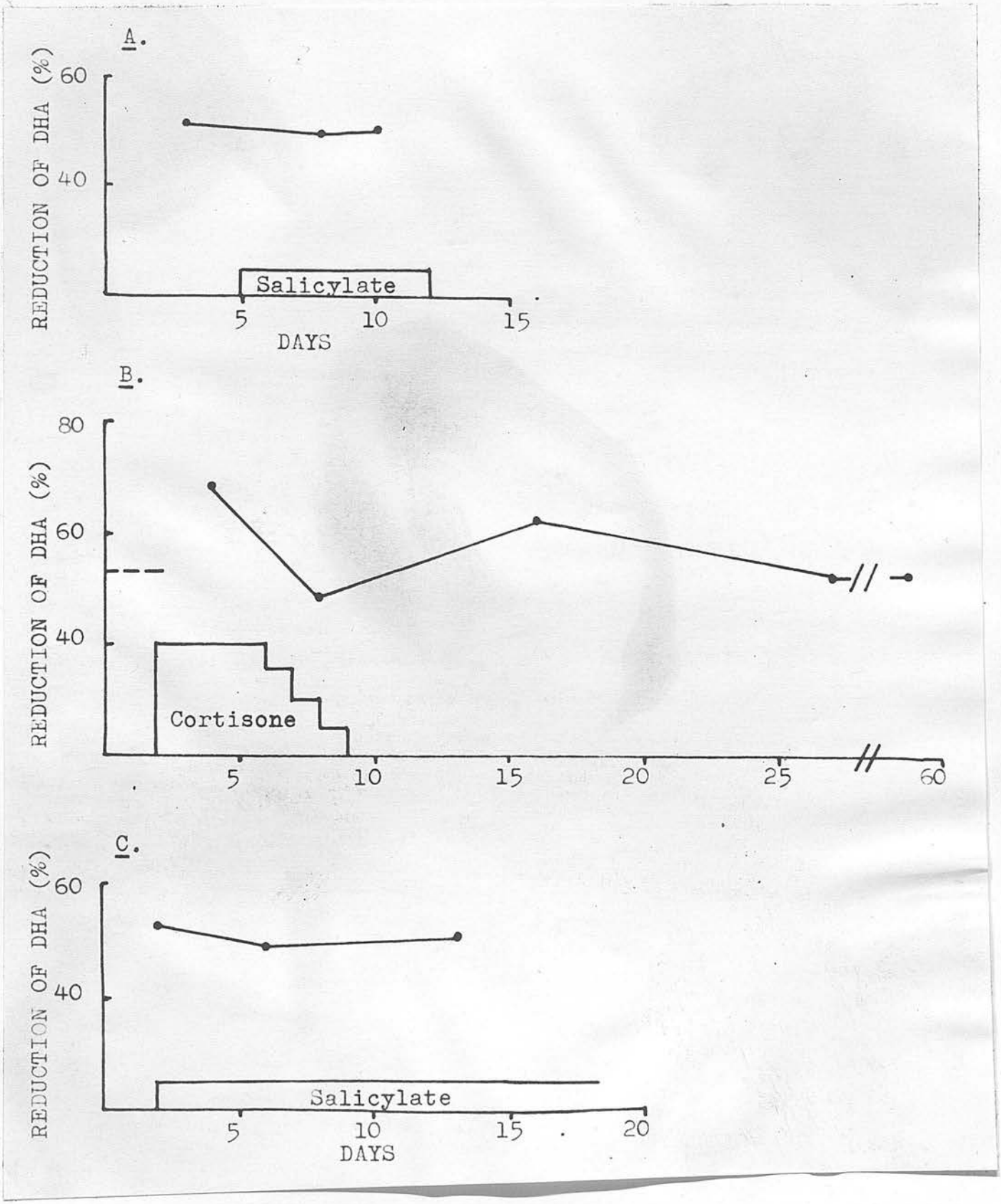


Fig.28. The effect of cortisone and salicylate on the percentage reduction of DHA by erythrocytes. A, salicylate on normal cells; B, cortisone on normal cells; C, salicylate on cells from a case of rheumatic fever.

TABLE 35. The effect of cortisone on the reduction of DHA by normal erythrocytes

Day	Vitamin C (mg/day)	Cortisone (mg/day)	Blood (ml)	Reduction of DHA (%)
1	50	-	-	-
2	50	-	-	-
3	50	150	-	-
4	50	150	-	-
5	50	150	20	69
6	50	150	-	-
7	50	150	-	-
8	50	100	-	-
9	50	75	20	48
10	50	50	-	-
11	50	25	-	-
12	50	-	-	-
13	50	-	-	-
14	50	-	-	-
15	50	-	-	-
16	50	-	-	-
17	50	-	-	-
18	50	-	20	60
1 month later	-	-	20	53

Diet:- Low in vitamin C; no fruit, green vegetables or turnip.
Vitamin C supplement and cortisone given after breakfast.
Blood withdrawn before breakfast.

in the percentage reduction was observed. The effect of these high doses of salicylate could not be followed over longer periods because of the unpleasant subjective symptoms associated with its continued ingestion.

(b) The effect of cortisone ingestion on the reducing capacity of normal erythrocytes. Blood was obtained from a subject in normal health 2 days and 6 days after the beginning of daily ingestion of 150 mg. of corticone acetate. Unfortunately a blood sample was not taken before cortisone treatment began. Blood was obtained 6 days and 40 days after the last dose of cortisone. It is considered that the percentage reduction of this latter sample represents the normal value for this individual. Details of the experiment are given in Table 35 and the results are plotted graphically in Fig.28b. After 2 days the percentage reduction of 68 was 10% above the upper limit of the normal range. It fell to 48% and then rose again and 6 days and 40 days after the last dose of cortisone it was within the normal range. These results show that cortisone had a marked effect on the metabolism of the erythrocytes but it is not possible to explain the result.

(c) The effect of salicylate on the reducing capacity of erythrocytes from a patient with acute rheumatic fever. Blood was obtained from a patient with acute rheumatic fever with no previous history of salicylate treatment. Treatment with salicylates (80gr. per day) was begun and blood samples were obtained on the third and the tenth day after the beginning of treatment. As shown in Fig.28c, salicylates had no effect on the DHA reducing

capacity of the erythrocytes.

(d) The effect of AA on the reducing capacity of rheumatoid erythrocytes. In one patient with rheumatoid arthritis the DHA reducing capacity was followed during treatment with 500 mg. of AA daily for 8 days. Two fasting blood samples were obtained before treatment and further samples were taken 1, 3, and 6 days after treatment began. Five days after the administration of AA had been stopped another sample was taken. The results are given in Fig.29. There was a large rise in the plasma AA following AA administration and a sharp fall on its withdrawal. The small fluctuations in the DHA reducing power of the erythrocytes are considered to be no more than the normal variations in a patient of this type, so that the reducing capacity appears to be unaffected by the plasma AA level.

It is never possible to withhold analgesics completely from patients with rheumatoid arthritis, so that in all experiments where salicylate was withheld it was replaced by codeine phosphate. It is unlikely that codeine phosphate has any marked effect since 2 months later when the patient was again on salicylate treatment the percentage reduction of DHA was unchanged.

(e) The effect of cortisone on the reducing capacity of rheumatoid erythrocytes. The reducing capacity of erythrocytes from a patient with rheumatoid arthritis was followed during a short period of cortisone therapy. This experiment was of particular value because it was possible to repeat it on the same individual; first with cortisone supplemented with a high AA intake and then replacing the cortisone with ACTH. Before

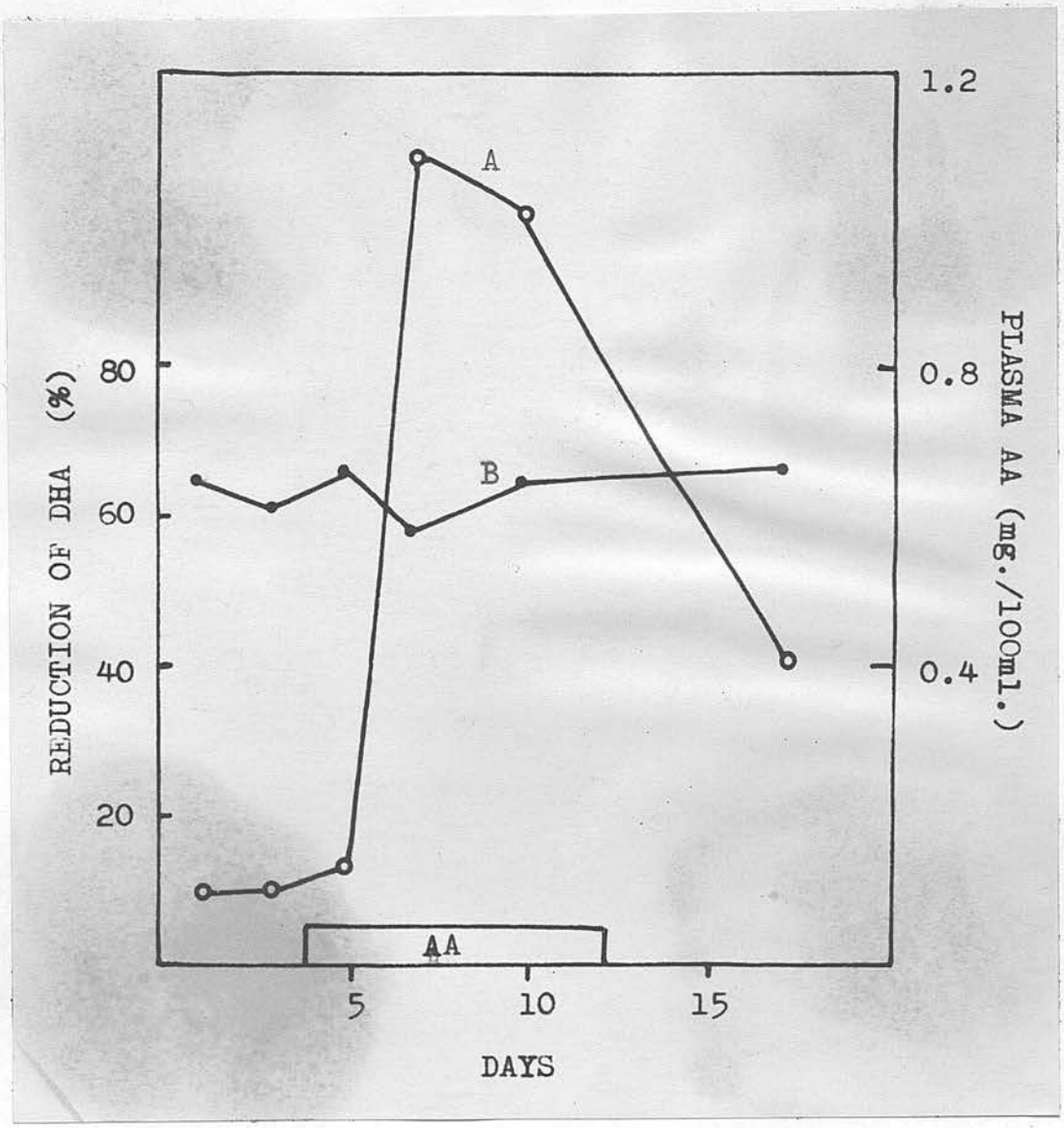


Fig.29. The effect of daily ingestion of 500mg. of AA on the percentage reduction of DHA by rheumatoid erythrocytes. Patient, J.M., female, rheumatoid arthritis. A, total AA in plasma (mg./100ml) B, percentage reduction.

TABLE 36. Protocol for the experiments on the effect of cortisone on the reduction of DHA by rheumatoid erythrocytes

Day	Period of low AA			Period of high AA		
	Vitamin C (mg/day)	Cortisone (mg/day)	Blood (ml)	Vitamin C (mg/day)	Cortisone (mg/day)	Blood (ml)
1	50	-	-	500	-	20
2	50	-	-	500	-	-
3	50	-	-	500	-	-
4	50	-	-	500	-	-
5	50	-	-	500	-	-
6	50	-	20	500	-	-
7	50	-	-	500	-	20
8	50	-	20	500	-	-
9	50	150	-	500	150	-
10	50	150	20	500	150	20
11	50	150	-	500	150	-
12	50	150	20	500	150	-
13	50	150	-	500	150	20
14	50	100	-	500	100	-
15	50	75	20	500	75	20
16	50	50	-	500	50	-
17	50	25	20	500	25	20
18-21	50	-	-	500	-	-
22	50	-	20	500	-	20

Diet:- Low in vitamin C; no fruit, green vegetables or turnip.
Vitamin C supplement and cortisone given after breakfast.
Blood withdrawn before breakfast.

cortisone therapy was begun 2 samples of blood were taken. Samples were also taken on the first, third, sixth and eighth day after it was started. A final specimen was collected 5 days after the last dose of cortisone. The experimental details and results are given in Table 36 and Fig.30 respectively. Cortisone administration produced wide fluctuations in the values for the percentage reduction of DHA. After 2 days the value had fallen to 35% and 3 days later it was 85%. Cortisone treatment was then gradually stopped and the value for the percentage reduction fell to the original value.

These changes were so dramatic that the experiment was repeated one month later with the difference that a supplement of 500 mg. of AA per day was given. The experimental details are given in Table 36 and the results in Fig.30. The control level, 7 days after AA administration was started but before cortisone was given, was exactly the same as in the previous experiment. This result confirms the conclusion that the level of AA intake does not affect the reducing capacity of erythrocytes. The response to cortisone is strikingly similar in the 2 experiments. A sudden fall at the third day is followed in both experiments by a rapid rise to a very high level, with a return to the original level after the cortisone is stopped. The duplication of the cycle together with the size of the responses suggests that in this patient cortisone elicited a definite response of the reducing mechanism in the erythrocyte. Since the response was the same on both a low and a high level of AA intake it is concluded that the changes were due to

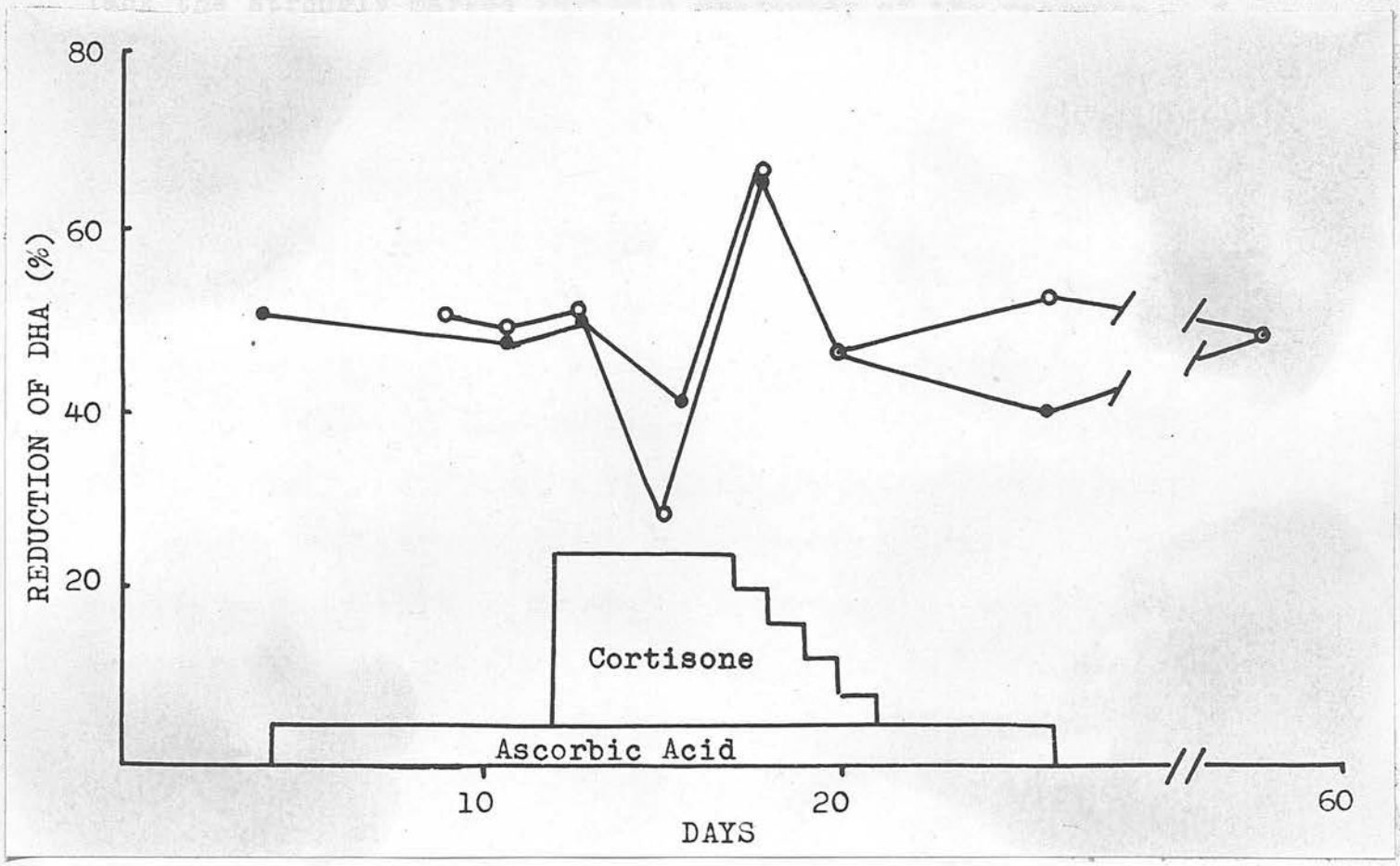


Fig. 30. The effect of cortisone on the percentage reduction of DHA by rheumatoid erythrocytes. Patient, W.M. male, rheumatoid arthritis. o - o low AA diet (50 mg. AA/day); ● - ● high AA diet (500mg. AA/day).

cortisone. Although no explanation of the changes is offered it is likely that changes in the activity of the adrenal cortex in response to cortisone administration in some way cause the changes in erythrocyte metabolism. Any direct effect on the erythrocyte might be expected in less than 3 days and would probably lack the strongly marked rhythmic character of the response.

(f) The effect of ACTH on the reducing capacity of rheumatoid erythrocytes. The response of the reducing mechanism to ACTH treatment was followed in the same patient (W.M.) at daily intervals for 12 days. Daily injections of 40 mg. ACTH were given after the specimens of fasting blood had been withdrawn. The results are plotted in Fig.31. The initial value for the percentage reduction was the same as in the 2 prior experiments. Within 24 hr. of the first ACTH injection the value had fallen to the very low level of 30%. In the next 24 hr. it rose sharply to 74% then fell to 60%. After this it rose progressively to 75% when the ACTH administration was discontinued. The cycle of changes following ACTH administration was similar to that elicited by cortisone treatment with the difference that the changes occurred sooner with ACTH therapy. The clinical effects of ACTH treatment are also earlier in onset than those due to cortisone therapy. The fact that ACTH has a similar effect to cortisone supports the hypothesis that the action is an indirect one operating through changes in suprarenal activity.

(g) The effect of meticorten on the reducing activity of rheumatoid erythrocytes. The short term effect on the

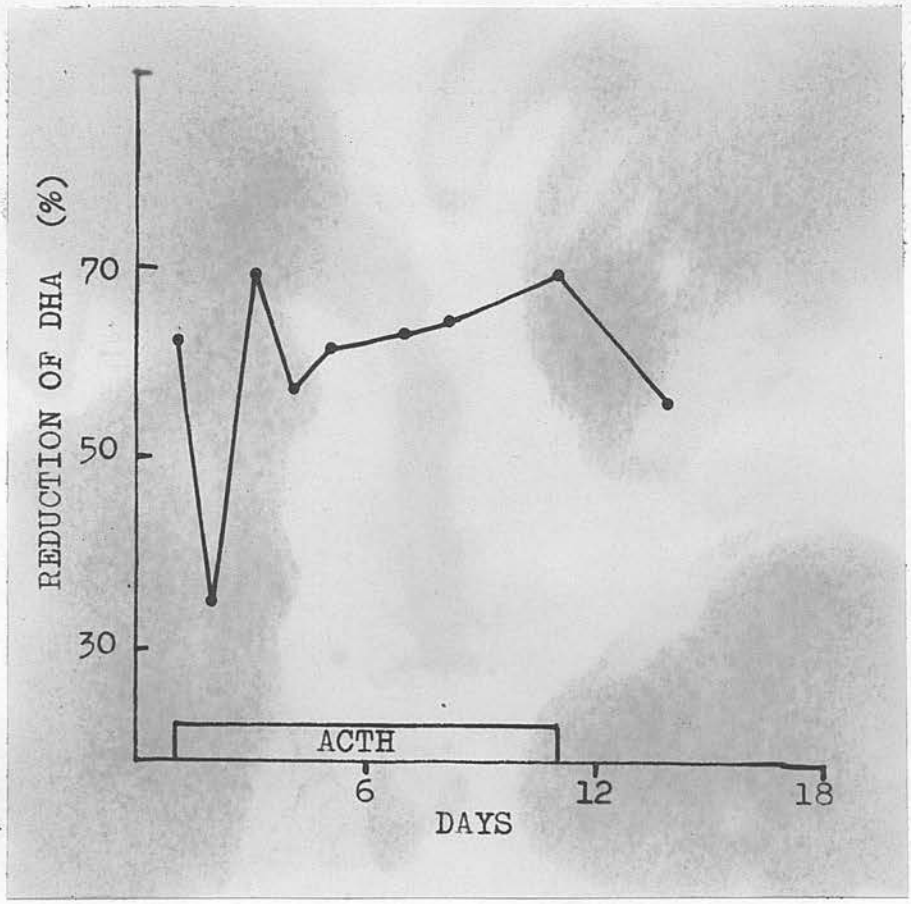


Fig. 31. The effect of ACTH on the percentage reduction by rheumatoid erythrocytes. Patient, W.M., male, rheumatoid arthritis. Dosage: 40 mg. ACTH per day.

TABLE 37. The effect of Meticorten on the reduction of DHA by rheumatoid erythrocytes

Time (hr)	Meticorten	Reduction of DHA (%)
0	-	64
12	50 mg	-
24	-	73
48	-	64

TABLE 38. Protocol of the experiments on the effect of salicylate on the reduction of DHA by rheumatoid erythrocytes. Patients, P.J., B.W. and M.T.

Days	AA supplement (mg/day)	Salicylate (gr/day)	No. of blood samples taken
1 - 14	None	-	2
15 - 28	500	-	2
29 - 42	500	80	2
43 - 49	500	-	1
50 - 57	None	-	2

TABLE 39. Protocol of the experiments on the effect of cortisone on the reduction of DHA by rheumatoid erythrocytes. Patients, A.McL. and A.C.

Days	AA supplement (mg/day)	Cortisone (mg/day)	No. of blood samples taken	
			A.McL.	A.C.
1 - 14	-	-	2	2
15 - 28	500	-	2	2
29 - 38	500	150	4	4
39	500	100	-	-
40	500	75	1	-
41	500	50	-	1
42	500	25	-	-
43	500	-	1	1
44 - 49	500	-	1	1
50 - 57	-	-	1	1

percentage reduction of DHA of a single dose of meticorten was examined in a patient with rheumatoid arthritis. Meticorten is a synthetic adrenocortical drug reported to have very little effect on electrolyte metabolism and to have a more powerful effect on steroid metabolism than cortisone.

A specimen of blood from the fasted patient was obtained and the percentage reduction of DHA was determined. Twelve hr. later a dose of 50 mg. of meticorten was given. Further blood samples were taken 12 and 36 hr. after the meticorten was given. Twelve hr. after dosage, the percentage reduction increased from 64 to 73% and 24 hr. later it had fallen to the original level (Table 37).

It is concluded that the effect of adrenocortical drugs on erythrocyte reducing capacity is probably not associated with their effects on electrolyte metabolism.

(h) Comparison of the effects of salicylate and cortisone during a long period of administration of large doses of AA to patients with rheumatoid arthritis. Protocols of the experiments are given in Tables 38 and 39. The effect of salicylate was examined in 3 patients and the effect of cortisone in 2 patients. Specimens of fasting blood on which the percentage reduction of added DHA was determined were taken during each of the following 5 periods of the experiment.

(a) a preliminary control period of 15 days during which none of the drugs were given;

(b) a period of 14 days when 500 mg. of AA was given daily;

(c) a period of 14 days during which either cortisone or

TABLE 40. Details of treatment, plasma AA, haematological values and erythrocyte reduction of DHA of patient J.S. (scurvy)

Day	Treatment	Plasma AA (mg/100ml)	Hb (%)	p.c.v.	M.C.H.C	Reduction of DHA (%)
1	None	0	42	20	27	71
2	500mg. AA/day	-	-	-	-	-
9	↓	1.18	50	29	29	74
15	↓	-	68	33	30	-
16	AA stopped	-	-	-	-	-
19	100mg Nicotinic Acid	-	-	-	-	-
20	50mg AA/day	-	-	-	-	-
23	↓	1.18	85	38	33	44
27	50mg AA + Vit B daily	0.88	90	41	33	63
35	↓	0.67	90	41	33	57
56	↓	0.45	-	41	-	58
83	↓	0.52	-	41	-	57

salicylate was given in addition to the AA. On the last 4 days of this period the cortisone dose was gradually reduced;

(d) a period of 7 days when only AA was given;

(e) a final period during which none of the drugs were given.

In the salicylate experiments (Fig.32 a,b,c,) the percentage reduction of DHA varied considerably. Although these changes were as much as 10% they were not causally related to the administration or withdrawal of either salicylate or AA. A composite graph in which the mean values for the 3 experiments are plotted is shown in Fig. 32d. It is evident from this graph that the fluctuations in percentage reduction were random.

In the 2 cortisone experiments (Fig.33a and b) the values for the percentage reduction of DHA rose steadily throughout the period of cortisone treatment. Furthermore, the high level was sustained for at least one week after cortisone was withdrawn and then fell slowly to the original level. The composite graph of the results (Fig.33c) shows clearly that the increase in the percentage reduction was related to the administration of cortisone and not to the AA.

Changes in the erythrocyte reducing capacity of a patient with frank scurvy.

The details of the treatment with AA, nicotinic acid, and a vitamin "B complex" preparation, together with the values for plasma AA, haemoglobin, p.c.v., MCHC and percentage reduction of DHA are given in Table 40 and Fig.34. Unfortunately treatment was sometimes given without warning, e.g. in

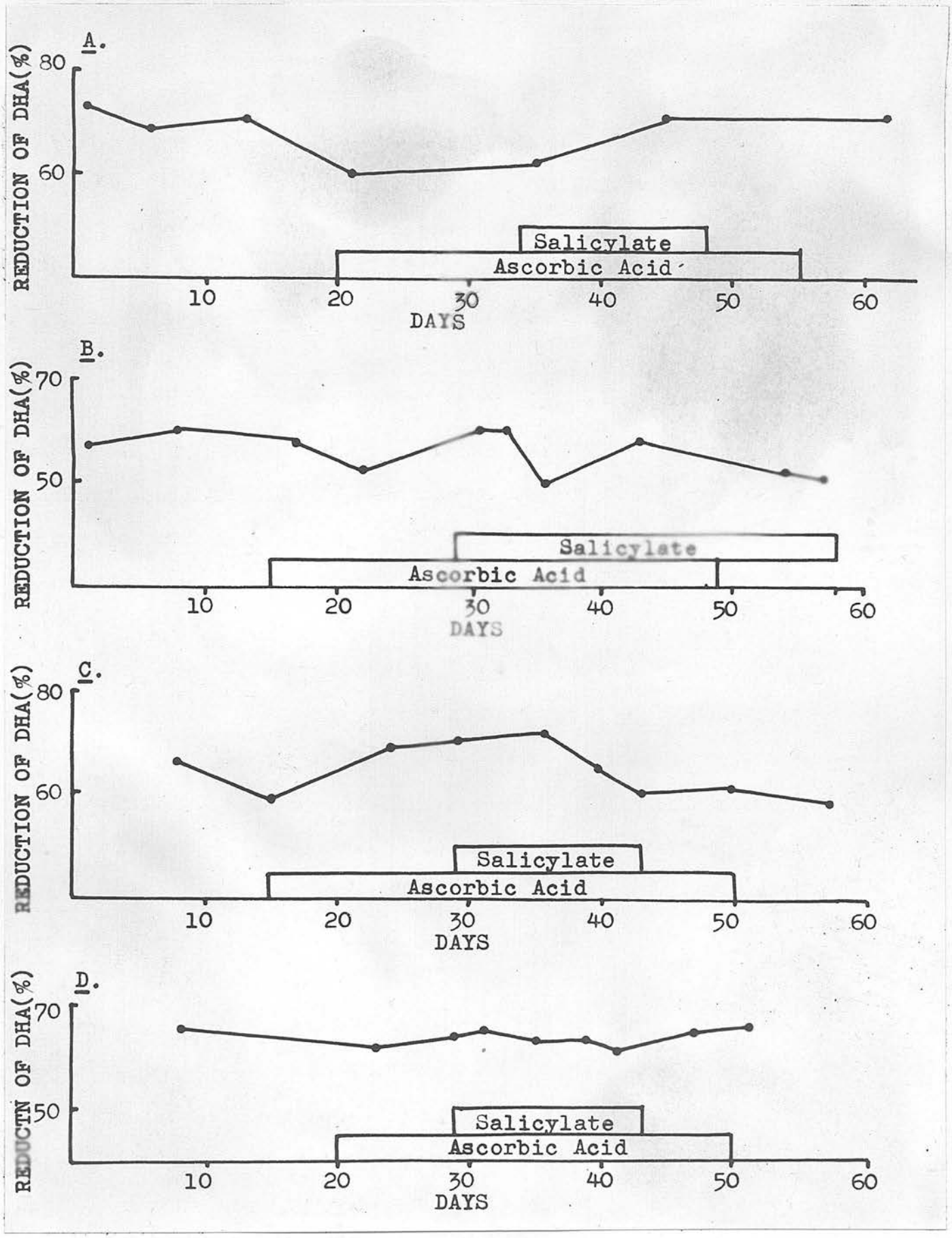


Fig.32. The effect of salicylate on the percentage reduction of DHA by erythrocytes from patients with rheumatoid arthritis. A, P.J., male; B, B.W., female; C, M.T., female; D, composite graph of A,B and C. Dosage:- salicylate, 80gr./day; AA, 500mg./day.

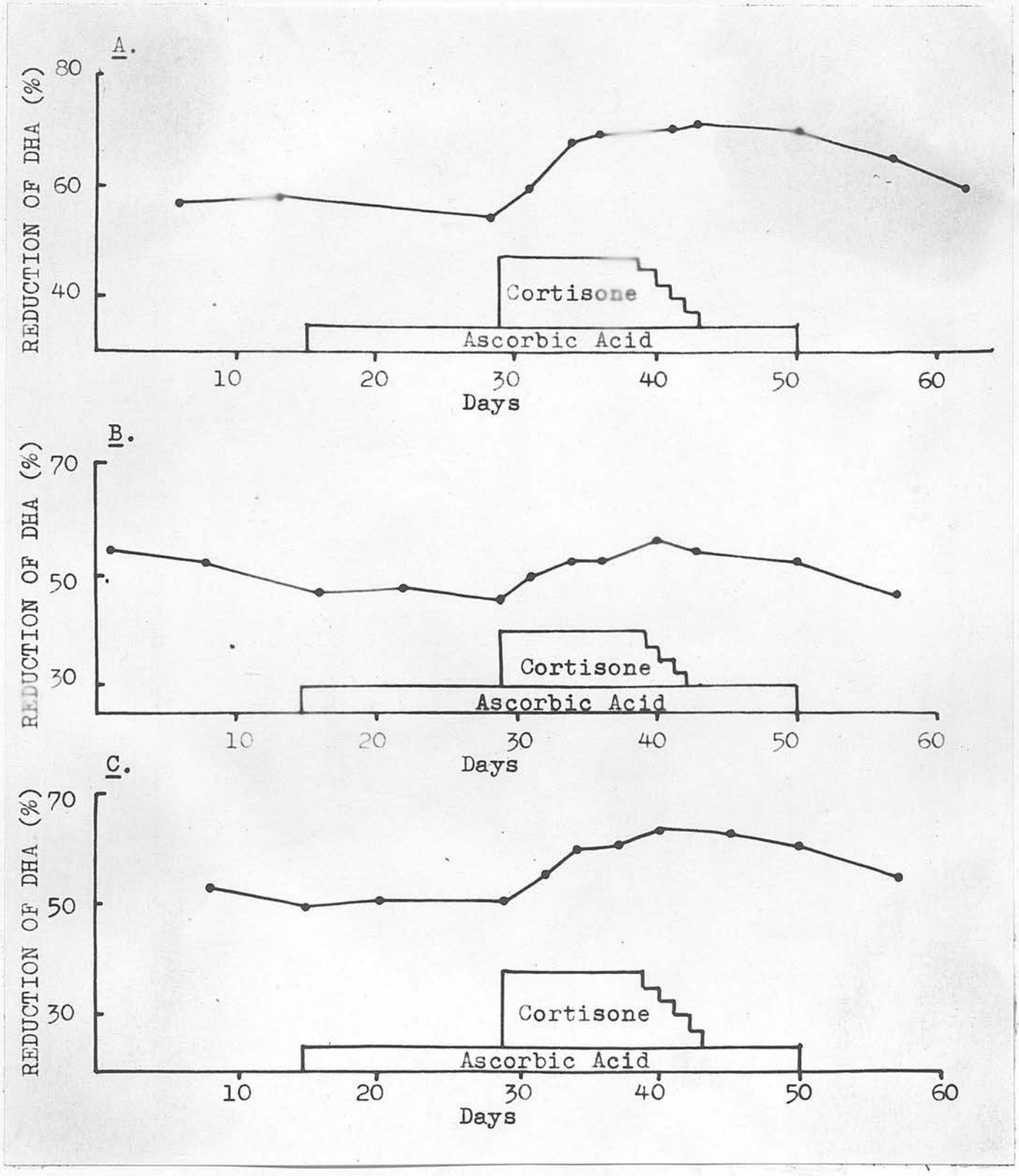


Fig. 33. The effect of cortisone on the percentage reduction of DHA by erythrocytes from patients with rheumatoid arthritis. A, A.C., male; B, A.McL., male; C, composite graph of A and B. Dosage:- cortisone, 150 mg./day, reduced gradually during the last 4 days; AA, 500 mg./day.

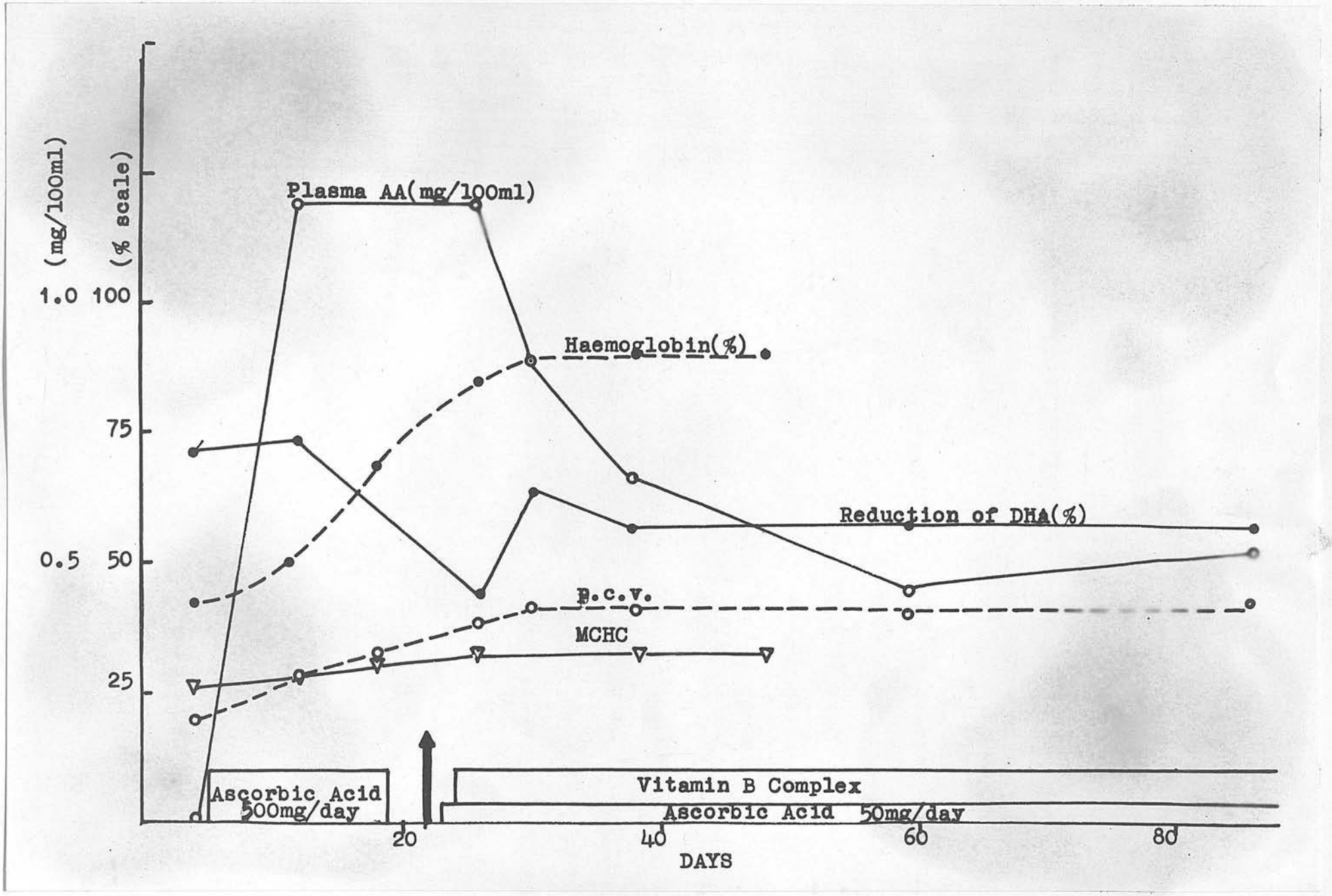


Fig. 34. Haematological data, plasma AA and percentage reduction of DHA over 80 days for patient J.S., male, with acute scurvy. Base line shows treatment.

the case of the intravenous injection of nicotinic acid, and a value for the percentage reduction could not be determined immediately before this. No record of any treatment with iron is recorded so it is assumed that the correction of the marked iron deficiency anaemia was due to the improved diet and the AA supplements, since this occurred before vitamin B was given. Initially the percentage reduction was very high and is similar to that found in cases of iron deficiency anaemia. On the twenty-third day the percentage reduction had fallen to 44. It was then discovered that alarge dose of nicotinic acid had been injected 4 days prior to this. It cannot be asserted with certainty that this very marked fall in the reducing capacity was due to the nicotinic acid and not due to the improvement in the anaemia. However 4 days before the injection the MCHC of 30 was still below the normal value for males (i.e.33) and the anaemia was therefore not fully corrected. Furthermore, when the DHA reducing capacity of the erythrocytes was determined again, it had increased during the next 4 days to 63. By this time the MCHC was 33. It is probable therefore that the nicotinic acid did contribute to a temporary lowering of the reducing capacity. During the next 8 weeks the patient was treated with a proprietary preparation of vitamin B. After 8 days the value for the percentage reduction had fallen further to 57 and remained at this level. No details of the amounts of the individual vitamins of the vitamin "B complex" preparation were available; however it would undoubtedly contain some nicotinic acid. The fall in the percentage reduct-

TABLE 41. The effect of nicotinic acid (100mg. i.v.) on the reduction of DHA by erythrocytes

Subject	Plasma AA (mg/100ml)	Reduction of DHA before nicotinic acid (%)	Reduction of DHA 2 days after nicotinic acid (%)	Reduction of DHA 4 days after nicotinic acid (%)
N. (rheumatoid)	0.22	64	61	54
O. (rheumatoid)	0.12	60	54	55
C. (rheumatoid)	0.26	71,67	54	58
F. (rheumatoid)	0.10	67,63	52	64
S. (scurvy)	1.18	63 - 73	-	44

ion from 63 to 57, which is in the upper range of normality, could be attributed either to vitamin B or to the correction of the anaemia.

The effect of nicotinic acid on the erythrocyte reducing capacity.

The results of the previous experiment suggested that nicotinic acid might have an effect on the erythrocyte reducing mechanism. Since this was the only experiment in which a fall to the normal value for the percentage reduction had been observed it was considered of interest to examine the effect of nicotinic acid more closely. The percentage reduction of DHA was therefore determined in patients with rheumatoid arthritis before and after the injection of 100 mg. of nicotinic acid. Rheumatoid patients were selected because it has been shown that the value for the percentage reduction is high in this disease. The results are summarised in Table 41. In every case a fall in the reducing capacity was observed between 2 and 4 days after the injection. It is therefore probable that in the previous experiment, nicotinic acid was responsible for the low value observed 4 days after its administration. Whether injection of nicotinic acid would bring about a fall in the erythrocyte reducing capacity of normal erythrocytes is not known. No figures are available for the blood nicotinic acid levels of normal subjects and patients with rheumatoid arthritis.

DISCUSSION

Three aspects of the ability of human erythrocytes to reduce DHA have been investigated; (a) the mechanism of the reduction, (b) changes in the reducing capacity during various diseases and (c) the effect of the administration of certain drugs on the reducing capacity.

The mechanism of the reduction of DHA. Preliminary experiments confirmed the reports in the literature that solutions of DHA at pH 7.0 are rapidly converted to DKG. At a lower pH e.g. 5.5, DHA is relatively stable for a period of at least 2.5 hr. At neither pH does any reduction to AA occur. Ascorbic acid is also known to be unstable in pure solution. In plasma, added DHA is very rapidly changed to substances which no longer form phenylhydrazine derivatives. This oxidation however is probably to be ascribed to the presence of a few leucocytes.

When added to human blood from which the leucocytes have been removed as completely as possible, DHA rapidly passes into the erythrocytes and is converted to AA. Erythrocytes, however are relatively impermeable to AA and therefore AA formed in the cells diffuses back into the plasma only slowly. These phenomena have been previously reported by Schultz et al. (1937), Heinemann (1941) and Lloyd (1951).

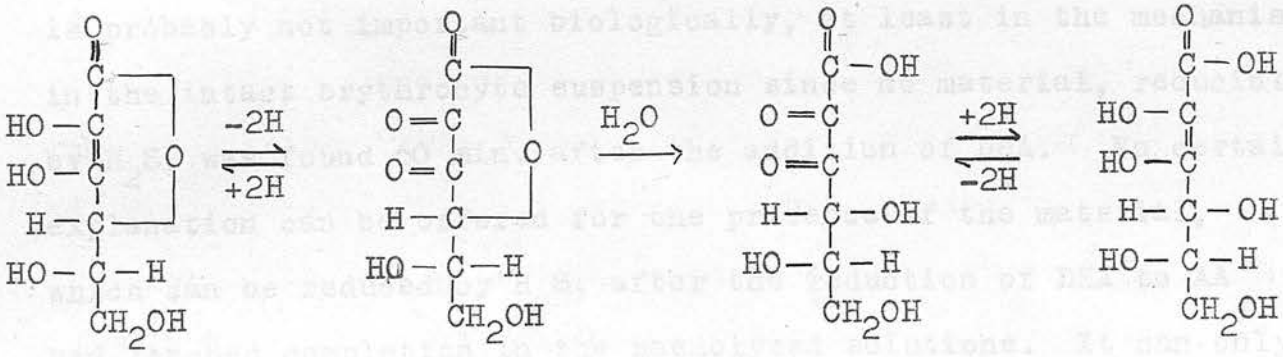
Examination of the course of the reduction in intact erythrocytes over 2 hr. shows that rapid formation of AA occurs in the erythrocytes during the first 30 min. after adding 10 mg. % (w/v) DHA. After this time very little more AA is formed. The concentration of total AA in the blood remains

constant for 30 min. but a slow decay to substances which do not form ozazones with phenylhydrazine sets in after this time. Furthermore, after 30 min. practically none of the total AA remaining in the plasma can be reduced by H_2S. Dehydroascorbic acid can therefore be metabolised by two processes. It can diffuse into the erythrocytes and there be reduced to AA or it can be converted to DKG in the plasma. This latter process no doubt limits the amount of DHA reduced by the erythrocytes to AA, since no further DHA would be available in the plasma after 30 min. These reactions proceed simultaneously and, under the standard conditions used, DHA is converted at equal rates to AA and DKG in normal blood. The formation of DKG from DHA may not be a function of the plasma itself but rather of the leucocytes since it was never possible to remove all of them by the differential centrifugation method used.

The mechanism for the reduction of DHA can be isolated almost completely from the mechanism which converts DHA to DKG by using erythrocytes haemolysed with water. Under these conditions the same amount of AA is formed during the first 30 min. as is formed by the intact erythrocytes suspended in plasma. However reduction continues for a further 60 min. in the haemolysed solutions and at the end of this time between 70 and 90% of the added DHA is reduced. A small amount of DHA remains in the haemolysed solution even after 160 min. Some DKG is also formed but both reactions reached completion in 90 min. It is possible that an equilibrium was attained between the remaining DHA and DKG since a zero level of DHA was never observed.

On the other hand the methods for differentiating between DKG and DHA in the blood depends on the reduction of the latter compound with H_2S, and this procedure has never been found to be entirely satisfactory. Penney & Zilva (1943b) have shown that some indophenol reducing material is produced by H_2S treatment of DKG. The slow conversion of DHA to DKG may have been due to the presence of a few leucocytes in the erythrocyte suspension but on the other hand it has never been demonstrated that a small conversion of DHA to DKG does not occur in erythrocytes.

Considering the structures of the compounds concerned the following reactions can be enumerated:-



Ascorbic acid — Dehydroascorbic acid — Diketogulonic acid

Both the reversible reduction of DHA to AA and the conversion of DHA to DKG are reactions which have been firmly established. A hypothetical intermediate, monodehydroascorbic acid, in the

reaction AA DHA has been postulated by Bezssonoff & Woloszyn, (1936). Lloyd & Sinclair (1953) have suggested that a compound of this type might be the form of the vitamin which is active in metabolic reactions.

The possibility of the formation of a substance related to DKG in the same way that AA is related to DHA has never been fully explored. Rheichstein & Gussner (1934) consider that this substance, which is the tautomeric form of 2-ketogulonic acid, is capable of conversion to DHA. Such a substance presumably could reduce indophenol and might therefore be responsible for the increase in indophenol reducing activity which Penney & Zilva (1943b) found after treating DKG with H_2S. These workers did not consider that the increased reducing activity was due to the conversion of DKG to AA. Such a reaction however is probably not important biologically, at least in the mechanism in the intact erythrocyte suspension since no material, reducible by H_2S, was found 60 min. after the addition of DHA. No certain explanation can be offered for the presence of the material, which can be reduced by H_2S, after the reduction of DHA to AA had reached completion in the haemolysed solutions. It can only be suggested that an equilibrium between DKG and DHA or between DKG and the tautomeric compound such as that suggested above was established.

Several workers have reported that the reduction of DHA in erythrocytes occurs directly by the oxidation of GSH and fixed -SH groups (Schultz *et al.*, 1937; Thomson, 1955). However the concentration of total -SH groups does not fall during the

course of DHA reduction. The reduction of DHA must therefore be governed by a mechanism which regenerates GSSG since it has been reported that erythrocytes can reduce enormous quantities of DHA when it is added in successive amounts (Lloyd & Parry, 1954; Thomson, 1955).

Thomson (1955) has observed that the reducing mechanism in erythrocytes exhibits some features of an enzyme system. Since GSH can directly reduce DHA it is suggested that these characteristics are related to an enzyme system concerned with the regeneration of thiol groups. In the course of the present work several experiments were carried out in an attempt to establish some of the properties of such a system. The levels of reduced pyridine nucleotides were followed during the reduction of DHA. Although no fall was observed, the participation of the coenzymes is not eliminated since reduced coenzymes are rapidly regenerated. TPN is concerned in the reduction of GSSG by glutathione reductase in rat liver (Rall & Lehninger, 1952). The presence of glutathione reductase in blood has not yet been reported. Indirect evidence that DPN takes part in the reduction in plant tissue has also been put forward (James & Cragg, 1943, 1944). Inhibitors such as CMB, iodoacetate and arsenite which are known to inhibit -SH enzymes partially inhibited the reduction of DHA added to blood. The concentrations of these substances were much in excess of the concentrations usually required to inhibit an enzyme system. Since the protein content of the erythrocyte solutions is high it is not surprising that large amounts of the inhibitors were required to produce an effect on the reduction.

However, the fact that a partial inhibition was observed indicates that thiol groups are concerned in the reduction mechanism. Cyanide also inhibited the reduction but this is a relatively non-specific inhibitor. Azide, fluoride, diethyldithiocarbamate and urethane had no effect on the reduction of DHA.

The reduction of GSSG or of oxidised pyridine nucleotides must eventually involve the coupled oxidation of some substrate. Eddy (1952) has reported that reduction of DHA by bacteria is stimulated by the addition of alcohol, malate, succinate, glucose, glycine, formate and lactate. However the rate of DHA reduction in haemolysed erythrocytes is not increased by the addition of glucose, alcohol, formate, tartrate, lactate, succinate or pyruvate.

Erythrocytes from a number of species reduce DHA. The failure of Panteleva (1950) to observe reduction in erythrocytes from horse, cat, pig, dog and rabbit is undoubtedly due to the fact that he used intact erythrocytes. Rapid reduction in haemolysed solutions of erythrocytes from rabbits and rats was found in the present study. Since the erythrocyte membrane of these animals is impermeable to DHA, reduction does not occur when it is added to suspensions of intact cells. Of the species examined only the erythrocytes of man, monkey and young guinea pigs are readily permeable to DHA. This observation is of interest since it is only these species which are unable to synthesise adequate supplies of vitamin C for normal body requirements. The significance of the permeability of the erythrocyte to DHA in the general metabolism of vitamin C and

in the production of scurvy is quite unknown.

The evidence collected in this report on the metabolism of vitamin C in normal human blood confirms the reports in the literature. Dehydroascorbic acid when added to blood is rapidly destroyed by reduction to AA in the erythrocytes and by conversion to DKG in the plasma or the leucocytes. It is therefore not surprising that the presence of DHA could not be demonstrated in the large series of plasmas which were investigated and reported in Section I.

In addition to the direct action of -SH groups in bringing about the reduction of DHA a supplementary mechanism must be postulated for the regeneration of these groups. Further information on the reactions involved in this mechanism will be more readily obtained when isolation of the system from the many other metabolic processes in erythrocytes has been achieved.

The change in the reducing capacity of erythrocytes during some diseases.

The mean percentage reduction of DHA in intact erythrocytes from patients suffering from rheumatoid arthritis is significantly higher than that in erythrocytes from individuals in normal good health. That this difference is a function of the erythrocytes and not of the supporting plasma is established by the fact that the difference in reducing capacity is still maintained when the erythrocytes are haemolysed with water. It is also clear from this experiment that the high reducing power of intact rheumatoid cells cannot be due to an increased permeability of the erythrocyte membrane. The most character-

istic difference between normal erythrocytes and those of rheumatoid patients is the low haemoglobin content of the latter. No correlation between the MCHC and the percentage reduction could be found within the rheumatoid group. To test the hypothesis that there may be an inverse relation between normal and hypochromic cells the percentage reduction was examined in patients with iron deficiency anaemia. In this group of patients the percentage reduction was also high. The possibility that the difference in reducing capacity might be due to a chronic illness or infection was examined by measuring the reducing capacity of erythrocytes from patients with untreated tuberculosis. The mean percentage reduction in this group was a little higher than normal but in only one case was the percentage reduction clearly beyond the normal range. The mean value for the MCHC was also normal. From these results it appears that erythrocytes with a low haemoglobin content reduce DHA more rapidly than do normal cells. However, in a small group of patients with pernicious anaemia Thomson (1955) found a high erythrocyte reducing capacity.

No explanation can be offered for the finding that erythrocytes with a low haemoglobin content reduce DHA more rapidly than normal erythrocytes.

<u>The effect of the administration of drugs on the erythrocyte reducing capacity</u>.

It has been claimed from studies based on the examination of the concentration of AA in the plasma and on the response to saturation doses of AA that the metabolism of vitamin C in

rheumatoid arthritis is not normal. However most of these studies have been carried out in North America where the normal plasma levels are much higher than in Great Britain. In the present study it was found that there was no difference in the plasma levels of total ascorbic acid in a group of normal individuals and in groups of patients suffering from rheumatoid arthritis, tuberculosis and iron deficiency anaemia.

The administration of AA has been reported to have beneficial effects on patients with rheumatoid arthritis (Massell et al., 1950). In the present study daily administration of 500 mg. of AA did not alter the capacity of erythrocytes to reduce DHA. This abnormality in vitamin C metabolism in rheumatoid arthritis is not corrected by AA therapy and may therefore simply reflect a generalised increased catabolism of the vitamin.

The effect of large doses of acetyl salicylate on the erythrocyte reduction of DHA was also examined, since salicylate administration is the standard therapy for rheumatoid arthritis. Salicylate administration did not increase the percentage reduction in normal erythrocytes nor did it alter the rate of reduction in rheumatoid cells. Cortisone on the other hand produced a temporary increase in the rate of reduction in normal erythrocytes and it increased the already high reduction in rheumatoid cells. The suggestion that cortisone causes tissues to become "more reducing" has already been made by Loxton & Le Vay (1953). This has been confirmed for erythrocytes in the present investigation. The mechanism by which the effect is brought about is unknown but from the results of a short experiment it is thought

that the wide fluctuations in the percentage reduction may reflect changes in the activity in the adrenal cortex in response to cortisone. These fluctuations could be repeated in the same individual. It is possible that following cortisone administration cyclic changes occur in the adrenal gland in which periods of great activity alternate with ones of low activity until a stable level is reached. Certainly in the long term experiments the rate of DHA reduction in the erythrocytes gradually reached a high stable level after 6 days. Similar fluctuations in the DHA reducing activity of erythrocytes were found after the administration of ACTH, which would support the hypothesis that the effect is an indirect one related to the adrenal gland. A single dose of meticorten, which is reported to have little effect on electrolyte metabolism but a very powerful effect on steroid metabolism, also produced a sharp rise in the erythrocyte reducing capacity.

The only drug which was found to have any effect on lowering thereducing capacity of erythrocytes was nicotinic acid. Following an intravenous injection of nicotinic acid a temporary lowering of the reduction rate was observed in erythrocytes from 4 patients with rheumatoid arthritis and one patient with acute scurvy. No explanation can be offered for this effect.

Only tentative conclusions can be drawn from the results on the effects of certain drugs on the reducing mechanism. It is suggested that in some way the adrenal cortex can control the oxidation reduction potential of cells and that drugs which may modify the activity of the gland have an indirect effect on

the reducing capacity of erythrocytes. Not only the rate of the reduction of DHA is affected but also probably the rates of many other reducing systems in the erythrocytes. Loxton & Le Vay (1953) interpret their results on the effects of cortisone on tissue potentials as a general increase in the reducing capacity of cells. It is difficult to imagine how such an effect could be due to the direct action of cortisone on cells and not be an effect mediated through some hormonal mechanism.

SUMMARY

1. A critical examination of the chemical methods for the estimation of vitamin C in biological materials has been carried out. The limitations of the methods are discussed.

2. The reaction of various sulphur compounds with indophenol and the interference which their presence would cause in the estimation of ascorbic acid by indophenol, has been examined.

3. A method has been developed for increasing the specificity of the indophenol method for the determination of ascorbic acid in mammalian extracts by the use of CMB.

4. It is concluded from the comparison of results obtained by different methods that the DHA content of human plasma is less than is generally believed and it constitutes an insignificant part of the total AA.

5. DHA added to human blood undergoes two metabolic processes. It is reduced to ascorbic acid by the erythrocytes and it is converted to DKG and to substances which do not form ozazones with dinitrophenylhydrazine, probably by the leucocytes. The mechanism for the reduction of DHA has been investigated. Although thiol groups are involved directly or indirectly in the reduction mechanism, they are rapidly regenerated, probably by an enzyme system. The enzyme system may involve phosphopyridine nucleotides but the ultimate hydrogen donor is unknown.

6. A significant difference from the normal capacity of erythrocytes to reduce added DHA was found in certain diseases. The mean percentage reduction of DHA by normal erythrocytes was 48 (n = 41 S.D. = 5.9, range = 32 to 59). In rheumatoid arthritis the mean percentage reduction was 62 (n = 31 S.D. = 9, range = 44 to 89). The mean percentage reduction of erythrocytes from patients with tuberculosis and iron-deficiency anaemia was 54 (n = 8) and 62 (n = 10) respectively. Three patients with acute scurvy had a high erythrocyte reducing rate. There was a tendency for the rate of reduction of DHA to be inversely related to the haemoglobin content of the erythrocyte.

7. Salicylate administration had no effect on the reducing mechanism. Adrenocortical drugs such as cortisone, meticorten and ACTH increased the rate of reduction of DHA by rheumatoid erythrocytes. Injection of nicotinic acid caused a temporary fall in the reduction rate of erythrocytes from patients with rheumatoid arthritis and scurvy.

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REFERENCES

Abbasy,M.A.,Gray Hill,N. & Harris,L.J. (1936). Lancet, ii, 1413.

Abbasy,M.A.,Harris,L.J. & Ellman,P. (1937). Lancet, ii, 181.

Andreae,W.A. & Browne,J.S.L. (1946). Canad.med.Ass.J. 55, 423.

Bacchus,H. (1951). Endocrinology, 49, 789.

Banerjee,S. & Belavady,B. (1953). Lancet, ii, 912.

Barron,E.S.G.,Barron,A.G. & Klemperer,F. (1936). J.biol.Chem. 116, 563.

Barron,E.S.G.,De Meio,R.H. & Klemperer,F. (1936). J.biol.Chem. 112, 625.

Barron,E.S.G. & Singer,T.P. (1945). J.biol.Chem. 157, 221.

Bessey,O.A. (1938a). J.biol.Chem. 126, 771.

Bessey,O.A. (1938b). J.Amer.med.Ass. 111, 1290.

Bessey,O.A.,Lowry,O.H. & Brock,M.J. (1947). J.biol.Chem. 168, 197.

Bezssonoff,N. & Woloszyn,M. (1936). C.R.Acad.Sci.,Paris, 203, 275.

Bhattacharya,S.K.,Robson,J.S. & Stewart,C.P. (1955). in the Press.

Bolin,D.W. & Book,L. (1947). Science, 106, 451.

Bolomey,R.A. & Kemmerer,A.R. (1946). J.biol.Chem. 165, 377.

Bolomey,R.A. & Kemmerer,A.R. (1947). J.biol.Chem. 167, 781.

Booker,W.M.,Dent,F.M.,Hayes,R.L,Harris,W. & Green,S. (1951). Proc. Soc.exp.Biol.,N.Y., 78, 170.

Borsook,H.,Davenport,H.W.,Jeffreys,C.E.P. & Warren,R.C. (1937). J. biol.Chem. 117, 237.

Bülow,M. (1936). Hoppe-Seyl.Z. 242, 40.

Butler,A.M. & Cushman,M. (1940). J.clin.Invest. 19, 459.

Bywaters,E.G.L.,Dixon,A.St-J. & Wild,J.B. (1950). Lancet, i, 158, 951.

Chen,S.D. & Schuck,C. (1950). Fed.Proc. 9, 354.

Clayton,B.E.,McSwiney,R.R. & Prunty,F.T.G. (1954). Biochem.J. 58, 542.

Crook,E.M. (1941). Biochem.J. 35, 226.

Damron,C.M.,Monier,M.M. & Roe,J.H. (1952). J.biol.Chem. 195, 599.

Daubenmerkl,W. (1949). Acta pharm.tox.,Kbh. 5, 270.

Davey,B.L.,Wu,M-L.,& Storvick,C.A. (1952). J.Nutr. 47, 341.

De Caro,L. & Giani,M. (1934). Hoppe-Seyl.Z. 228, 13.

De Ritter,E.,Cohen,N. & Rubin,S.H. (1951). Science, 113, 628.

Discombe,G. (1954). Brit.med.J. i, 326.

Eddy,B.P. (1952). Biochem.J. 50, 601.

Emmerie,A. & Van Eekelen,M. (1934). Biochem.J. 28, 1153.

Evelyn,K.A.,Malloy,H.T. & Rosen,C. (1938). J.biol.Chem. 126, 645.

Farmer,C.J. & Abt,A.F. (1936). Proc.Soc.exp.Biol.,N.Y., 34, 146.

Fox,F.W. & Levy,L.F. (1936). Biochem.J. 30, 211.

Fujita,A. & Ebihara,T. (1937). Biochem.Z. 290, 201.

Geschwind,I.I.,Williams,B.S. & Li,C.H. (1951). Acta endocr., Copenhagen, 8, 247.

Giri,K.V. & Krishnamurthy,P.V. (1941). Nature,Lond., 147, 59.

Glick,D.,Alpert,M. & Stecklein,H.R. (1953). J.Histochem.Cytochem. 1,326.

Golden,R. & Sargent,F. (1952). Arch.Biochem.Biophys. 39, 138.

Gould,B.S. & Shwachman,H. (1943). J.biol.Chem. 151, 439.

Green,D.E. (1933). Biochem.J. 27, 1044.

Hall,M.G.,Darling,R.C. & Taylor,F.H.L. (1939). Ann.intern.Med. 13, 415.

Harkness,J. & Donovan,C. (1953). J.clin.Path. 6, 330.

Harris,L.J. & Mapsom,L.W. (1947). Brit.J.Nutr. 1, 7.

Heinemann,M. (1938). J.clin.Invest. 17, 751.

Heinemann,M. (1941). J.clin.Invest. 20, 467.

Hensch,P.S.,Kendall,E.C.,Slocumb,C.H. & Polley,H.F. (1950). Arch. intern.Med. 85, 545.

Hirst,E.L. & Zilva,S.S. (1933). Biochem.J. 27, 1271.

Hochberg,H.M.,Melnick,D. & Oser,B.L. (1943). Industr.Engng Chem. (Anal.Ed.), 15, 182.

Hopkins,F.G. & Morgan,E.J. (1936). Biochem.J. 30, 1446.

Hopkins,F.G. & Slater,B.R. (1935). Biochem.J. 29, 2803.

James,W.O. & Cragg,J.M. (1943). New Phytol. 42, 28.

James,W.O. & Cragg,J.M. (1944). New Phytol. 43, 62.

Johnson,S.W. (1933). Biochem.J. 27, 1287.

Johnson,S.W. & Zilva,S.S. (1934). Biochem.J. 28, 1393.

Kellie,A.E. & Zilva,S.S. (1935). Biochem.J. 29, 1028.

Kellie,A.E. & Zilva,S.S. (1936). Biochem.J. 30, 361.

King,C.G. (1936). Physiol.Rev. 16, 238.

King,C.G. (1941). Industr.Engng Chem. (Anal.Ed.), 13, 225.

Kinkawa,T. (1944). J.Jap.biochem.Soc. 18, 167. in Chem.Abstr. (1950). 44, 10763.

Knox,W.E. & le May-Knox,M. (1951). Biochem.J. 49, 686.

Kolthoff,I.M. & Harris,W.E. (1946). Industr.Engng Chem. (Anal.Ed.), 18, 161.

Lemberg,R. (1942). Austr.J.exp.Biol.med.Sci. 20, 111.

Lemberg,R.,Legge,J.W. & Lockwood,W.H. (1939). Biochem.J. 33, 754.

Lemberg,R.,Legge,J.W. & Lockwood,W.H. (1941). Biochem.J. 35, 339.

Levenson,S.M.,Rosen,H. & Hitchings,G.H. (1951). Arch.Biochem.Biophys. 33, 50.

Levitas,N.,Robinson,J.,Rosen,F.,Huff,J.W. & Perlsweig,W.A. (1947). J.biol.Chem. 167, 169.

Lewin,E. & Wassén,E. (1949). Lancet, ii, 993.

Linkswiler,H. (1954). Fed.Proc. 13, 464.

Lloyd,B.B. (1951). J.Physiol. 112, 49P.

Lloyd,B.B. & Parry,H.V. (1954). J.Physiol. 126, 50P.

Lloyd,B.B. & Sinclair,H.M. (1953). Biochemistry and Physiology of Nutrition, Vol.1., edited by G.H.Bourne & C.W.Kidder. New York: Academic Press Inc.

Lloyd,B.B.,Sinclair,H.M. & Webster,G.R. (1945). Biochem.J. 39, xvii.

Loeffler,H.J. & Ponting,J.D. (1942). Industr.Engng Chem. (Anal.Ed.), 14, 864.

Lowry,O.H.,Lopez,J.A. & Bessey,O.A. (1945). J.biol.Chem. 160, 609.

Loxton,G.E. & Le Vay,D. (1953). Nature,Lond., 171, 524.

Lubschez,R. (1945). J.clin.Invest. 24, 573.

Lugg,J.W.H. (1942). Austr.J.exp.Biol.med.Sci. 20, 273.

Mack,G.L. & Tressler,D.K. (1937). J.biol.Chem. 118, 735.

MacLean,K.S. (1951). Lancet, i, 444.

McSwiney,R.R.,Clayton,B.E. & Prunty,F.T.G. (1954). Lancet, i, 178.

Mann,T. & Leone,E. (1953). Biochem.J. 53, 140.

Mapson,L.W. (1943). J.chem.Soc. 62, 223.

Mapson,L.W. (1953). In Vitamin C Requirement of Human Adults. Med.Res.Coun.Spec.Rep.Series, No.280.

Mapson,L.W. & Goddard,D.R. (1951). Nature,Lond., 167, 975.

Mapson,L.W. & Ingram,M. (1951). Biochem.J. 48, 551.

Margolis,H.M. & Caplan,P.S. (1951). Ann.intern.Med. 34, 61.

Massell,B.F.,Warren,J.E.,Patterson,P.R. & Lehmus,H.J. (1950). New Engl.J.Med. 242, 614.

Matthews,M.B. (1951). J.biol.Chem. 189, 695.

Matusis,I.I. (1951). Biokhimya, Leningr. 16, 420. in Chem.Abstr. (1952). 46, 2156.

Mawson,C.A. (1935). Biochem.J. 29, 569.

Meyer,K.H.,Haselbach,C.H. & Boissonnas,R.A. (1952). Helv.chim.Acta. 35, 1781.

Mills,M.B. & Roe,J.H. (1947). J.biol.Chem. 170, 159.

Mindlin,R.L. & Butler,A.M. (1937-38). J.biol.Chem. 122, 673.

Nichol,C.A. & Welch,A.D. (1950). Proc.Soc.exp.Biol.,N.Y., 74, 52.

Owen,J.A. & Iggo,B.J. (1955). Biochem.J. in the Press.

Owen,J.A.,Iggo,B.J. & Horn,D.B. (1954). Nature,Lond., 174, 701.

Panteleva,N.S. (1950). Trudy Leningr. Obshschestva Estestvoipytatelei, Otdel,Fiziol.i.Biokhim., 69, 143. in Chem.Abstr. (1952). 46, 4093.

Parrot,J.L. & Gazave,F.M. (1951). C.R.Soc.Biol.,Paris. 145, 823.

Patterson,J.W. (1949). Endocrinology, 45, 344.

Patterson,J.W. (1950). J.biol.Chem. 183, 81.

Penney,J.R. & Zilva,S.S. (1943a). Biochem.J. 37, 39.

Penney,J.R. & Zilva,S.S. (1943b). Biochem.J. 37, 403.

Penney,J.R. & Zilva,S.S. (1945). Biochem.J. 39, 392.

Pirani,C.L. (1952). Metabolism, 1, 197.

Plaut,F. & Bülow,M. (1935). Hoppe-Seyl.Z. 236, 241.

Quastel,J.H. & Wheatley,A.H.M. (1934). Biochem.J. 28, 1014.

Rall,T.W. & Lehninger,A.L. (1952). J.biol.Chem. 194, 119.

Rheichstein,T. & Gussner,A. (1934). Helv.chim.Acta, 17, 311.

Rhinehart,J.F.,Connor,C.L. & Mettier,S.R. (1934). J.exp.Med. 59, 97.

Rhinehart,J.F.,Greenberg,L.D. & Baker,F. (1936). Proc.Soc.exp.Biol., N.Y., 35, 347.

Rhinehart,J.F.,Greenberg,L.D. & Christie,A.U. (1936). Proc.Soc. exp.Biol.,N.Y., 35, 350.

Roe,J.H. & Barnum,G.L. (1936). J.Nutr. 11, 359.

Roe,J.H. & Kuether,C.A. (1943). J.biol.Chem. 147, 39.

Roe,J.H.,Mills,M.B.,Oesterling,M.J. & Damron,C.M. (1948). J.biol.Chem. 174, 201.

Scarborough,H. & Stewart,C.P. (1937). Biochem.J. 31, 2232.

Schaffert,R.R. & Kingsley,G.R. (1955). J.biol.Chem. 212, 59.

Schroll,W. (1938). Pflug.Arch.ges.Physiol. 240, 642.

Schultze,M.O.,Stotz,E. & King,C.G. (1937-38). J.biol.Chem. 122, 395.

Shive,W.,Bardos,T.J.,Bond,T.J. & Rogers,L.L. (1950). J.Amer.chem. Soc. 72, 2817.

Smythe,C.V. & King,C.G. (1942). J.biol.Chem. 142, 529.

Snow,G.A. & Zilva,S.S. (1943). Biochem.J. 37, 630.

Snow,G.A. & Zilva,S.S. (1944). Biochem.J. 38, 458.

Spies,T.D.,Stone,R.E.,de Maeyer,E. & Niedermeier,W. (1949). Lancet, ii, 1219.

Stewart,C.P.,Horn,D.B. & Robson,J.S. (1953). Biochem.J. 53, 254.

Stotz,E.,Harrer,C.J.,Schultze,M.O. & King,C.G. (1937-38). J.biol. Chem. 122, 407.

Tillmans,J. (1927). Z.Untersuch.Lebensmitt. 54, 33. In The Vitamins, Vol.1 . (1954), edited by W.H.Sebrell & R.S.Harris. New York: Academic Press Inc.

Tillmans,J.,Hirsch,P. & Siebert,F. (1932). Z.Untersuch.Lebensmitt. 63, 21. In Brit.Chem.Abstr. (1932). A. 310.

Thomson,L.C.G. (1955). Ph.D. Thesis, University of Edinburgh.

Todhunter,E.N.,McMillan,T. & Ehmke,D.A. (1950). J.Nutr. 42, 297.

Van Eekelen,M. & Emmerie,A. (1936). Biochem.J. 30, 25.

Van Eekelen,M. ,Emmerie,A.,Josephy,B. & Wolff,L.K. (1933). Nature, Lond., 132, 315.

Vinokurov,S.I. & Silakova,A.I. (1944). Byull.eksp.biol.Med. 18, 65. In Chem.Abstr. (1946). 40, 1212.

Vogt,M. (1948). J.Physiol. 107, 239.

Waksman,B.H. (1949). Medicine,Baltimore, 28, 143.

Waygood,E.R. (1950). Canad.J.Res. 28, 7.

Whitmore,F.L. & Woodward,G.E. (1946). Org.Synth.Coll. Vol.1, 159.
edited by M.Gilman & A.H.Blatt.

Young,F.G. & Mitolo,M. (1934). Nature,Lond., 133, 572.

Zilva,S.S. (1928). Biochem.J. 22, 779.