XXIII. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOCHROMOGENS

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BY combined action of atmospheric oxygen and various reducing substances on haemochromogens green compounds are obtained which still contain iron, but less firmly bound than in haematin. Lemberg [1935] showed that these "green haemins" are bile pigment haemochromogens, closely related to biliverdins; they have been termed verdohaemochromogens.[1]

The process consists in oxidative cleavage of the porphyrin nucleus with removal of one of the α-methene groups, i.e. the same methene group as is removed in the biological process. When protoverdohaemochromogen is treated with acids biliverdin (dehydrobilirubin) results, and this substance is readily reduced to bilirubin by various tissue dehydrogenase systems [Lemberg & Wyndham, 1936]. A protoverdohaematin compound is therefore a possible intermediate substance between blood pigment and bile pigment.

A great variety of reducing substances appear to be able to induce verdohaemochromogen formation when pyridine-haemochromogen is shaken with air in their presence at about 50°, e.g. hydrazine, polyhydric phenols and, under certain conditions, SH compounds. Fischer & Lindner [1926] found extracts of yeast, minced plants or animal tissue (particularly liver) to be equally active; the reducing principle in these extracts was thermostable. That ascorbic acid acts rapidly in the same way even at room temperature was first observed by Karrer *et al.* [1933].

Barron, de Meio & Klemperer [1936] showed quite recently that ascorbic acid, when free from Cu and Fe, is not autoxidizable at $p\mathrm{H} < 8$, and that haemochromogens strongly catalyse the oxidation of ascorbic acid in neutral or slightly acid solutions. Simultaneous oxidative decomposition of the haemochromogens during this process was not noticed by these authors. If the results of Barron *et al.* and those of Karrer *et al.* are both correct, the oxidation of ascorbic acid and of haematin in pyridine is a coupled oxidation; neither ascorbic acid nor haematin alone undergoes oxidation under the conditions of the experiment.

It is important to investigate whether or not the coupled oxidation of haematin compounds and ascorbic acid is likely to occur under physiological conditions; and if so, how far it can account for haemoglobin breakdown in the animal body, and how far it can contribute to the destruction of vitamin C in the body. Secondly, it is desirable to study the mechanism of the coupled oxidation. In the present paper are described the technique with which we have studied the coupled oxidation of pyridine-haemochromogen and ascorbic acid quantitatively, and the results which we have obtained. Further evidence with regard to the mechanism of the reaction will be adduced in the second paper.

[1] Roche & Bénévent [1936] have recently suggested restriction of the use of the terms "haemin", "haematin" and "haemochromogen" to the iron complex salts of porphyrins. We do not find this suggestion practical and propose to use these terms in a wider sense, including the iron complex salts of all tetrapyrrolic compounds, of those with closed rings (porphyrins, chlorins, rhodins etc.) as well as of those with open chain (*iso*biliverdins, biliviolins).

Spectroscopic study of the reaction of pyridine-haematin with ascorbic acid

A dilute solution of haematin in 20% aqueous pyridine displays absorption bands at 565·2 and 530·7 mμ., both rather diffuse, the second band being the stronger. This spectrum is characteristic of ferric haemochromogen (pyridine-parahaematin). When 1 ml. of 1% ascorbic acid (B.D.H.) in water is added to a solution of 0·5 mg. haematin in 3 ml. 20% pyridine (prepared from crystalline haemin), the colour turns rapidly yellowish red and the typical absorption spectrum of ferrous pyridine-haemochromogen is seen. (Two absorption bands: I, 557·6; II, 525·2 mμ.) The first of the two bands is by far the stronger and both bands are stronger than those of the ferric haemochromogen. Therefore, the strength of absorption at about 557 mμ. of the ferric compound is negligible when compared with that of the ferrous compound, a fact which is of importance for some spectrocolorimetric determination methods used in this and the following paper.

If atmospheric O_2 is excluded, ascorbic acid causes no other alteration; when, however, the solution is shaken with air at room temperature (20°), it turns green in 2–3 min. The solution now displays the absorption spectrum of verdohaemochromogen: I, about 655; II, 531; III, 500 mμ. Intensities I (very strong); II, III. $Na_2S_2O_4$ causes the solution to turn yellow; band I persists, but bands II and III disappear and are replaced by general absorption in the blue; cautious shaking with air restores the green colour and the verdohaemochromogen spectrum. When HCl is added to the green solution, the colour is changed to blue-green and the absorption bands disappear and are replaced by absorption in the distant red end of the spectrum (biliverdin). The solution now gives the Gmelin reaction with nitric acid. These observations confirm Karrer *et al.* [1933] and show that ascorbic acid reduces ferric haemochromogen to ferrous haemochromogen, which undergoes oxidation to verdohaemochromogen when shaken with air in the presence of ascorbic acid. We can also confirm the observation of these authors that $Na_2S_2O_4$ protects haematin from oxidation to verdohaemochromogen, a fact of which we make use in our technique.

Non-autoxidizability of ascorbic acid in pyridine

We found that O_2 uptake and ascorbic acid destruction are negligible in a solution prepared by dissolving crystalline ascorbic acid (B.D.H.) in 20% pyridine (redistilled over KOH). The solutions had *p*H 6·9–7·9, varying with the amount of ascorbic acid.

A solution of 20·0 mg. of ascorbic acid in 1 ml. of distilled water was added to 9 ml. 20% pyridine. After 10 min. shaking in a Warburg manometer the solution contained the original amount of ascorbic acid, and after 30 min. only 0·03 mg. had been oxidized.

Technique

Reagents. Crystalline ascorbic acid (B.D.H.) was used throughout. Haemin (α-chlorohaemin) crystals were prepared from horse blood and ox blood in the usual way and recrystallized from pyridine-chloroform-glacial acetic acid. The pyridine was pure pyridine of Schering-Kahlbaum, redistilled over KOH.

Estimation of haematin

Principle of the method. For determination of total protohaematin in presence of verdohaemochromogen we have employed reduction with $Na_2S_2O_4$, followed by spectrocolorimetric comparison with a similarly reduced standard solution of

haematin. The first absorption band of haemochromogen at 557 mμ. is suitable for this purpose, since verdohaemochromogen does not absorb in this region and therefore does not interfere.

Since the absorption of ferric haemochromogen at 557 mμ. is negligible when compared with that of ferrous haemochromogen, the concentration of ferrous haemochromogen can be measured with sufficient accuracy even in presence of ferric haemochromogen. This allows approximate determination of the ratio of ferrous to ferric protohaemochromogens during the experiment.

The apparatus consists of a Leitz Universal Colorimeter (with micro-cups and micro-plungers), on the top of which a Watson Microspectroscope was mounted. One micro-cup contains the solution to be examined, the other the standard solution. The field of vision shows the two spectra side by side. The thickness of the layer of the unknown solution is adjusted until the first absorption band is of the same strength as that given by the haemochromogen standard solution.

As standard a freshly prepared 100-fold dilution of a stock solution of 100 mg. haemin in 100 ml. 20% pyridine is reduced with $Na_2S_2O_4$ immediately before measurement. The stock solution keeps for weeks, but was not used for more than 1 week; the reduced standard remains unaltered for at least 30 min. It is essential to use pure pyridine. After some experience the readings can be taken with an accuracy of $\pm 3\%$.

The reaction under study is rapid and for accurate measurements speed in sampling and particularly in arresting the reaction is absolutely essential. We found that for the latter purpose $Na_2S_2O_4$ served well. A measured volume of the unknown solution was pipetted into a 5 ml. measuring flask containing some pyridine (20%) and a small knife-point of $Na_2S_2O_4$. The volume was then made up to 5 ml. with 20% pyridine, and the solution poured into one micro-cup of the colorimeter. The amount of the solution pipetted off was varied so that the ultimate haematin concentration was close to that of the standard. The spectro-colorimetric determination was done within 3 min. after sampling, but no appreciable change in haematin concentration was observed over much longer periods.

Estimation of ascorbic acid

After removal of haematin with trichloroacetic acid, ascorbic acid was usually titrated with $M/100$ iodine. Titration with 2:6-dichlorophenolindophenol gave quite similar results (see Table I). A small blank (0·06 ml. iodine per 15 ml. titrated solution) was subtracted. In some experiments in which glutathione was present the indicator method was used. A sharp end-point was always obtained unless the final ascorbic acid concentration was higher than 10 mg. per 100 ml., in this case the solution had to be diluted with 5% trichloroacetic acid before titration.

The ascorbic acid oxidation was arrested by precipitating the haematin by addition of 9 vol. 7% trichloroacetic acid to the 20% pyridine solution followed by filtration. Even so there was always some loss of ascorbic acid (Table I), which, with more haematin, was still more pronounced. Evidently the precipitated haematin still catalysed ascorbic acid oxidation. Additional oxidation was caused by ionized iron set free from verdohaemochromogen by the action of trichloroacetic acid. This could be prevented by addition to the trichloroacetic acid of cyanide sufficient to produce an ultimate concentration of $M/1000$. In agreement with other authors we found only 90–95% of the real ascorbic acid concentration in the trichloroacetic acid filtrate, even when haematin was absent. There

was, of course, some oxidation of ascorbic acid due to the almost instantaneous stoichiometric reaction between ferric haemochromogen and ascorbic acid. This could usually be neglected, however, since in most experiments a great molar excess of ascorbic acid is used. In the experiments of Table I the oxidation of ascorbic acid due to the stoichiometric reaction would be 0·02 mg. and thus within the limits of the error of estimation.

Table I

To 2 ml. of a solution containing 2·5 mg. ascorbic acid (and in the haematin experiments 0·31 mg. haematin) in 20% pyridine, 18 ml. of 7% trichloroacetic acid (containing 0·2 ml. *M*/10 NaCN in the cyanide experiments) were added immediately after the addition of the haematin. 10 ml. of the filtrate contained the following amounts of reduced ascorbic acid (in mg.):

	Trichloroacetic acid filtrates			
	Ascorbic acid present	Blank without haematin	With haematin	With haematin and cyanide
Iodine titration	1·24	1·14	0·92	1·15
Indicator titration	1·24	1·14	0·99	1·14

Reaction of ascorbic acid with pyridine-haematin in nitrogen

A solution of haematin in 20% pyridine contains ferric haemochromogen, which is quantitatively reduced to ferrous haemochromogen by excess of ascorbic acid. The first question to be considered is whether the oxidation of ascorbic acid by haematin is reversible or irreversible.

An aqueous solution of 1·27 mg. ascorbic acid was added under nitrogen to a solution of excess of haematin (25 mg.) in 20% pyridine. After 30 sec. 9 vol. of trichloroacetic-HCN mixture were added rapidly, and the haematin removed by filtration. The precipitate was washed several times with dilute trichloroacetic acid and one-half of the filtrate titrated with iodine directly, the other half after reduction with H_2S and removal of the H_2S by N_2. In the first half no ascorbic acid was found, in the second half the original amount of ascorbic acid had been fully restored by the treatment with hydrogen sulphide indicating that dehydroascorbic acid was the probable oxidation product. To investigate whether 1 or 2 mol. of haematin are required for the oxidation of 1 mol. ascorbic

Table II

Haematin γ	Ascorbic acid γ	Ratio ascorbic acid /haematin	Colorimeter reading	Haematin reduced γ	γ haematin which should be reduced provided the ratio be 1 : 2	γ haematin which should be reduced provided the ratio be 1 : 1	% of theoretical (1 : 2) reduction
			A. *Method* 1				
448	$Na_2S_2O_4$	—	43·0	448	—	—	100
224	120	2 : 1	18·9	197	224	224	88
448	120	2 : 2	33·1	345	445	448	77
224	60	1 : 1	16·3	170	222	224	76
224	60	1 : 1	15·6	162	222	224	72
448	60	1 : 2	30·4	316	222	445	70
224	30	0·5 : 1	16·6	173	111	222	77
448	30	0·5 : 2	14·0	146	111	222	65
			B. *Method* 2				
1·00	7·41	1 : 2	0·58	1·00	2·00	58	
2·00	7·41	1 : 1	0·80	1·00	2·00	80	
2·00	3·70	2 : 1	0·55	0·50	1·00	110	
2·00	1·76	2 : 0·5	0·29	0·24	0·48	120	

acid two methods were used. The excess of ascorbic acid remaining after reaction with a known amount of ferric haemochromogen was determined; secondly the concentrations of ferrous haemochromogen produced by insufficient amounts of ascorbic acid were measured.

Tables II A & B give the results, which show that 1 mol. of ascorbic acid reduces 2 mol. of haematin.

THE COUPLED OXIDATION OF ASCORBIC ACID AND PYRIDINE-HAEMOCHROMOGEN

Technique

The concentrations of ascorbic acid and haematin decrease during the coupled oxidation and, since the velocities of oxidation of both substances depend, as will be seen, on both concentrations, it is essential to measure the initial velocities in order to have clearly defined conditions.

The speed of the reaction is considerable, and cannot, as in other cases of haematin catalysis, be decreased by lowering the concentration of the catalyst, because it would then become impossible to make accurate haematin estimations. Particular care must be taken to ensure that the O_2 content of the solution is replaced with sufficient rapidity, so that the diffusion velocity of O_2 into the solution may not become a factor determining the speed of the reaction. Actually with low shaking velocities we found the speed of the oxidation of both haematin and ascorbic acid to depend on the velocity, particularly in the Warburg apparatus in which the horizontal shaking does not cause a sufficient contact of the liquid and gaseous phases. Rapid perpendicular manual shaking of about 3 ml. liquid phase in Thunberg tubes, however, gave satisfactory results. The rates of haematin and ascorbic acid oxidation under these conditions were considerably greater than in the experiments in Warburg flasks, particularly with high haematin and ascorbic acid concentrations (see Figs. 1–4) and were not increased by further acceleration of the shaking.

Thunberg tubes with bent, hollow stoppers were used throughout. They allow the reaction to be started suddenly by mixing the ascorbic acid solution in the stopper with the solution of haematin in dilute pyridine in the bottom part of the tube, and they also make it possible to work in various gas mixtures.

The tube was immersed in a thermostat to acquire the required temperature, the contents were mixed, the shaking begun at once and the stop-watch set going. The tubes were held by the side arms (so that the temperature equilibrium was not upset during the shaking) and they were shaken in such a way that the liquid remained in constant vigorous movement up and down the walls of the tube without splashing over into the hollow stopper. At the required time the shaking was stopped, the tube quickly opened and the samples of ascorbic acid and haematin measured out with pipettes. For each experiment a separate Thunberg tube was used. Usually ascorbic acid and haematin samples were taken from the same tube, one of us doing the experiment, the sampling and the ascorbic acid estimations, and the other the haematin estimations. Stopping the shaking retards the reaction velocity so much that no error is introduced in the few seconds which elapse between the taking of the two samples. No difference was found when the order of taking the samples was reversed. In experiments of short duration (up to 30 sec.) and with very small amounts separate operations for ascorbic acid and haematin were carried out; the total content of the tube was then available for one estimation. The curves showing the decrease of ascorbic acid and haematin concentrations with time are thus constituted of a

number of such separate experiments. Altogether more than 700 single experiments have been carried out, the reproducibility of the results being very satisfactory. Occasional stray values occurred, but in general, differences of the

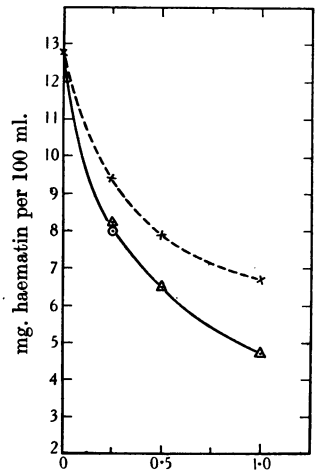

Fig. 1.

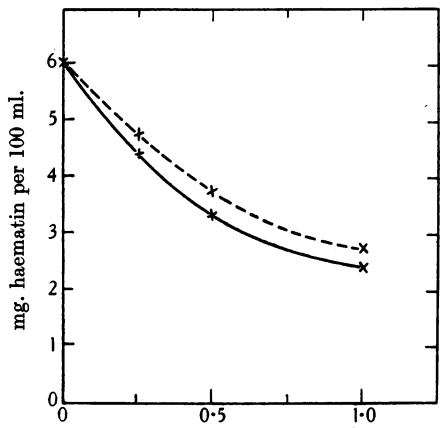

Fig. 2.

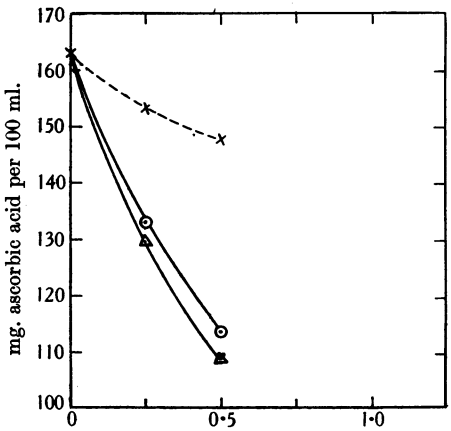

Fig. 3.

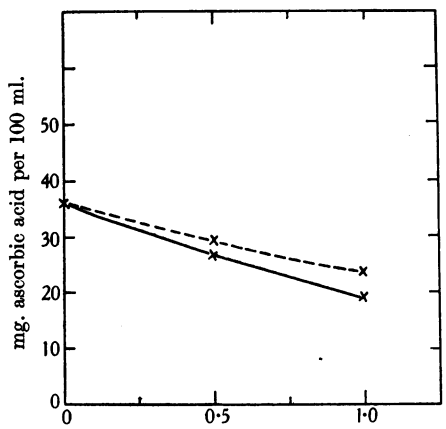

Fig. 4.

Figs. 1–4. Effect of shaking velocities on the rates of oxidation of haematin (Figs. 1 and 2) and of ascorbic acid (Figs. 3 and 4).

Figs. 1 and 3. 10 *mM*. ascorbic acid, 0·2 *mM*. haematin; Figs. 2 and 4, 2 *mM*. ascorbic acid, 0·1 *mM*. haematin.

—— Exps. in Thunberg tubes (shaking velocity 240 per min., ⊙; 350 per min., ◬).

– – – Exps. in Warburg flasks (shaking velocity 150 per min.; stroke, 2·5 cm.).

values for haematin and ascorbic acid concentrations between two separate experiments of the same kind did not exceed 10%, and the majority agreed within much closer limits. Each haematin or ascorbic acid value embodied in the curves is generally the average of two or three single values.

The products of the coupled oxidation

A solution containing 18·2 mg. ascorbic acid and 1·94 mg. haematin in 10 ml. 20% pyridine was shaken at 37° for 3 min.; 48·3% of the ascorbic acid was present in the reduced form and after reduction with H_2S 99·5% of the ascorbic acid was recovered. The oxidation of ascorbic acid during this time was therefore fully reversible. The product of the reversible oxidation is evidently rather stable under the conditions of our experiments. This is not in agreement with the observations of Borsook *et al.* [1937] that dehydroascorbic acid has only a very short life at neutral reaction; it is possible that pyridine stabilizes the lactone ring, the hydrolysis of which causes irreversible breakdown of dehydroascorbic acid. In the further course of the reaction there is some irreversible oxidation. After 20 min., when only 10% of the ascorbic acid was still in the reduced form and the haematin completely oxidized to verdohaematin, H_2S-reduction of the trichloroacetic acid filtrate restored only 64% of the original ascorbic acid.

It has been shown above that the product of the oxidation of pyridine-haemochromogen is verdohaemochromogen. The following experiment was carried out to see whether this substance is the only product of the reaction. 1·34 mg. haemin in 10 ml. 20% pyridine containing 30 mg. ascorbic acid were kept in the air for 5 hr. The solution was then green and protohaemochromogen had disappeared. A sample was diluted 12·5 times and compared with a similarly diluted standard solution of verdohaemochromogen [Lemberg, 1935] of the same molar concentration as the original protohaemochromogen solution. No difference was found in the strength of the bands in the red. From this it must be concluded that verdohaemochromogen is the only product of the reaction, at least when the reaction is carried out under mild conditions.

Measurements of the oxygen uptake

The oxidation of ascorbic acid to dehydroascorbic acid should require 0·5 mol. O_2, that of haemochromogen to verdohaemochromogen 1–2 mol. O_2 (according to whether the CH-group is removed as formaldehyde, as formic acid or as CO_2). In order to compare the actual O_2 uptake with that calculated from the destruction of ascorbic acid and of haemochromogen we have measured the O_2 uptake in Warburg manometers and determined in parallel experiments the destruction of ascorbic acid and haemochromogen occurring under the same conditions. The possibilities of such experiments are, however, limited. It will be seen below that the ratio of mol. ascorbic acid oxidized to mol. of haematin oxidized is always higher than 10. The O_2 uptake, due to the oxidation of haematin forms, therefore, only a small fraction of the total O_2 uptake and this makes it impossible to obtain conclusive evidence concerning the number of O_2 atoms required for the oxidation of haematin to verdohaematin. The evidence for the O_2 uptake of ascorbic acid, on the other hand, is conclusive, since the difference between O_2 uptake due to ascorbic acid and total uptake is small.

The experiments were carried out in Warburg manometers in flasks with a side bulb from which the ascorbic acid solution was tipped into the pyridine solution of haematin contained in the flask.

Table III shows that with initial concentrations of 0·2 *mM.* haematin and 9 *mM.* ascorbic acid the measured O_2 uptake is close to that calculated. With lower haematin and ascorbic acid concentrations however, particularly in the first minutes, the O_2 uptake is lower than it should be if the oxidation of ascorbic

acid involved one atom of O_2. These observations support the view of Bezssonoff & Woloszyn [1936; 1937] that under certain conditions a reversible oxidation product of ascorbic acid can be obtained which has only one H atom less than ascorbic acid. As yet we do not consider our results as conclusive evidence for the existence of this reversible oxidation stage of ascorbic acid, but they are certainly suggestive. This is further discussed in the following paper.

Table III

Initial haematin mg. in 3 ml.	Initial ascorbic acid mg. in 3 ml.	Time of oxidation min.	$-\Delta$ haematin mg.	$-\Delta$ ascorbic acid mg.	$-\Delta O_2$ haematin calculated ($1O_2$/mol.) μl.	$-\Delta O_2$ ascorbic acid calculated ($\frac{1}{2}O_2$/mol.) μl.	$-\Delta O_2$ sum calculated μl.	$-\Delta O_2$ found μl.
0·38	4·80	0–0·5	0·15	0·36	5·1	22·9	28	32
0·18	1·08	0–3	0·135	0·84	4·6	53·6	58	52
0·10	0·96	2–3	—	0·220	About 1	14·0	15	6·3
0·10	0·96	3–5	—	0·236	About 0·5	15·0	15·5	11·5

The O_2 uptake continues almost undiminished after the complete oxidation of haematin (in Fig. 5 after 10 min.) and also after the total ascorbic acid has been oxidized (after 19 min.). After this time oxidation products of ascorbic acid must be further oxidized.

We have chosen the conditions of the experiment in Fig. 5 exactly as in the experiment given by Barron and coworkers to demonstrate the catalytic effect of pyridine-haemochromogen on the oxidation of ascorbic acid. The curve of

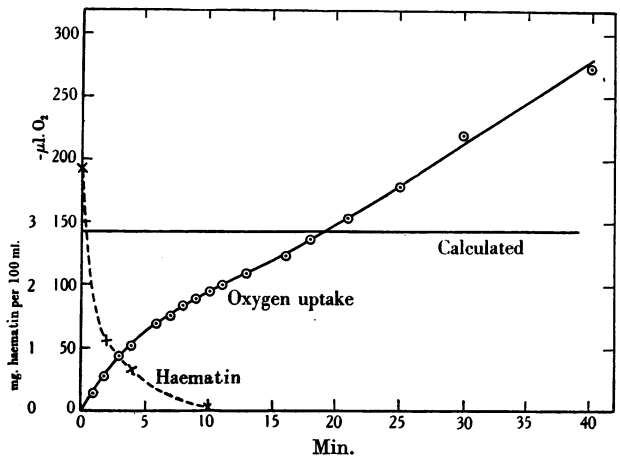

Fig. 5. Oxygen uptake in Warburg manometer. The horizontal line represents oxygen required for complete oxidation of ascorbic acid to dehydro-ascorbic acid and of haematin to verdohaematin, the broken line haematin concentration.

0·096 mg. haemin and 2·4 mg. ascorbic acid in a total volume of 3 ml. containing 1·63 ml. of $M/10$ phosphate buffer pH 6·34 and 500 ml. pyridine. $T = 37°$. Shaking 150 oscillations per min.

these authors (over 240 min.) showed thus largely the effect of verdohaemochromogen on ascorbic acid and its further oxidation products. This also demonstrates how essential it is in our case to study the reaction in the initial stage in which interference by the oxidation products of both substances is still negligible.

Effect of complete oxidation of one component on the oxidation of the other

Determinations of the remaining ascorbic acid and of the remaining haematin also showed that the oxidation of the ascorbic acid proceeds, though with somewhat decreased speed, when all haematin has been oxidized to verdohaematin (Fig. 6).

We must conclude from these facts that verdohaemochromogen, like haemochromogen, has a catalytic effect on the oxidation of ascorbic acid. Verdohaemochromogen, in turn, is also oxidized in the further reaction, but much less rapidly than haemochromogen; in experiments with a great excess of ascorbic acid the solution ultimately turns brown and verdohaemochromogen is no longer present.

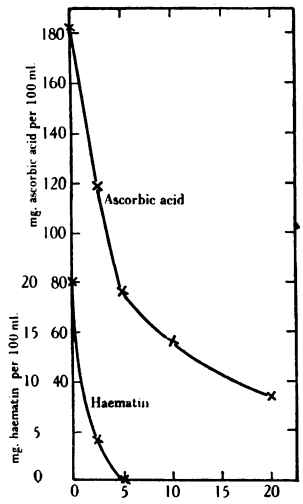

Fig. 6.

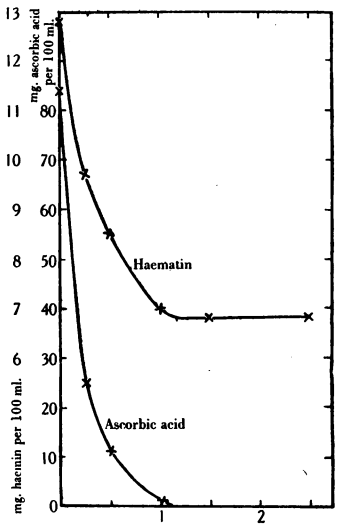

Fig. 7.

Fig. 6. Oxidation of ascorbic acid after complete oxidation of haematin. $T = 37°$.

Fig. 7. No oxidation of haematin after complete oxidation of ascorbic acid.

In experiments in which ascorbic acid became completely oxidized before the total haematin had been transformed into verdohaematin, the further oxidation of haematin ceased completely when ascorbic acid was no longer present, Fig. 7. At this moment the first absorption band of protohaemochromogen disappears and the remaining haematin is now in the ferric form.

Effect of various factors on the coupled oxidation. The oxidation ratio

It is to be expected that some factors might influence the rate of oxidation of one substance more than that of the other, so that the ratio of mol. ascorbic acid oxidized to mol. haematin oxidized will vary. We call this ratio the "oxidation ratio". The knowledge of its order of magnitude is evidently of interest in relation to the importance which a reaction of this type may have in the animal body.

The reaction velocity of the oxidation of either substance must depend on both haematin and ascorbic acid concentrations both of which undergo a constant change. A problem of this kind allows rigorous mathematical treatment only if

the oxidation velocity of one substance depends in a simple and constant manner on the concentration of the other, e.g. the mutual destruction of catalase and hydrogen peroxide. Here, however, no such simple relations exist, as will be seen below; this indicates that the reactions we are studying must be of a rather complicated nature, a supposition which will be supported by the evidence submitted in our second paper.

Effect of temperature. The influence of temperature variation on the rate of oxidation of haematin is shown in Fig. 8, and for ascorbic acid in Fig. 9.

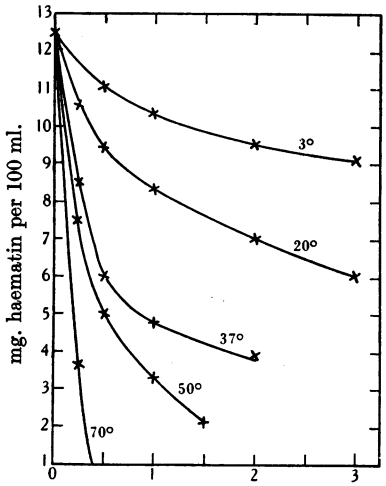

Fig. 8.

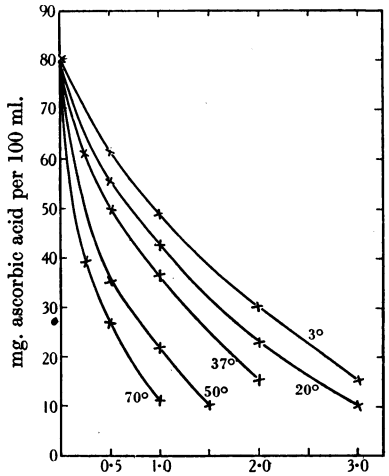

Fig. 9.

Fig. 8. Effect of temperature on oxidation of haematin.
Initial haematin concentration: ·02mM. Initial ascorbic acid concentration: 4·5mM.

Fig. 9. Effect of temperature on oxidation of vitamin.
Initial haematin concentration: 0·2mM. Initial ascorbic acid concentration: 4·5mM.

In Table IV the temperature coefficients of the initial rates of haematin and ascorbic acid oxidation are calculated. The initial velocities of the haematin oxidation have been taken from the curves graphically. For the velocities of the

Table IV

Temperature	Rate of haematin oxidation mg./100 ml. × min.	Q_{10} haematin	Rate of ascorbic acid oxidation k (unimol.) $= 1/t \log C_1/C_2$	Q_{10} ascorbic acid
	3·5		0·22	
3°		1·73		1·24
	8·9		0·32	
20°		1·46		1·20
	15·7		0·44	
37°		1·42		1·49
	24		0·74	
50°		1·23		1·21
70°	36		1·08	

ascorbic acid oxidation unimolecular reaction constants of ascorbic acid destruction have been used. At the first glance the latter procedure must seem wrong, since haematin catalyses the oxidation of ascorbic acid and the considerable alteration of the haematin concentration even in the early stages of the reaction should, therefore, not be neglected. It will be seen later, however, that the oxidation of ascorbic acid proceeds, indeed, rather closely according to the unimolecular law and that this apparently paradoxical fact is due to verdohaemochromogen, the oxidation product of haemochromogen, being also a catalyst of ascorbic acid oxidation, not very inferior to haemochromogen.

The average Q_{10} of ascorbic acid oxidation in all the experiments done at 20 and 37° was 1·30, that of haematin oxidation 1·86, somewhat higher than in the experiment shown in Table IV. From these data the apparent activation energies of haematin and ascorbic acid oxidations are 10,000 cals. for haematin oxidation and 4300 cals. for ascorbic acid oxidation. These values are given with the utmost reserve, particularly the activation energy for the haematin oxidation (cf. also the second paper). At higher temperatures Q_{10} haematin appears to decrease considerably.

From the experiments embodied in Figs. 8 and 9, the size of the oxidation ratio can be deducted. It is seen that the oxidation ratio decreases with increasing temperature; after 1 min. at 3° 59 mol. of ascorbic acid are destroyed per mol. haematin, at 37° only 20; a great molar excess of ascorbic acid is thus oxidized in the reaction.

Effect of oxygen pressure. Of all factors the pO_2 has the most striking effect on the oxidation ratio. When 0·2 *mM.* haematin and 4·5 *mM.* ascorbic acid were oxidized in 20% pyridine at 20° the following oxidation ratios were found after 1 min.: in O_2: 59; in air: 32; in 4% O_2–96% N_2: 12. Fig. 10 shows that this is due to the fact that the haematin oxidation does not depend on the pO_2 in a range of 30–760 mm. mercury.

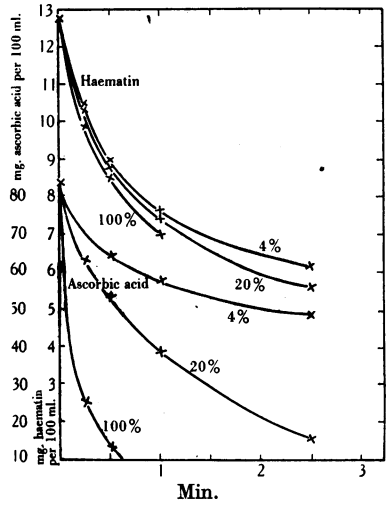

Fig. 10. Effect of oxygen pressure on the rate of oxidation of haematin and ascorbic acid. $T = 20°$

The rate of ascorbic acid oxidation, however, is proportional to the pO_2, as shown by the data of Table V.

Table V

% oxygen of gas mixture	k_{uni} of ascorbic acid oxidation	k_{uni} in % of k_{uni} in oxygen
100	1·71	100
20	0·34	19·9
4	0·087	5·1

Effect of p*H.* The range over which the influence of the *p*H on the reaction can be studied is limited by the insolubility of pyridine-haemochromogen at *p*H < 6·5 and by the autoxidizability of ascorbic acid in solutions having *p*H > 8. A solution of 8 mg. ascorbic acid and 1·3 mg. haematin in 10 ml. 20% pyridine has *p*H 7·61. In the experiments summarized in Fig. 11 the *p*H of the solution was altered by addition of small amounts of *N*/50 NaOH or 1% acetic acid, the concentrations of ascorbic acid, haematin and pyridine remaining the same; the

*p*H was measured with the double hydrogen electrode. In Fig. 11 the rate of oxidations of ascorbic acid and haematin (decrease of concentration of haematin in mg. per 100 ml. and of ascorbic acid in mg. per 10 ml. during 30 sec.) is plotted against the *p*H.

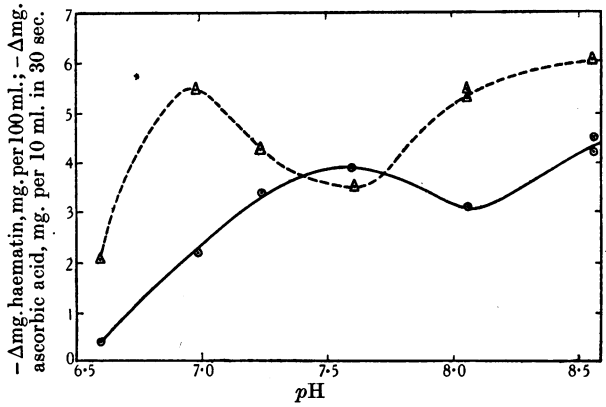

Fig. 11. Effect of *p*H on the rate of oxidation of haematin (solid line, ⊙) and ascorbic acid (broken line, △). Ascorbic acid: 80 mg.; haematin: 13 mg. per 100 ml.

The oxidations of ascorbic acid and of haematin are not affected by the *p*H in the same way. The oxidation of ascorbic acid reaches a maximum near neutrality; it falls off towards a minimum near *p*H 7·6 and then rises with increasing *p*H. The oxidation of ascorbic acid catalysed by haemochromogen thus depends on the *p*H in a way quite different from that of its autoxidation, which rises from about *p*H 8 towards the alkaline side.

The optimum *p*H for haematin oxidation is 7·6, distinctly more towards the alkaline side than that of the ascorbic acid oxidation; the velocity decreases somewhat to *p*H 8, but increases again above *p*H 8.

Effect of various initial concentrations of haematin and ascorbic acid. When the initial concentration of ascorbic acid is kept constant and that of haematin varied, the initial velocities of the oxidation both of haematin and ascorbic acid are roughly proportional to the initial concentration of the former (Figs. 12–14).

When the initial concentration of haematin is kept constant and that of ascorbic acid varied, the initial velocity of haematin oxidation increased with increasing ascorbic acid concentration, but not quite proportionally (Fig. 15). By varying the ascorbic acid concentration the *p*H is slightly altered, but this can be corrected by making use of the data of Fig. 11. After such correction the initial rate of haematin oxidation is roughly proportional to the initial ascorbic acid concentration in the range from 2 to 20 *mM*. ascorbic acid.

With equal initial concentrations of haematin the initial rate of oxidation of ascorbic acid is proportional to the initial concentration of the latter up to a concentration of about 9 *mM*. (Fig. 16); at higher concentrations the velocity of ascorbic acid oxidation decreased. The shift of *p*H caused by the variations in vitamin concentration does not greatly affect this result; in Table VI the decrease of ascorbic acid concentration during 30 sec. oxidation has been corrected for the *p*H shift, using the data of Fig. 11.

The decrease of the rate of oxidation with high ascorbic acid concentrations is thus real, and not caused by a *p*H shift. We have at present no explanation to offer for this phenomenon.

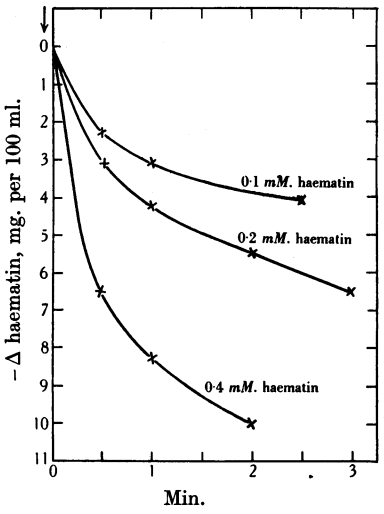

Fig. 12.

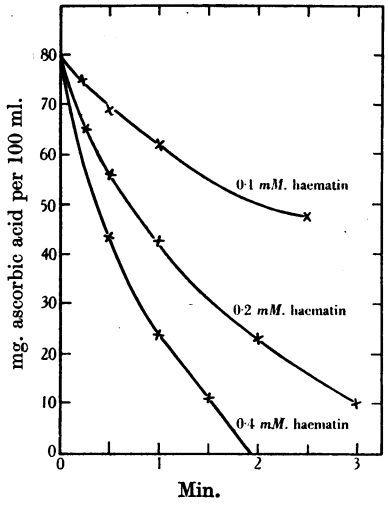

Fig. 13.

Fig. 12. Effect of initial haematin concentration on oxidation of haematin. Ascorbic acid: 4·5 *mM.* $T = 20°$.

Fig. 13. Effect of initial haematin concentration on oxidation of ascorbic acid. Ascorbic acid 4·5 *mM.* $T = 20°$.

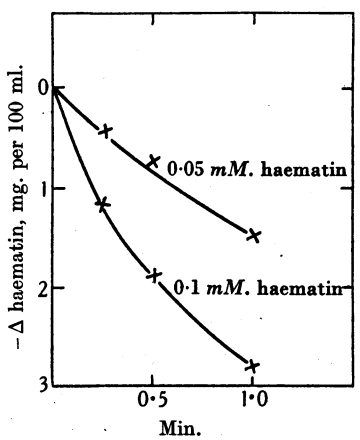

Fig. 14. Effect of initial haematin concentration on oxidation of haematin. Ascorbic acid: 1 *mM.* $T = 37°$.

Table VI

Initial ascorbic acid conc. *mM.*	*p*H	Correction factor from Fig. 11	- Δ mg./100 ml. ascorbic acid per 30 sec. Uncorr.	Corr.
2·2	7·89	0·75	16	12
4·5	7·61	1	27	27
9	7·25	0·82	51	42
18	6·99	0·64	39	25

From these observations it appears to be nearly correct that the velocities of both haematin and ascorbic acid oxidations are proportional to both haematin and ascorbic acid concentrations in the initial stage of the reaction. If they

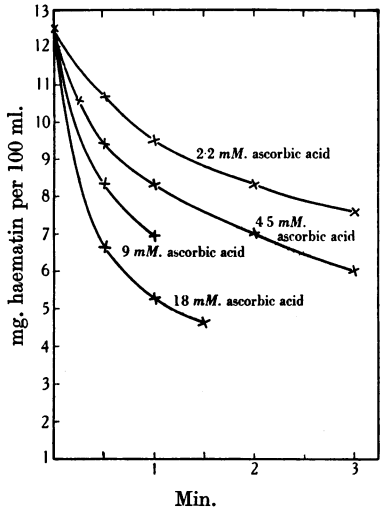

Fig. 15.

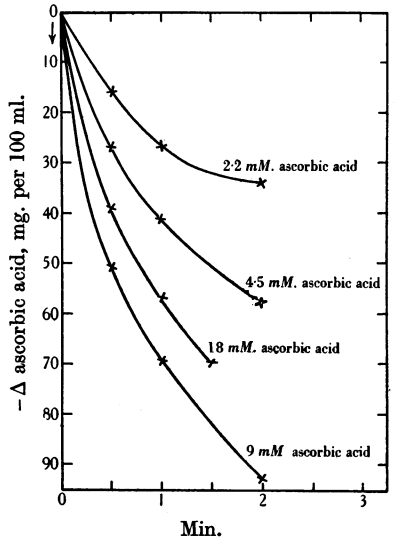

Fig. 16.

Fig. 15. Effect of initial ascorbic acid concentration on rate of haematin oxidation. Haematin: 0·2 mM. $T = 20°$

Fig. 16. Effect of initial ascorbic acid concentration on oxidation of ascorbic acid. Haematin: 0·2 mM. $T = 20°$.

remained so during the coupled oxidation, the mutual effect of pyridine-haemochromogen and ascorbic acid would be kinetically the same as the mutual effect of catalase and hydrogen peroxide, and we should be able to express the course of the concentration-time curves mathematically. The "unimolecular reaction constant" (mean reaction velocity) $k_h = \frac{1}{t_2 - t_1} \log \frac{h_1}{h_2}$, where h_1 and h_2 are the haematin concentrations at the times t_1 and t_2, ought then to be proportional to the mean concentration of ascorbic acid $v' = \frac{v_1 + v_2}{2}$; and correspondingly the unimolecular reaction constant of the ascorbic acid oxidation k_v should be proportional to the mean haematin concentration $\left(h' = \frac{h_1 + h_2}{2}\right)$. These laws do not however hold, as Table VII clearly shows.

Table VII

Time min.	$T = 3°$				$T = 20°$				$T = 37°$			
	k_k	$k_h/v' \times 10^3$	k_v	$k_v/h' \times 10^3$	k_h	$k_h/v' \times 10^3$	k_v	$k_v/h' \times 10^3$	k_h	$k_h/v' \times 10^3$	k_h	$k_v/h' \times 10^3$
0–0·25	0·130	1·73	0·23	19·3	0·286	3·95	0·36	31·2	0·67	9·46	0·46	43·5
0·25–0·5	0·092	1·37	0·225	19·9	0·209	3·60	0·26	25·9	0·60	10·9	0·38	52·5
0·5–1	0·057	1·07	0·20	18·5	0·108	2·19	0·24	27·2	0·21	4·9	0·26	49·1
1–2	0·035	0·89	0·21	21·5	0·074	1·74	0·27	34·8	0·07	2·7	0·39	88·8
2–3	0·019	0·82	0·29	30·8	0·067	4·16	0·36	54·7	—	—	—	—

k_h/v' and k_v/h' are not constant, k_h drops much more rapidly in the initial stages of the reaction than the mean concentration of ascorbic acid. Later, its decrease becomes less rapid, sometimes proportionally to the decrease of ascorbic acid concentration and sometimes even independently of it. That the later stages of the haematin oxidation do not depend on the ascorbic acid concentration becomes particularly clear by comparison of the two curves in Fig. 17, which show the decrease of haematin concentration with two different initial concentrations of ascorbic acid at 3°.

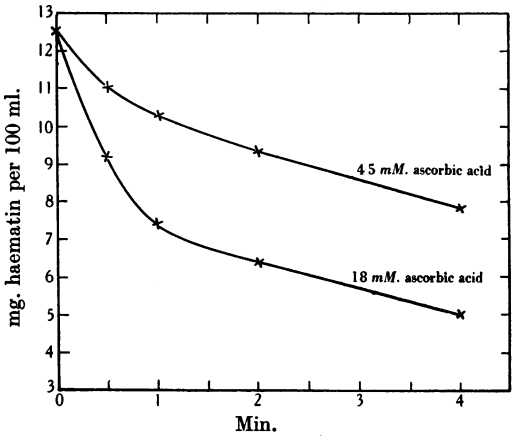

Fig. 17. Effect of initial ascorbic acid concentration on oxidation of haematin. Haematin: 0·2 *mM*. $T = 3°$.

Whereas the initial rates of haematin oxidation are proportional to the initial ascorbic acid concentrations, the later course of haematin oxidation is apparently independent.

These facts can only be explained by assuming that there are at least two reactions involved in the destruction of haematin: a rapid initial reaction, the rate of which depends on ascorbic acid concentration, and a slower reaction following in the later stages and being less dependent on or even independent of ascorbic acid concentration. In our second paper we shall present evidence for the existence of two different reactions which may account for these phenomena.

We have demonstrated above that the initial rate of ascorbic acid oxidation is proportional to the initial haematin concentration up to an ascorbic acid concentration of 9 *mM*. From Table VII, however, we see that k_v seems to remain definitely more constant, though with considerable variations, than k_v/h'; the reaction seems to be unimolecular and not dependent on haematin concentration. Here the correct explanation appears to be that when haematin is oxidized the catalytic action does not disappear but is replaced by that of verdohaematin which is also catalytically active though somewhat less so. A slight initial drop of k_v is indeed noticeable throughout. The tendency of k_v to increase again later is perhaps caused by a partial removal of iron from verdohaematin, producing iron catalysis of the vitamin oxidation.

We can now understand why the oxidation ratio varies somewhat with the time during which it is determined; it is lowest at first and rises later. Variations of the haematin concentration have little effect on the oxidation ratio; increase of the ascorbic acid concentration (up to 9 *mM*.) increases it a little in the initial stages and to a somewhat greater extent later.

Variation of the pyridine concentration between 5 and 20% of pyridine has no effect on the rate of oxidation of either haematin or ascorbic acid.

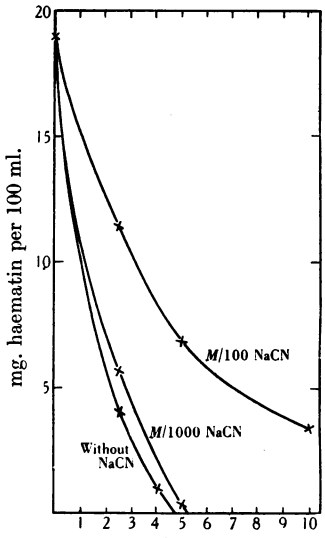

Fig. 18.

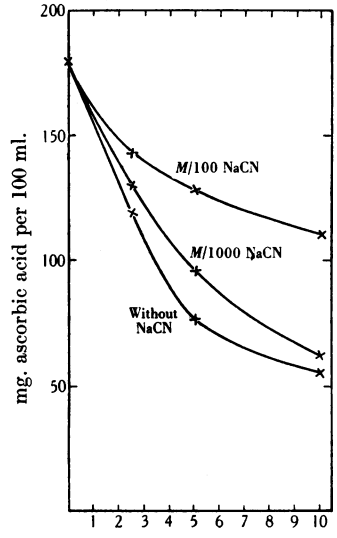

Fig. 19.

Fig. 18. Effect of cyanide on oxidation of haematin. Ascorbic acid: 10$mM.$; haematin: 0·3$mM.$ $T=30°$.

Fig. 19. Effect of cyanide on oxidation of ascorbic acid. Ascorbic acid: 10$mM.$; haematin: 0·3$mM.$ $T=20°$.

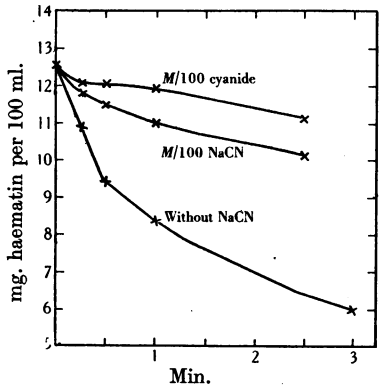

Fig. 20.

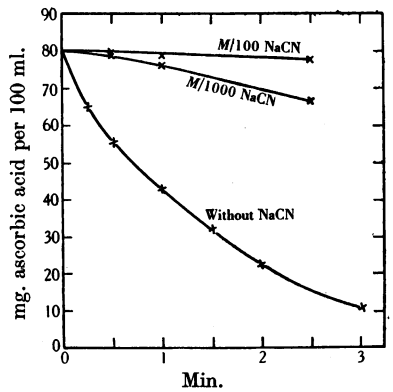

Fig. 21.

Fig. 20. Effect of cyanide on oxidation of haematin. Ascorbic acid: 4·5$mM.$; haematin: 0·2$mM.$ $T=20°$.

Fig. 21. Effect of cyanide on oxidation of ascorbic acid. Ascorbic acid: 4·5$mM.$; haematin: 0·2$mM.$ $T=20°$.

Effect of cyanide. With 10 $mM.$ ascorbic acid and 0·3 $mM.$ haematin $M/1000$ cyanide inhibits the oxidation of both haematin and ascorbic acid very little (about 10%), $M/100$ cyanide somewhat more (about 50%) and both

haematin and ascorbic acid oxidations in approximately the same proportion (Figs. 18 and 19). When the concentration of ascorbic acid is smaller, the inhibition by the same concentration of cyanide is considerably greater (Figs. 20 and 21).

The influence of the ascorbic acid concentration on the degree of cyanide inhibition is explained by the following experiment. Cyanide was added to a 0·4 *mM.* solution of haematin in 20 % pyridine to *M*/100 concentration. The two bands of pyridine-parahaematin had then disappeared and were replaced by a single diffuse band of pyridine-cyanhaematin. This solution was placed in the bottom part of Thunberg tubes, and various amounts of ascorbic acid solution were filled into the hollow stoppers. The tubes were then evacuated and filled with N_2, the contents mixed and the degree of reduction of pyridine-cyanhaematin to haemochromogen judged by the strength of the two absorption bands of haemochromogen which appeared. There was evidently complete or almost complete reduction when the ascorbic acid was 10 *mM.* When, however, the ascorbic acid concentration was only thrice the theoretical (0·6 *mM.*) the haemochromogen bands were just visible, showing that the reduction was very incomplete. When the tube with the larger amount of ascorbic acid was opened and shaken with air, the spectrum again displayed the pyridine-cyanhaematin band in addition to weaker haemochromogen bands and soon the verdohaemochromogen band in the red appeared. There is evidently an equilibrium between pyridine-cyanhaematin, pyridine-haemochromogen, cyanide and ascorbic acid, and the greater the concentration of vitamin, the less cyanide is bound to the haematin. A high concentration of ascorbic acid can thus counteract the influence of cyanide. It remains, however, to be explained why occasionally the ascorbic acid oxidation is inhibited more strongly by cyanide than the haematin oxidation.

Effect of reduced glutathione. Hopkins & Morgan [1936] have shown that reduced glutathione protects ascorbic acid from oxidation by "hexoxidase", an enzyme oxidizing ascorbic acid, found in *Brassica* by Szent-Györgyi [1931]. This has been confirmed quite recently by Borsook *et al.* [1937] who showed that dehydroascorbic acid is reduced rapidly by glutathione. It was therefore of interest to see whether glutathione can protect ascorbic acid from the catalytic oxidation by pyridine-haemochromogen, and, if so, whether the verdohaemochromogen formation was also inhibited by glutathione.

For these experiments we used the technique described above with the only difference that the trichloroacetic acid filtrate was divided in two equal parts, in one of which ascorbic acid was determined with 2:6-dichlorophenolindophenol, while in the second part the sum of glutathione and ascorbic acid was determined by iodine titration.

When 0·2 *mM.* haematin in 20 % pyridine containing 200 mg. of glutathione per 100 ml. (6·5 *mM.*) was shaken with air in a Thunberg tube at 37°, the haematin content decreased only from 12·8 to 9·5 mg. per 100 ml. in 10 min. Verdohaemochromogen was not detectable, and after reduction with $Na_2S_2O_4$ a weak band at 584 mμ. was seen in addition to the haemochromogen bands. There is also no considerable oxidation of glutathione under these conditions (2·6 mg. per 100 ml. in 10 min.).

When, however, glutathione is added to a solution of pyridine-haemochromogen containing some ascorbic acid, formation of verdohaemochromogen takes place.

In Table VIII an experiment with added glutathione is compared with a control experiment without glutathione. It is seen that glutathione somewhat

Table VIII

Haematin: 0·1 *mM.*; ascorbic acid: 2·3 *mM.*; glutathione: 7·1 *mM.* in 20% pyridine. $T = 37°$. Shaken in Thunberg tubes in air. Control without glutathione.

Time min.	$-\Delta$ haematin mg. per 100 ml.	Control: $-\Delta$ haematin mg. per 100 ml.	$-\Delta$ ascorbic acid mg. per 100 ml.	Control: $-\Delta$ ascorbic acid mg. per 100 ml.	$-\Delta$ glutathione mg. per 100 ml.	$-\Delta$ glutathione in terms of mg. per 100 ml. ascorbic acid	$-\Delta$ ascorbic acid saved mg. per 100 ml.
0–1	0·43	0·36	0·52	2·08	6·38	1·83	1·56
0–2	0·60	0·39	1·24	2·85	6·74	1·93	1·61

increases the rate of oxidation of haematin in presence of ascorbic acid, but decreases considerably the oxidation of ascorbic acid. A somewhat greater amount of glutathione is oxidized than corresponds to the ascorbic acid protected from oxidation. This experiment and several others which gave identical results prove that glutathione preserves the ascorbic acid by back-reduction of its oxidation product, and that ascorbic acid acts in this way as hydrogen carrier between haematin and glutathione. The protective effect of glutathione is certainly much more pronounced when the oxidation of ascorbic acid proceeds less rapidly than in our experiment or when glutathione is present in a greater molar quantity. The *p*H in this experiment was 7·5, and this proves that glutathione can protect ascorbic acid from oxidation by haemochromogens at physiological reactions.

Effect of the nitrogenous haemochromogen base. Barron *et al.* [1936 1, 2] have shown that other haemochromogens (nicotine-, pilocarpine-) also catalyse the oxidation of ascorbic acid, but that haematin in phosphate buffer does not.

Likewise the formation of verdohaemochromogen is not limited to pyridine-haemochromogen though other haemochromogens react less rapidly. Nicotine-haemochromogen which is the strongest catalyst for the oxidation of ascorbic acid is much less oxidized to verdohaemochromogen. With 4·5 *mM.* ascorbic acid and 0·2 *mM.* haematin at *p*H 7·7 and 37° nicotine-haemochromogen gives after 1 min. in air an oxidation ratio $\frac{\text{mol. ascorbic acid}}{\text{mol. haematin}}$ of 107, whereas that of pyridine-haemochromogen is 20. With pilocarpine-haemochromogen the oxidations of both ascorbic acid and haematin are slower.

Denatured globin-haemochromogen is very little reduced by ascorbic acid, and when it was shaken with air for 5 hr. in the presence of ascorbic acid, no formation of verdohaemochromogen took place. After 24 hr. at room temperature, however, a distinct absorption band in the red was found.

Discussion

In the present paper we have shown that ascorbic acid and haematin undergo coupled oxidation in dilute pyridine solutions. Pyridine-haemochromogen catalyses the reversible oxidation of ascorbic acid, which is not autoxidizable under these conditions, and ascorbic acid induces the oxidation of haematin to verdohaematin, a bile pigment iron complex.

The catalysed oxidation of ascorbic acid leads mainly to dehydroascorbic acid and in the first stages with low concentrations of ascorbic acid and haematin probably to a monodehydrogenation product. The rate of the reaction depends on the O_2 pressure. It has a low temperature coefficient and as in many catalysed reactions the activation energy is low. Ferric haemochromogen oxidizes ascorbic

acid rapidly to dehydroascorbic acid, and ferrous haemochromogen is autoxidizable and readily gives ferric haemochromogen. In these results there is nothing conflicting with the assumption that the valency change of haemochromogen catalyses the ascorbic acid oxidation. The same explanation has been given by Barron *et al.*

Cyanide, which blocks the action of other haematin enzymes even in low concentration by uniting with the ferric form giving a stable and inactive compound, is less effective as an inhibitor in our case. This might suggest that the mechanism of the haematin catalysis cannot be explained by a valency change, and that the haemochromogen remains in the ferrous state throughout the reaction. It has been shown, however, that ascorbic acid reduces pyridine-cyanhaematin in the presence of pyridine to ferrous haemochromogen, and the fact that the degree of cyanide inhibition depends on the ascorbic acid concentration provides further evidence that the low degree of cyanide inhibition must be explained in this way.

If the oxidation of ascorbic acid is due to the oscillations of the valency of haematin alone, it can be calculated that we have in our experiments a "turnover number" (number of complete cycles of valency change) of between 20 and 50 per min. in air, and about 50–100 in O_2; the turnover number of nicotine-haemochromogen is still greater (45 as compared with 25 with pyridine-haemochromogen under the same conditions).

The catalytic effect of haematin compounds on ascorbic acid oxidation has also direct physiological interest. Barron *et al.* [1936] recently adduced evidence that the hexoxidases of cabbage [Szent-Györgyi, 1931] and of Hubbard squash [Tauber *et al.* 1935] are of haemochromogen nature. $M/200$ cyanide inhibited the oxidation of ascorbic acid in the presence of enzymes from the juices of cabbage, yellow squash and watercress considerably, whereas typical inhibitors of copper catalysis (8-hydroxyquinoline; glutathione) had no effect. (In these experiments there was not sufficient glutathione to make the ascorbic acid-protecting effect of glutathione demonstrable.) In some fruits and other vegetables (apple, peach, lettuce) copper may be active in addition to haemochromogens. We have attempted to concentrate the enzyme of the Hubbard squash sufficiently to see the haemochromogen bands in the spectroscope, and have, indeed, observed weak bands in the green. The α-band was measured with some difficulty and its position fixed at about 560 mμ.

Haemochromogens might be thought to be unsuitable as catalysts of ascorbic acid oxidation, since they also undergo destruction during the process. There are, however, considerable differences in the stability of various haemochromogens in this reaction, some of them being only slowly attacked. It is also not impossible that these enzymes may be haemochromogens of bile pigment type; verdohaemochromogen, the product of the oxidation of protohaemochromogen, which has been shown above to possess considerable catalytic activity for ascorbic acid oxidation, is very stable under the conditions of the reaction. The occurrence of small amounts of verdohaemochromogen in the liver has been demonstrated by Lemberg & Wyndham [1936] and its occurrence in plants is quite likely.

How far haemochromogen catalysis may contribute to normal or pathologically increased ascorbic acid destruction in the animal body, cannot yet be estimated. The protective action of glutathione will, as a rule, probably suffice to counteract any ascorbic acid oxidation by haemochromogens which might occur. It seems, however, not quite unreasonable to assume that pathologically increased formation of haemochromogens or haemolysis with simultaneous

lowering of the protective glutathione mechanism may cause the increased requirement of ascorbic acid which recent investigations show to be a typical feature of many infections.

As to the catalytic action of ascorbic acid on the oxidation of haematin to bile pigment haematin there can be no doubt that this is an oxidation, and it may seem surprising that the rate of the reaction does not depend on the O_2 pressure within large limits, though some O_2 is, of course, necessary. The explanation must clearly be that only ferrous haemochromogen undergoes this oxidation. The reaction velocity might then be proportional to the O_2 pressure, but it will also be proportional to the concentration of ferrous haemochromogen and this will be inversely proportional to the O_2 pressure within certain limits. This explains also the necessity of reducing agents for the reaction. The temperature coefficient, though rather low, is still of the order of normal non-catalysed reactions, e.g. the destruction of catalase by hydrogen peroxide, and this indicates that the formation of verdohaemochromogen is due to autoxidation of the haemochromogen, the role of ascorbic acid being only that of a reducer.

Why then cannot all reducing agents cause verdohaemochromogen formation? For example $Na_2S_2O_4$ is quite unsuitable, although it reduces ferric to ferrous haemochromogen; when such a solution is shaken with air ferric haemochromogen and not verdohaemochromogen results. This may be explained by the assumption that each time only a small proportion of the molecules of ferrous haemochromogen undergoes oxidation to verdohaemochromogen, whilst the greater number yield ferric haemochromogen. When the turnover number (calculated from the amount of ascorbic acid oxidized per min. per mole haematin present) and the percentage of haematin oxidized during the same time are compared, our experiments show that with pyridine-haemochromogen at least 100 oxidation cycles are necessary for the complete oxidation of haematin to verdohaematin; each time 99% of the haemochromogen is oxidized to the ferric state and only 1% to verdohaematin. In the presence of $Na_2S_2O_4$, which undergoes a more rapid oxidation than ferrous haemochromogen, the oxidation of the latter substance practically begins only after the $Na_2S_2O_4$ has been completely oxidized, so that there are only a few oxidation-reduction cycles, forming practically no verdohaemochromogen. Ascorbic acid, however, does not react with oxygen directly, but only with ferric haemochromogen and this is therefore repeatedly reduced and oxidized. Although each time only a small part may form verdohaemochromogen the cumulative amount becomes considerable. We shall bring evidence in the second paper that the process is not specific for ascorbic acid, but can be produced by other reducing systems which fulfil the same conditions.

Nicotine-haemochromogen which, to judge from its effect on ascorbic acid, has a greater turnover number than pyridine-haemochromogen, is much less rapidly oxidized and requires about 210 repeated oxidation and reductions for complete transformation to verdohaematin, about four times as many as pyridine-haemochromogen. Considering the complicated nature of the process leading to the formation of verdohaemochromogen, there are various ways in which such a difference can be explained, so that the discussion will be deferred until more is known of the nature of this process.

From the evidence of results described in this paper we can make the statement that the transformation of a haematin compound into a bile pigment iron complex which readily loses its iron proceeds rapidly by the reaction which we have studied, at physiological temperature and under physiological con-

ditions. It is possible to advance this as a hypothesis which may help to explain the problem of physiological bile pigment formation.

The main part of bile pigment formation certainly takes place in the cells of the reticulo-endothelial system, which probably contain ascorbic acid and glutathione. This fact has to be kept in mind in discussing an objection which may be raised against our hypothesis. Our experiments have been carried out with pyridine-haemochromogen, and pyridine can hardly be considered as a physiological cell constituent in spite of the fact that nicotinamide has already been stated to be an important physiological substance. It has been shown that the reaction is not limited to pyridine-haemochromogen, but so far no haemochromogen has been found which reacts with reasonable rapidity and which is likely to occur in the cell. Haemoglobin itself reacts slowly, though in the same direction, with ascorbic acid. It might very well be that still another hydrogen carrier has to be inserted between haemoglobin and ascorbic acid to bridge the considerable gulf between the redox potentials of the two substances, and that this substance occurs in the reticulo-endothelial cells. There is still another possibility. It is suggestive that the redox potential of pyridine-haemochromogen ($E_0' = -0{\cdot}050$ at pH 9·15 according to Barron *et al.* [1936, 2]) stands midway between that of haemoglobin ($E_0' = +0{\cdot}15$) and of denatured globin-haemochromogen (cathaemoglobin) which is very little reduced by ascorbic acid and thus has a redox potential rather close to that of haematin itself ($E_0' = -0{\cdot}24$ at pH 9·16) which behaves similarly. It does not appear unreasonable to assume that the process of haemoglobin breakdown in the recitulo-endothelial cells is initiated by a denaturation of haemoglobin which does not lead to normal cathaemoglobin, but to a protein-haemochromogen of somewhat higher redox potential and a behaviour similar to that of pyridine-haemochromogen. Recent work has revealed the great importance of specific proteins for many biological processes, and evidence is available indicating that the denaturation of globin proceeds in stages. In support of this hypothesis it may be recalled that the verdohaemochromogen, found by Lemberg & Wyndham [1936] accompanying catalase in horse liver extracts, has as protein component not globin, but a protein of a different nature, similar to that to which haematin is bound in catalase; moreover bilirubin in serum is combined with a protein having the properties of serum albumin, and the chemical composition of globin [Pedersen & Waldenström, 1937].

Summary

Pyridine-haemochromogen and ascorbic acid undergo a coupled oxidation by atmospheric oxygen, the oxidation of 1 mg. of ascorbic acid at 37° being coupled with that of about 0·2 mg. haematin. The product of the haematin oxidation is verdohaematin, a bile pigment iron complex; the main product of the oxidation of ascorbic acid is dehydroascorbic acid.

Other haemochromogens and haemoglobin react in the same way, though less rapidly.

Temperature variations affect haematin oxidation more than ascorbic acid oxidation (Q_{10} haematin = 1·8, Q_{10} ascorbic acid = 1·3).

Variations of oxygen pressure from 4 to 100% of an atmosphere have little effect on haematin oxidation, whereas the rate of ascorbic acid oxidation is proportional to the oxygen tension.

The optimum pH is 7·0 for oxidation of ascorbic acid and 7·6 for that of haematin.

The initial velocities of haematin and ascorbic acid oxidations are proportional to the initial concentrations of haematin and (within certain limits) of ascorbic

acid. The rate of ascorbic acid oxidation decreases in the course of the reaction, however, less than it should according to these laws, owing to the catalytic activity of verdohaemochromogen, and the rate of haematin oxidation decreases initially more than it should, owing to the complex nature of this process.

The degree of cyanide inhibition depends on the ascorbic acid concentration.

Glutathione, which slightly accelerates verdohaemochromogen formation with ascorbic acid, though alone it does not cause it, saves ascorbic acid from oxidation by back-reduction of its oxidation product. Ascorbic acid and glutathione thus transport hydrogen from activated substrates to ferric haemochromogen.

The bearing of these observations on the theory of the mechanism of the reaction and on the problem, whether the physiological breakdown of haemoglobin and the bile pigment formation may proceed in a similar way, is discussed.

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