The results (Table 2) show clearly that definite relationships exist between sterol content and soil type. Deposition of sterols must depend primarily upon the nature and number of the animal and plant inhabitants, so that deposition will be at a maximum in soils favourable to the maintenance of a flourishing population. A characteristic of such soils, however, is the rapidity with which humus material undergoes disintegration. Since the sterol content of these soils shows no continued increase, this component of the humus is presumably decomposed or disposed of in some other manner. In soils of the opposite type there is a marked accumulation of sterols. Extreme acidity, lack of aeration, and excessive water content are all factors which, either individually or collectively, inhibit the decomposition of humus and its constituent sterols, and are thus directly responsible for the high sterol contents of bog and fen peats. In arable soils these adverse factors have been counteracted, and, whilst it is found that sterols are present in varying amount, the content of any particular soil shows a gradual decrease unless replenished by organic manuring.

Agencies responsible for sterol disappearance

In view of the insolubility of the sterols, and of their association with natural fats and oils, their disappearance from soils cannot be attributed to the leaching action of rain-water, but is probably the result of degradation of the sterol molecule by the co-ordinated activities of a variety of soil micro-organisms. Evidence in favour of this theory has been obtained experimentally in the following manner.

Six 0.05 g. quantities of cholesterol were accurately weighed, and each intimately mixed with 25 g. soil. The mixtures were placed in separate porous flower pots in which the drainage holes had been closed with watch-glasses, and the pots buried with their tops 1 in. below the surface of a well-worked loamy garden soil. One group of three pots was watered weekly with rainwater, whilst the second group was treated with a dilute solution of HgCl_2 .

Table 3. *Rate of disappearance of sterols from soil under different conditions*

Time months	Sterol content (mg.)	
	Group I (water)	Group II (HgCl_2)
0*	50.0	50.0
3	42.7	49.2
6	29.6	49.8
12	18.3	48.9

* The sterol content of the untreated 25 g. sample of soil is negligible.

At intervals of 3, 6 and 12 months respectively, one pot from each group was removed, and the sterol content of the soil determined (Table 3). In the first group sterol disappeared, but the sterilizing action of the HgCl_2 inhibited microbiological activity in the second group so that the sterol content altered little. Similar experiments, using 'organic' cholesterol in the form of brain tissue and egg yolk, gave comparable results although disappearance of sterol was here less rapid than with the crystalline material.

The nature of the biological agents responsible for sterol breakdown is being investigated, and there are indications that decomposition is initiated by certain acid-tolerant, aerobic bacteria.

SUMMARY

1. A method is described for the determination of soil sterols.
2. The sterol content of a wide range of British soils has been determined.
3. Accumulation of sterols in soils occurs under conditions of extreme acidity, waterlogging and lack of aeration.
4. Where these conditions are reversed, as in arable soils, the sterol content gradually decreases.
5. The disappearance of soil sterols has been demonstrated experimentally, and has been shown to result from the activities of microbiological agents.

The author wishes to express his thanks to colleagues who have assisted in obtaining soil samples.

REFERENCES

- Kelsey, F. E. [1939]. *J. biol. Chem.* **127**, 15.
 Schreiner, O. & Shorey, E. C. [1909]. *J. Amer. chem. Soc.* **31**, 116.
 Schreiner, O. & Shorey, E. C. [1911]. *J. biol. Chem.* **9**, 9.
 Windaus, A. [1909]. *Ber. dtsch. chem. Ges.* **42**, 238.

Liver Catalase

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Stern [1935-6] isolated biliverdin (dehydrobilirubin) from the split products of horse liver catalase; the pigment was identified by Lemberg [cf. Lemberg & Wyndham, 1936-7]. Biliverdin was not present in

the catalase preparation as such, but as a bile pigment haem compound bound to a protein similar to or identical with that characteristic of catalase [Lemberg & Wyndham, 1936-7]. After reduction

and transformation to pyridine-haemochromogen it gave the absorption spectrum of verdohaemochromogen. At that time it was assumed that the bile pigment haemoprotein was an impurity.

Sumner & Dounce [1937] obtained ox liver catalase in crystalline form. After repeated recrystallization fission with HCl in acetone of the enzyme yielded haemin and a blue compound, which was believed to contain iron and to be different from both biliverdin and verdohaemochromogen. While one-half of the catalase iron was firmly bound as protohaemin, the other half ('blue iron') was easily ionized by treatment with HCl, but unlike the iron of ferritin, which sometimes accompanies catalase, it was not precipitated with the protein. Iron of bile pigment haem compounds is readily set free by acid.

Lemberg, Norrie & Legge [1939] reported the isolation of crystalline biliverdin hydrochloride from crystalline ox and horse liver catalases. In view of the constant molar ratio of biliverdin to haemin (about 1 to 3), it was then assumed that the bile pigment haem formed an essential part of the catalase molecule. Another possibility, however, was mentioned, i.e. that the protohaem group in catalase may be oxidized to bile pigment haem by H_2O_2 , leaving the protein component unaltered. At that time we were unable to find experimental support for this assumption.

Stern [1940] accepted this latter explanation of the presence of bile pigment haem in crystalline catalase as the correct one, while Agner [1939] and Theorell [1940] claimed that iron-free biliverdin was admixed with catalase. Agner based this claim on the isolation of a highly active horse liver catalase (of Kat.-f. 62,000),* which contained 0.086 % protohaem iron (83 % of the total iron in the form of protohaem and only 10 % in the form of 'blue iron'), but still gave biliverdin on splitting with acid. No quantitative estimations of the bile pigment were carried out, however, nor was any explanation given for the lack of success in separating catalase and biliverdin by fractional precipitation with $(NH_4)_2SO_4$, crystallization and cataphoresis.

Sumner, Dounce & Frampton [1940] obtained horse liver catalases in which the content of the iron accompanying biliverdin was inversely proportional to the specific activity. This indicated that the bile pigment haemoprotein is inactive and present in catalase in varying proportion.

We arrived at the same conclusions independently and with other methods. While Sumner studied the

ionizable iron fraction, we investigated the molar ratio of bile pigment to haemin obtained by splitting catalase with acids, and the absorption spectra of the CO-haemochromogens obtained from catalase.

We have also shown that the bile pigment haem content of catalase can be increased by treatment with H_2O_2 in the presence of ascorbic acid, and that ascorbic acid inhibits catalase by accelerating its destruction by H_2O_2 .

EXPERIMENTAL

Isolation and purification of catalase

Crystalline ox liver catalase was readily prepared by the method of Sumner & Dounce [1937]. The crystals can be further purified by washing with distilled water, and are dissolved by dialysing the suspension in distilled water against *M*/50 phosphate buffer pH 7.8; they are then recrystallized according to the method of Sumner & Dounce.

The crystals have the form of fine needles or small platelets described by Sumner, but on long standing in the refrigerator strong solutions of ox liver catalase occasionally deposit very large thick hexagonal plates which are no longer easily soluble in phosphate buffer.

Horse liver catalase. The dioxan method of Sumner *et al.* [1940] was not consistently successful in our hands, and we used the earlier stages of the method of Zeile & Hellström [1930]. The difficulty due to phosphate in the eluates [Agner, 1938] is easily overcome by removal of the phosphate by thorough dialysis at 0° against distilled water. After this the fractionation of catalase and ferritin even in strong solutions offers no difficulties. Crystallization and recrystallization were carried out according to Sumner *et al.* [1940]. Horse liver catalase crystals are more readily soluble in water than ox liver catalase.

Amorphous horse liver catalase was prepared by the methods of Zeile & Hellström [1930] and of Keilin & Hartree [1936].

Methods

Total protein was estimated with a micromodification of the method of Bick [1936].

Catalase activity. The activity was determined by the method of Zeile & Hellström [1930]. We can confirm that the values of $-(\Delta \log C_{H_2O_2})/\Delta t$ show a noticeable decrease with time even under their conditions. Sumner extrapolates these values for $t=0$, but does not mention his method of extrapolation. By a different calculation one can determine the exact value of k_0 .

According to Nosaka [1927] and Maximowitsch & Awtonomova [1928] the kinetics of destruction of H_2O_2 by blood catalase are given by the equations $-dC = k EC dt$ and $-dE = k' EC dt$, where C is the concentration of H_2O_2 ,

* Kat.-f. (Katalasefähigkeit) was used by Euler to designate the activity of a solution containing 1 g. of catalase in 50 ml. We have previously used the translation 'catalase factor' in order to avoid the grammatical difficulties connected with the use of Kat.-f. A better translation is specific catalase activity (Sp.C.A.) and this will be used in this paper.

E that of catalase, k the reaction constant of the destruction of H_2O_2 by the enzyme and k' that of the destruction of the enzyme by H_2O_2 . Integration yields the equation

$$\ln \frac{C_0}{C} - \ln \frac{C_0 + A}{C + A} = k' t A, \quad (1)$$

where A is an integration constant defined as

$$A = \frac{k E_0}{k'} - C_0. \quad (2)$$

If the H_2O_2 concentrations C_0, C_1, C_2 and C_3 at the times t_0, t_1, t_2 and t_3 , where $t_3 - t_2$ equals $t_2 - t_1$, are known, kE_0 , the required value for the activity at $t=0$, can be calculated. The simplest way of finding A is by approximation from the equation

$$\ln \frac{C_1}{C_2} - \ln \frac{C_2}{C_3} = \ln \frac{C_1 + A}{C_2 + A} - \ln \frac{C_2 + A}{C_3 + A}. \quad (3)$$

Inserting the value of A into equation (2) yields k' and from equation (1) kE_0 is then found as $(A + C_0)k'$. We have followed, however, the customary procedure of using $\log_{10}$ for calculation of the catalase reaction constant.

Table 1 shows that the course of H_2O_2 destruction by liver catalase can be satisfactorily calculated by assuming the validity of these equations, and that the action of liver catalase, therefore, obeys the same kinetic laws as that of blood catalase.

Table 1. *Kinetics of H_2O_2 destruction by liver catalase*

$$A = 12, k' = 0.0045$$

t min.	$C_{H_2O_2}$ found as average of 2 estimations	$C_{H_2O_2}$ according to theory
0	9.03	9.03
0.5	8.11	8.07
1.5	6.62	6.64
3.5	4.59	4.55
4.5	3.86	3.85
6.5	2.81	2.75
7.5	2.41	2.35
9.5	1.80	1.75
10.5	1.56	1.53
20.0	0.43	0.45

In several instances we have observed a slight fall of A with time which would indicate that the speed of destruction of the enzyme does not decrease as much as the H_2O_2 concentration. The curvature of the graph of $C_{H_2O_2}$ plotted against time is slightly less than expected from theory.

A simpler calculation of k_0 can be derived from the equations: $-dC = kCE dt$ and $-dE = k'E$. It follows

$$\ln C_0 - \ln C = \frac{k}{k'} E_0 (1 - e^{-k't}). \quad (4)$$

The destruction of the enzyme does, of course, depend on the H_2O_2 concentration, and the curvature of the $C_{H_2O_2}$ graph is greater than would be expected from equation (4). Under the conditions of the estimations, however, with a low enzyme concentration and a small fall of H_2O_2 concentration, the equation

$$k_0 = \text{antilog.} \left\{ \frac{\frac{1}{2} (t_1 + t_2) \log k_{t_1-t_2} - \frac{1}{2} (t_2 + t_3) \log k_{t_2-t_3}}{\frac{1}{2} (t_3 - t_1)} \right\}, \quad (5)$$

which is derived from equation (4) yields results which agree with those found by the formulae (1)–(3) within the experimental error.

In order to compare our results with those of Sumner, we have calculated k_0 from the examples given by Sumner & Dounce [1939, p. 442] with our formulae. The values given by these authors are 0.0510 and, with half the enzyme concentration, 0.0247. With the formulae (1)–(3) we find 0.0511 and 0.0254, with formulae (5) 0.0505 and 0.0248. Our results are, therefore, strictly comparable with those of Sumner. Sumner & Dounce do not mention the volume of diluted catalase solution added to 50 ml. of H_2O_2 , but if this was 0.5 ml., as used by Zeile, the specific activities were 24,000 and 23,200, and not 35,000 and 34,500 as given by them.

Protohaem in catalase was determined spectrophotometrically by measurement of the absorption at 558 $m\mu$ of the haemochromogen formed on adding NaOH, final conc. 1.25 %, followed by a few mg. $Na_2S_2O_4$. We have found no difference in the height of the absorption band on the addition of pyridine, nor any cloudiness of the solution, except with partly denatured catalase. The concentration of protohaemin in mg./ml. is $(\epsilon_{555 m\mu} \times 10^3)/47.5l$, where l is the length of layer through which the light passes, and 47.5 the extinction coefficient of a solution of protohaemochromogen containing 1 mg. haemin/ml.

Estimation of bile pigment content and bile pigment:haemin ratio. The isolation of bile pigments from catalase treated with acetic acid was by the method of Lemberg, Lockwood & Legge [1941b]. About 5 ml. of catalase solution of $k = 5000$ sufficed. No ascorbic acid was added, since acidified catalase does not oxidize biliverdin. The 66% acetic acid solution was diluted with the same volume of peroxide-free ether, and the almost colourless protein removed by washing with Na acetate solution and water. These washings were saturated with $(NH_4)_2SO_4$, and re-extracted with ether [cf. Lemberg *et al.* 1941b, p. 369]. The bile pigments were extracted by repeated shaking with 20 % HCl.

The isolation of crystalline biliverdin hydrochloride from crystalline liver catalase has been described [Lemberg *et al.* 1939]. The crystals possessed the typical form and optical and solubility properties, and on oxidation of the complex zinc salt with iodine [cf. Lemberg, 1935] the characteristic fluorescence and absorption band of the biliverdin zinc complex salt were obtained.

In our first estimations we determined biliverdin in the acid extracts colorimetrically, by comparison with a biliverdin standard prepared from pure crystalline biliverdin. Later it was recognized that the extracts contain small amounts of biliviolin (usually about 20 % of total bile pigment). The values for bile pigment content and for the molar ratio (bile pigment Fe)/(bile pigment Fe + protohaem Fe), obtained by this method, are therefore too small, but can be compared among themselves. The results obtained by this method are given in Table 2.

In the later experiments, summarized in Table 3, biliverdin and bilviolin were measured spectrophotometrically according to method III [Lemberg *et al.* 1941b].

After extraction of the bile pigments the ethereal solution of haemin was washed once with water, and protohaem was then extracted with NH_3 and determined spectrophotometrically as pyridine-haemochromogen [Lemberg, Legge & Lockwood, 1941a]. Usually 50–70% of the total protohaem of catalase was thus recovered. The remainder clings to the denatured protein. We shall give below evidence showing that the bile pigment in catalase is present in the form of a bile pigment haem compound, from which bile pigment is split off quantitatively. The ratio (bile pigment Fe)/(bile pigment Fe + protohaem Fe), the latter being called 'total haem Fe', given in Tables 2 and 3, was calculated assuming that the recovery of bile pigment was as complete as that of protohaem.

*Bile pigment and haemin yields
from liver catalases*

With the earlier method we found the ratio of bile pigment Fe to total haem Fe to be close to 0.25 for a number of preparations of ox and horse liver catalase of varying purity (Table 2). This suggested that catalase contained three protohaem groups and one bile pigment haem group in the molecule. With the more exact later method, however, a higher ratio was established, varying between 0.30 and 0.50 with an average of 0.38 (Table 3). This value does not suggest a stoichiometric ratio of bile pigment haem and protohaem, and the assumption of compounds between catalase and bile pigment haemoprotein, which contain one, two or three bile pigment haem groups in addition to three, two or one protohaem groups respectively, is thus not supported by experimental evidence.

Tables 2 and 3 show that the protohaem contents of the catalase preparations increase with their specific activities. Laszkowski & Sumner [1941] have isolated crystalline ox erythrocyte catalase of the specific activity 48,000, and have found it to be free from bile pigment haem; the latter is, therefore, not necessary for catalase action. At first sight the experiments summarized in Tables 2 and 3 seem to support the view that the bile pigment haemoprotein is catalytically as active as the protohaem compound. The ratio of bile pigment Fe to total haem Fe does not vary with the specific activity over a wide range. A closer analysis reveals, however, that the bile pigment haemoprotein is inactive. The variations of the specific activity in our experi-

ments are partly associated with factors other than that of the varying bile pigment content. The last column of Table 3 shows that the percentage of total haem Fe in catalase only occasionally approaches (e.g. preparations D2a, D2c and F2 of ox liver catalase and D1 of horse liver catalase) the theoretical value of 0.099%, calculated for the presence of four haem groups in a catalase molecule of the molecular weight 225,000 [cf. Sumner *et al.* 1940]. The preparations with a lower total haem Fe content presumably still contain some inactive protein without haem groups, although some are crystalline. By multiplication with a correction factor (0.099 divided by percentage total haem Fe found) the true specific activity of the haemoproteins is obtained. If this is plotted against the ratio protohaem Fe/total haem Fe (Fig. 1), it is clearly seen that specific activity increases with decreasing bile pigment content. All but one of the points lie close to a straight line. This line cuts the 0-point, showing that the bile pigment haemoprotein is inactive, and reaches 52,000 for pure catalase without bile pigment haem. There is no significant difference between the activities of ox and horse liver catalases, and their variations with bile pigment haem content. The value 52,000 is close to that found by Sumner for the specific activity of ox erythrocyte catalase (48,000), which is free from

Fig. 1. Increase of specific activity with decreasing bile pigment haem content. $\triangle$ Ox liver catalase. $\circ$ Horse liver catalase.

bile pigment haem, and for the purest horse liver catalases.

Sumner *et al.* [1940] worked with a number of catalase preparations with more widely varying bile pigment haem contents, most of them of approximately equal purity, as the total haem Fe values (Table 2 of their paper) show. The specific activities, therefore, decrease with increasing bile pigment content without any correction for the presence of non-haematin compounds. It is more difficult to

Table 2. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation	Specific activity	Protein conc. %	Haemin % of total protein	$\mu\text{g. per ml.}$					Ratio: bile pigment Fe total haem Fe	$k/\text{proto-haem Fe}$
					Ox liver catalase	Extracted					
						Proto-haem Fe	Bili-verdin	Bile pigment Fe	Proto-haem Fe		
K 2	0.1 %NH ₃ eluate from Al ₂ O ₃ *	1,800	3.40	0.07	1.94	0.94	3.26	0.31	0.25	1,600	
L 1	Crude extract	6,700	0.60	0.25	1.27	0.48	1.70	0.16	0.25	1,580	
A 2b	Eluted with NH ₃ after A 2a	10,700	0.90	0.22	1.70	0.84	3.10	0.30	0.26	2,640	
C 1	Crystalline	—	—	—	—	9.32	33.5	3.21	0.25	—	
A 2	"	12,500	0.60	0.31	1.58	0.56	3.19	0.30	0.35	2,380	
A 2a	A 2 adsorbed to Al ₂ O ₃ and re-eluted	—	—	—	1.57	0.81	3.81	0.36	0.31	3,120	
A 1	Crystalline	17,500	1.90	0.31	5.06	3.25	13.0	1.25	0.27	3,260	
B 2	Mother liquor of B 1	17,000	0.90	0.33	2.57	1.67	4.7	0.45	0.21	2,960	
B 1	Crystalline	—	—	—	—	8.45	36.4	3.48	0.29	—	
B 1'	B 1 recrystallized	33,000	1.20	0.73	7.53	4.28	14.4	1.38	0.24	2,630	
Horse liver catalase											
A 1	Crude extract	5,000	4.6	0.26	10.1	1.68†	4.6	0.44	0.21	1,140	
A 2	After adsorption to Al ₂ O ₃	25,700	3.82	0.50	19.2	12.3	46.4	4.43	0.27	2,560	
B 1	Amorphous	20,000	4.28	0.48	20.7	9.9	44.0	4.20	0.28	2,080	
B 2a	1st fraction of ammonium sulphate ppt.	—	—	—	20.8	11.6	33.6	3.21	0.22	—	
B 2b	2nd fraction	—	—	—	17.0	9.0	32.4	3.10	0.25	—	
C 2	Crystalline	—	—	—	8.75	4.93	16.0	1.53	0.24	—	
Zeile, Agner, Sumner											

* Eluate after main part of catalase had been eluted with secondary phosphate.

† The small percentage of haemin recovery after splitting makes the result uncertain.

Table 3. *Bile pigment and haemin yields from ox and horse liver catalases*

No.	Preparation	Specific activity	Protein conc. %	Haemin % of total protein	$\mu\text{g. per ml.}$				Bile pigment Fe	Ratio: $\frac{\text{bile pigment Fe}}{\text{total haem Fe}}$	$k/\text{proto-haem Fe}$	Total haem Fe % of total protein
					Protein conc. %	Proto-haem Fe	Bili-verdin	Bili-violin				
H 2	Sumner & Dounce	2,500	2.07	0.66	Ox liver catalase 11.75	4.70	14.7	20.9	3.40	0.42	220	0.098
H 1												
E 2	Crystalline	15,000	—	—	1.18	0.33†	2.30	0.88	0.30	0.45	2,920	0.050
E 3	2nd fraction of crystals	19,700	1.60	0.36	4.96	2.16	12.3	1.5	1.32	0.38	2,510	0.050
D 1	Amorphous	21,800	0.62	0.43	2.28	0.81†	5.40	1.33	0.64	0.44	2,680	0.066
J 1	Amorphous	24,600	0.78	0.50	3.29	2.00	11.2	0.95	1.16	0.37	2,580	0.067
D 2c	Crystalline	24,600	0.50	—	—	1.36	4.06	0.91	0.48	0.26	—	—
D 2b	"	24,600	0.88	0.54	4.08	2.36	7.03	4.37	1.09	0.32	2,650	0.068
E 1	"	25,700	1.44	0.55	6.76	1.98†	15.0	6.9	1.94	0.49†	2,740	0.093
D 2a	"	26,200	0.82	0.59	4.16	2.04	8.6	3.3	1.14	0.36	2,580	0.079
F 2	"	27,300	0.20	0.58	0.99	0.61	4.15	1.03	0.50	0.45	2,770	0.090
	"	36,400	0.90	0.70	5.39	3.45	11.9	4.3	1.55	0.31	3,040	0.087
					Horse liver catalase							
M 1	Zeile, Agner, Sumner	—	—	—	—	1.45	6.9	1.0	0.75	0.34	—	—
C 2	Crystals	—	—	—	—	3.42	28.8	6.2	3.35	0.49	—	—
G	Mother liquor of crystals	13,800	0.92	0.31	2.45	1.58	10.3	1.9	1.15	0.42	2,500	0.047
H	Crystalline	19,000	1.29	0.47	5.20	2.80	17.0	6.5	2.24	0.44	2,350	0.073
F 1b	"	18,400	0.84	—	—	2.49	13.6	2.4	1.53	0.38	—	—
F 2	Amorphous	23,200	1.01	0.51	4.41	2.20	8.8	1.65	1.00	0.31	2,580	0.064
F 1	"	24,000	1.95	0.51	8.53	4.22	24.0	4.2	2.69	0.39	2,750	0.072
F 1a	"	24,000	1.80	0.57	8.68	5.42	18.9	7.8	2.56	0.32	2,350	0.071
D 1	Crystalline	28,000	1.32	0.60	6.75	1.84†	16.6	0.4	1.62	0.47	2,730	0.091
F 1a	"	30,600	0.72	—	—	2.72	11.8	0.7	1.19	0.30	—	—
F 1b	"	31,200	0.43	—	—	1.57	5.09	2.71	0.74	0.32	—	—

* After long standing of the crude extract; remarkably much inactive haematin and biliviolin.

† The small percentage of haemin recovery after splitting makes the result less certain.

‡ According to the absorption spectrum of the CO-haemochromogen: 0.38.

Table 6. *Increase of bile pigment haem in catalase by treatment with H_2O_2 in the presence of ascorbic acid*

Liver catalase		Ascorbic acid		H_2O_2 g.		$\text{mg. H}_2\text{O}_2$ mg. active enzyme	Total activity decrease %	Activity decrease due to oxidation of prosthetic group %	dC/dE destroyed mg. enzyme inactivated	Specific activity	$k/\text{protohaem Fe}$
Species	Vol. ml.	mg. per 100 ml.	t°	H_2O_2 g.	$\text{mg. H}_2\text{O}_2$ mg. active enzyme	Total activity decrease %	Activity decrease due to oxidation of prosthetic group %	dC/dE destroyed mg. enzyme inactivated	Specific activity	Before oxidation	After oxidation
Ox	15	6,150	5	30	1,700	83	44	2,000	18,300	25	—
Horse	10	12,200	5	30	900	65	9	1,400	27,000	15	1,650
Horse	10	4,530	5	30	1,200	49	30	2,350	13,800	6	1,850
Horse	22	6,340	25	5	1,200	34	10	3,300	13,000	—	2,400
Horse	22.2	6,340	30	23	1,200	19	7	6,500	—	—	2,500
Horse	20	6,340	40	37	600	74	2	800	—	—	2,090

draw a straight line through the points of Sumner's table in a diagram similar to Fig. 1, with or without correction for presence of non-haematin compounds, and the line does not appear to go through the 0-point. This is probably owing to the way in which the bile pigment haem Fe is determined by Sumner as 'blue iron' fraction. Even the best catalase preparations still contain small amounts of ferritin, a protein with high iron content, and it is likely that a small part of the ferritin iron is not precipitated with the protein, but is determined as 'blue iron'. Our method takes into consideration only haem and bile pigment haem, and the presence of iron in any other form, e.g. as ferritin, does not interfere with the results. Apart from this minor difference, however, our results independently confirm those of Sumner by different methods.

Spectrophotometric study of the catalase haemochromogen

While Zeile [1933] claimed that the haemochromogen prepared from catalase is identical with that from protohaem, Lemberg & Wyndham [1936-7] observed an absorption band at $651\text{m}\mu$ with the pyridine haemochromogen prepared from highly active, but amorphous horse liver catalase.

The same band is observed, when crystalline preparations of ox and horse liver catalase are studied. NaOH, final conc. 1%, is added to catalase and after the addition of a few mg. $\text{Na}_2\text{S}_2\text{O}_4$ the absorption spectrum is immediately observed. With the spectrophotometer the ratio $\epsilon_{650} : \epsilon_{557}$ is found to be 0.25, while for protohaemochromogen, prepared in the same manner from haemoglobin, this ratio is only 0.015. This absorption in the red part of the spectrum is not due to unspecific background absorption, as Stern [1937] assumed, since a definite maximum of absorption at $650\text{m}\mu$ is found. It cannot be due to biliverdin either, since biliverdin is reduced by $\text{Na}_2\text{S}_2\text{O}_4$ in alkaline solution to a yellow compound which does not absorb in the red.

The presence of another haemochromogen in addition to protohaemochromogen is confirmed by examination of the CO-haemochromogen. By bubbling coal gas through the solution, previously saturated with the gas, during the addition of $\text{Na}_2\text{S}_2\text{O}_4$ and NaOH, the danger of secondary oxidation by H_2O_2 formed from $\text{Na}_2\text{S}_2\text{O}_4$ is prevented. A strong absorption band at $630\text{m}\mu$ is found, which is usually about half as high as that of CO-protohaemochromogen at $570\text{m}\mu$ (Fig. 2).

The absorption curve differs considerably from that of CO-protohaemochromogen prepared from haemoglobin.

While the haemochromogen accompanying protohaemochromogen is similar to cholehaemochromogen in some properties, differences exist between

them. The position of the absorption band of the CO-compounds is the same, and removal of CO causes the same shift to $618\text{m}\mu$; the absorption band at $630\text{m}\mu$ of the catalase compound is, however, accompanied by absorption rising towards the infra-red. A further difference is that choleglobin gives only a poor yield of biliverdin, while bile pigment formation from the catalase haemoprotein appears to be quantitative. While such differences

Fig. 2. CO-haemochromogen absorption curve. — CO-haemochromogen from catalase. ---- CO-haemochromogen from haemoglobin.

may be due to the different proteins to which the bile pigment haem group is linked, the fact that the absorption band of the haemochromogen before the action of CO is found at $650\text{m}\mu$, not at $618\text{m}\mu$, suggests a prosthetic group in catalase slightly different from cholehaem. It seems to constitute a link between verdohaem and cholehaem.

The bile pigment haemochromogens have been found to possess approximately equal molar extinction coefficients at the respective maxima of absorption. We may therefore assume that the CO-haemochromogen from catalase has the same molar extinction at $630\text{m}\mu$ as CO-cholehaemochromogen, and can then calculate the ratio of bile pigment haem to total haem as

$$(\epsilon_{630}/\epsilon_{570} - 0.04)/(0.55\epsilon_{630}/\epsilon_{570} + 0.96).$$

This formula is in principle the same as that given by Lemberg *et al.* [1941a], except that it gives the ratio bile pigment haem/total haem instead of bile pigment haem/protohaem as in the earlier formula.

Table 4 shows that the ratio found in this way is the same as that found by determinations of bile pigment and haemin in the split products. With one exception (D 2b) the results agree very well. It can

Table 4
Molar ratio: $\frac{\text{bile pigment haem}}{\text{total haem}}$

Ox liver catalase preparation	Found by estimation of the split products	Found by spectrophotometric analysis
F2	0.31	0.35
D2c	0.32	0.33
E1	0.36	0.38
D1	0.37	0.40
E2	0.38	0.38
L	0.40	0.42
E3	0.44	0.44
H1	0.45	0.43
D2a	0.45	0.43
D2b	0.49	0.38

be concluded from these experiments that the biliverdin obtained from catalase by splitting with acids, occurs in catalase in the form of a bile pigment haemoprotein.

OXIDATION OF CATALASE TO BILE PIGMENT HAEMOPROTEIN BY H_2O_2

Calculation of the minimum amount of H_2O_2 required. We have reported [Lemberg *et al.* 1939] that we could not observe an increase in the bile pigment haem content of catalase when we allowed the enzyme to destroy gradually a large amount of H_2O_2 .

From the kinetics of the enzyme reaction given above we can now calculate how much H_2O_2 catalase can destroy before it is inactivated, and thus determine the minimum amount of H_2O_2 with which the enzyme must react in order to obtain an experimentally verifiable increase in its bile pigment haem content. These calculations show that the amounts of H_2O_2 required are so large that the experimental difficulties are insurmountable, even if the inactivation of catalase in dilute solution were due entirely to the oxidation of protohaem to bile pigment haem.

From the two equations

$$-dC = kEC dt \quad \text{and} \quad -dE = k'EC dt$$

it follows that

$$\frac{dC}{dE} = \frac{k}{k'} = \frac{A + C_0}{E_0},$$

where $A + C_0$ is measured in terms of ml. KMnO_4 /10 ml. H_2O_2 solution. C_0 ml. KMnO_4 are equivalent to 10 ml. $N/100$ H_2O_2 and $A + C_0$ therefore to $(A + C_0)/100C_0$ mol./l. E_0 in mol./l. is determined as $20k_0/225,000K$, where k_0 is the initial velocity constant of H_2O_2 destruction, and K the specific activity. Since we are interested only in the oxidation

of the active catalase molecules, the maximum specific activity (50,000) is used. Thus we have:

$$\frac{dC}{dE} = 5.62 \cdot 10^4 \frac{A + C_0}{C_0 k_0} \frac{\text{g. mol. } \text{H}_2\text{O}_2 \text{ destroyed}}{\text{g. mol. catalase inactivated}}$$

$$= 850 \frac{A + C_0}{C_0 k_0} \frac{\text{g. } \text{H}_2\text{O}_2 \text{ destroyed}}{\text{g. enzyme inactivated}}$$

$$\text{or} \quad 850 \frac{1}{C_0 k'} \frac{\text{g. } \text{H}_2\text{O}_2 \text{ destroyed}}{\text{g. enzyme inactivated}}$$

k' was not determined with great accuracy; at 0° it varied from 0.0007 to 0.0023 with an average of 0.0014. No significant difference was found between the k' of ox and of horse liver catalase. dC/dE varies from 20,000 to 75,000 g. H_2O_2 /g. catalase, with an average of 35,000.

In order to inactivate 10 mg. out of 50 mg. active enzyme (the minimum needed for estimation) more than 1100 ml. of 30 % H_2O_2 solution would have to be added. This would increase the ratio of bile pigment Fe to total haem Fe from 0.40 to 0.52, provided that the inactivation of the enzyme were entirely due to the oxidation of haematin to bile pigment haematin.

Effect of ascorbic acid on the destruction of catalase by H_2O_2 . We have found, however, a way to overcome these difficulties. Various authors have observed that the action of catalase is inhibited by reducing substances, e.g. glutathione and ascorbic acid. Marks [1936] found that ascorbic acid and reduced glutathione increase the rate of inactivation of catalase by atmospheric O_2 . By the action of ascorbic acid and H_2O_2 haemoglobin and haemochromogens are oxidized to bile pigment haem compounds. We have therefore investigated whether the inhibition of the enzyme action by ascorbic acid is due to a real diminution of k , or to a faster destruction of the enzyme by H_2O_2 (increase of k').

Ascorbic acid reacts only slowly with dilute H_2O_2 or KMnO_4 . If the first drop of $N/100$ KMnO_4 solution giving a colour lasting for more than 10 sec. is taken as the end-point, the presence of ascorbic acid in a concentration not greater than 10 mg./100 ml. does not interfere with the titration. Moreover there is no measurable reaction between H_2O_2 and ascorbic acid during 10 min.

Table 5 shows that k_0 is decreased little, if at all, by ascorbic acid, while k' is greatly increased. Ascorbic acid inhibits the enzyme action by accelerating the destruction of catalase by H_2O_2 . The ratio dC/dE is thus diminished from 35000 to 4000 by a concentration of 10 mg. ascorbic acid/100 ml.

Experiments with glutathione failed to yield clear-cut results. Reduced glutathione reacts too rapidly with H_2O_2 , and also with KMnO_4 . Nevertheless our experiments indicate that the inhibition of catalase activity by reduced glutathione is not due to increased destruction of the enzyme.

Table 5. *Destruction of catalase by H_2O_2 at 0° in the presence and absence of ascorbic acid*

	k_0	k'	$\frac{dC/dE}{\text{g. } H_2O_2 \text{ destroyed}} \times \text{g. catalase inactivated}$
Horse liver catalase without ascorbic acid	0.054	0.0018	29,000
Horse liver catalase with 2 mg./100 g. ascorbic acid	0.054	0.0074	7,100
Horse liver catalase with 10 mg./100 g. ascorbic acid	0.054	0.0127	4,100
Ox liver catalase without ascorbic acid	0.056	0.0017	27,000
Ox liver catalase with 4 mg./100 g. ascorbic acid	0.052	0.0088	4,700

Large-scale experiments. These were made to see if treatment of catalase with H_2O_2 in the presence of ascorbic acid increases the bile pigment haem content. Much higher concentrations of catalase and, at least locally, of H_2O_2 cannot be avoided in these experiments. Otherwise the large dilution of the enzyme would make impossible its recovery in the concentrated form required for bile pigment estimations. Even the use of concentrated (30%) H_2O_2 causes much dilution. Concentrated H_2O_2 , however, destroys the enzyme by denaturing the protein. The danger of this was minimized by gradual addition and thorough mixing.

30% H_2O_2 (Schering Kahlbaum *pro analysi*) was neutralized with NaOH and slowly dropped from a micro-burette, at a rate of one drop every 7–10 sec., into the dilute well-buffered (pH 6.8) catalase solution containing the ascorbic acid. The mixture was kept in a continuous swirling motion, and the drops of H_2O_2 were not allowed to fall into the froth; gentle swirling by hand was best. Occasionally octyl alcohol had to be added, but this caused some enzyme denaturation and was avoided if possible. It can be calculated that with the enzyme concentrations used all the H_2O_2 was almost completely destroyed before the next drop was added.

In the preliminary experiments, to 0.5 ml. of a catalase solution ($k = 7000$) 5 ml. of 30% H_2O_2 were added as described; temp. $< 5^\circ$. In a second experiment ascorbic acid was added (5 mg./100 ml.). Without ascorbic acid 26% of the enzymic activity was destroyed ($dC/dE = 4200$), with ascorbic acid 59% ($dC/dE = 1800$). Ascorbic acid thus increased the destruction of catalase by H_2O_2 under these conditions, but not as much as in the experiments in very dilute solutions (cf. Table 5). This suggests that in the large-scale experiments much catalase is destroyed in a different way, a way which is much less or not at all accelerated by ascorbic acid. If it is assumed that the enzyme destruction in dilute

solution in the presence of ascorbic acid is entirely due to the oxidation of protohaem to bile pigment haem and this reaction itself remained unaltered in the large-scale experiments, it can be calculated that the destruction of 60% of the activity of the enzyme in the experiment above ought to be accompanied by a significant increase in the bile pigment haem content. dC/dE in the large-scale experiment (1800) is about one-third of dC/dE in dilute solution (4700, cf. Table 5), i.e. three times as much enzyme is destroyed by the same amount of H_2O_2 in the large-scale experiments as in dilute solution, in which we assume the destruction to be due to the oxidation of protohaem to bile pigment haem. If, of the 60% total loss of activity, 20% is due to oxidation of protohaem to bile pigment haem, an original ratio of bile pigment haem to total haem of 0.40 ought to be increased to 0.52, which is far beyond the range of experimental error. The large-scale experiments were carried out at temperatures from 5 to 37° . Since the inactivation of the enzyme by H_2O_2 has a higher temperature coefficient than the catalytic destruction of H_2O_2 , higher temperatures ought to be favourable, up to the critical zone, where temperature inactivation of the enzyme becomes significant. After the addition of H_2O_2 the mixtures were worked up in the following way. The catalase activity was determined as usual. For the other estimations the catalase was concentrated by precipitation with $(NH_4)_2SO_4$. In spite of being finely dispersed the precipitate was easily collected on a Whatman no. 42 filter; the water-clear filtrate was practically inactive. The catalase was dissolved in secondary phosphate solution and dialysed at 0° in a collodion bag.

The experiments summarized in Table 6 (p. 122) show that in five of six experiments a definitely higher bile pigment haem content was found after treatment with H_2O_2 . In the one experiment in which no significant increase was found, H_2O_2 had been added more rapidly at 37° , and the fact that a smaller amount of H_2O_2 had led to the same degree of enzyme destruction (see columns 5 and 8), indicates greater denaturation of protein.

The quotient dC/dE and that part of enzyme inactivation due to the oxidation of protohaem to bile pigment haem vary considerably. This is not surprising in view of the experimental difficulties. The degree of denaturation of catalase by conc. H_2O_2 may alter according to froth formation, addition of octyl alcohol and efficiency of mixing. The ascorbic acid concentration is altered during the addition of H_2O_2 by dilution and by oxidation, and while the former was avoided by adding ascorbic acid from time to time, the degree of the latter was unknown.

We had expected that these experiments would supply further evidence that the bile pigment

haemoprotein is catalytically inactive, but no conclusive evidence was obtained. The determination of protohaem as haemochromogen is more difficult than with untreated catalase and is disturbed by cloudiness of the solution and incomplete reduction by $\text{Na}_2\text{S}_2\text{O}_4$. If the values for protohaem are correct, H_2O_2 causes a fall of the quotient $k/\text{protohaem Fe}$, i.e. some inactivation of catalase without precipitation or oxidation, and the picture is further complicated by the fact that the decrease of the specific activities of the recovered solutions is smaller than would correspond to the decrease of total activity. The latter must be explained by the denaturation and removal of inactive proteins in subsequent treatment.

No difficulties were, however, encountered in the determination of protohaem after acid splitting and of the ratio bile pigment Fe to total haem Fe. Duplicate estimations of this ratio gave results within ± 0.02 and usually ± 0.01 . With the exception of the experiment in the last row of Table 6, which may be considered as a blank, the increases of the bile pigment haem content are significant.

DISCUSSION

In earlier experiments we have found that by the action of H_2O_2 in the presence of ascorbic acid the protohaem group of haemoglobin and of haemochromogens is oxidized to bile pigment haem. Analogous reactions with a slightly modified mechanism (coupled oxidation), but of principally the same type, occur *in vivo*. They underlie the physiological formation of bile pigment from haemoglobin as well as the intracorporeal biliverdin formation by phenylhydrazine [Lemberg & Legge, 1942] and other drugs. Ox and horse liver catalases contain bile pigment haem, while ox erythrocyte catalase has been found by Laskowski & Sumner [1941] to contain protohaem alone. Does catalase undergo an oxidation *in vivo* similar to haemoglobin?

In the present paper we have shown that by the action of H_2O_2 in the presence of ascorbic acid the protohaem groups of catalase are oxidized to bile pigment haem groups. These experiments had to be carried out with unphysiologically high concentrations of enzyme and H_2O_2 but we have shown the effect of ascorbic acid on the rate of the enzyme destruction in low concentrations, and it is likely that the mechanism is the same.

The bile pigment haem content of the liver catalase can be explained in three ways: (1) it is an artefact and due to the oxidation of catalase in post-mortem changes or during its isolation from the liver; (2) bile pigment haem groups formed by the breakdown of haemoglobin in the liver are incorporated in the synthesis of catalase; or (3) catalase itself undergoes an oxidation in the liver.

With regard to the first possibility, it might be argued that the presence of larger amounts of ascorbic acid in the liver than in the red cells may explain why an oxidation does not occur during the isolation of catalase from erythrocytes. By autooxidation of ascorbic acid H_2O_2 is formed and may act together with ascorbic acid on liver catalase which was originally free from bile pigment haem. The amounts of H_2O_2 which can be formed during the isolation cannot, however, be large and appear to be insufficient to explain an oxidation of 30–50% of the prosthetic group. Even at 20° the inactivation of catalase by ascorbic acid is slow [Marks, 1936] and the isolation is carried out at 0°. Neither Sumner *et al.* [1940] nor we ourselves have found evidence supporting this assumption.

The second explanation does not appear probable if it is remembered how specific is the synthesis of haemoglobin. Porphyrins with side chains differing from those of protoporphyrin are not united with iron and globin, even though such compounds may still carry O_2 , while if the catalase haemoprotein were synthesized from bile pigment haem and catalase protein, it would be inactive.

This leaves the third hypothesis, an *in vivo* oxidation of catalase, as the most likely one. The difference between erythrocyte and liver catalases would be explained by the greater content of reducing substances in the liver cells and their greater respiration.

If this is the correct explanation, it leads to important implications. Catalase can then no longer be considered as a mere safety valve against occasional H_2O_2 formation. H_2O_2 must be destroyed by the enzyme *in vivo* on a scale which is not negligible. Even in the presence of ascorbic acid in concentrations found in the liver cells, each molecule of catalase must have destroyed at least 2×10^7 molecules of H_2O_2 before it became converted into bile pigment haemoprotein. 1000 g. of horse liver contain 0.5–1.0 g. of active protohaem catalase according to the activity of the extracts [Stern, 1935–6], which would mean the destruction of at least 500–1000 g. of H_2O_2 by 1000 g. of liver during the lifetime of the enzyme. We have not yet any definite data on the latter, but to judge from the fact that the liver catalase of a colt did not contain less bile pigment haem than that of the adult horse [Sumner *et al.* 1940] it is probably to be measured in weeks or months rather than in years.

The method of determining the bile pigment content of catalase may be used for studying the metabolism of catalase, but since large amounts of catalase are required for the estimations, other methods, e.g. with tracer elements, would be preferable.

Finally our experiments lend support to the findings of Keilin & Hartree [1938] that ferric

catalase iron is reduced to ferrous iron during its action on H_2O_2 . Only ferrous haem compounds are oxidized by H_2O_2 to bile pigment haem compounds. Ascorbic acid does not reduce catalase, but may protect the ferrous H_2O_2 -catalase compound against autoxidation.

SUMMARY

1. A simplified procedure for the isolation of crystalline horse liver catalase is described.

2. By splitting with acetic acid and extracting with ether, biliverdin and small amounts of biliverdin are obtained from crystalline ox and horse liver catalases.

3. The molar ratio of (bile pigment)/(bile pigment + haem) from impure and pure horse and ox liver catalases was found to vary from 0.3 to 0.5.

4. The CO-haemochromogens of these catalase preparations had, in addition to the absorption bands in CO-protohaemochromogen, an absorption band in the same position as that of CO-cholehaemochromogen. Spectrophotometric analysis showed that the height of this band was proportional to the bile pigment content as found by the splitting with acids, and that the same values for the ratio of

(bile pigment Fe)/(bile pigment Fe + haem Fe) were found by both methods. This shows that the bile pigments are derived from a bile pigment haemoprotein.

5. If the presence of inactive haem-free protein in the impure preparations is taken into consideration, it can be shown that the specific activity (Kat.-f.) increases with decreasing bile pigment haem content, being about 50,000 for catalase containing protohaem only, and nil for the bile pigment haemoprotein.

6. A theoretically sound method for measuring catalase activity is described, which gives also the rate of destruction of the enzyme by H_2O_2 .

7. Ascorbic acid inhibits catalase by accelerating the destruction of the enzyme by H_2O_2 .

8. By the action of H_2O_2 on catalase in the presence of ascorbic acid, bile pigment haemoprotein is formed from protohaem catalase.

9. The significance of these findings for the explanation of the bile pigment haem content in liver catalase is discussed.

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REFERENCES

- Agner, K. [1938]. *Biochem. J.* **32**, 1702.
 — [1939]. *Naturwissenschaften*, **27**, 418.
 Bick, M. [1936]. *Aust. J. exp. Biol. med. Sci.* **4**, 305.
 Keilin, D. & Hartree, E. F. [1936]. *Proc. roy. Soc. B*, **121**, 173.
 — [1938]. *Proc. roy. Soc. B*, **124**, 397.
 Laskowski, M. & Sumner, J. B. [1941]. *Science*, **94**, 615.
 Lemberg, R. [1935]. *Biochem. J.* **29**, 1322.
 — & Legge, J. W. [1942]. *Aust. J. exp. Biol. med. Sci.* **20**, 65.
 — Legge, J. W. & Lockwood, W. H. [1941a]. *Biochem. J.* **35**, 339.
 — Lockwood, W. H. & Legge, J. W. [1941b]. *Biochem. J.* **35**, 363.
 — Norrie, M. & Legge, J. W. [1939]. *Nature, Lond.*, **144**, 551.
 Lemberg, R. & Wyndham, R. H. [1936-7]. *J. roy. Soc. N.S.W.* **70**, 343.
 Marks, G. W. [1936]. *J. biol. Chem.* **115**, 299.
 Maximowitsch, S. M. & Awtonomova, E. S. [1928]. *Hoppe-Seyl. Z.* **174**, 233.
 Nosaka, N. [1927]. *J. Biochem., Tokyo*, **8**, 301.
 Stern, K. G. [1935-6]. *J. biol. Chem.* **112**, 661.
 — [1937]. *J. biol. Chem.* **121**, 561.
 — [1940]. *Ann. Rev. Biochem.* **9**, 20.
 Sumner, J. B. & Dounce, A. L. [1937]. *J. biol. Chem.* **121**, 417.
 — [1939]. *J. biol. Chem.* **127**, 439.
 — & Frampton, V. L. [1940]. *J. biol. Chem.* **136**, 343.
 Theorell, H. [1940]. *Ann. Rev. Biochem.* **9**, 666.
 Zeile, K. [1933]. *Ergebn. Physiol.* **35**, 531.
 — & Hellström, H. [1930]. *Hoppe-Seyl. Z.* **194**, 171.

The Technique of the Biological Vitamin A Assay

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The publications of Burn [1937], Irwin [1937], Coward [1938] and Bliss & Marks [1939], to cite a few of the more important papers, testify to the existence of a good measure of agreement on the

general principles of the graded-response biological assay. At the same time assays of any particular substance usually necessitate the use of some *ad hoc* technique. The rat-growth (curative) vitamin A