spectroscope the narrow width of the absorption band in the red led to an overestimate of its intensity. Others have also been misled by the narrowness of the first absorption band; thus Grinstein, Schwartz & Watson (1945) describe the relative intensities of the absorption bands of coproporphyrin I as IV, III, I and II in decreasing order.

The eluate containing the second porphyrin zone was evaporated to dryness, and after recrystallization from chloroform and methanol gave 7·1 mg. of the typical hair-like crystals of uroporphyrin I octamethyl ester (m.p. 288°). In chloroform, this ester showed absorption bands at 625·7, 576·7, 536·5 and 503·6 mμ., intensity IV>III>II>I (Hartridge reversion spectroscope). The third porphyrin zone has not yet been identified.

DISCUSSION

The isolation of coproporphyrin III ester in relatively enormous quantities in the large-scale production of purified diphtheria toxoid provides an abundant and hitherto unexplored source of coproporphyrin III, which will be of the greatest value in the investigation of the metabolic relationships of this material in the intact animal. The production of small quantities of uroporphyrin I provides yet another example of the so-called dualism of the porphyrins, attention to which was first drawn by Fischer (1937). In the light of present knowledge it is not possible to speculate on the significance of this dualism to the economy of the organism. The implications suggested by Pappenheimer (1947) regarding the relationship of the production of porphyrin to toxin formation will be the subject of a separate paper to be published elsewhere.

SUMMARY

Coproporphyrin III tetramethyl ester in large yield and uroporphyrin I octamethyl ester in very small yield have been prepared from toxic culture filtrates of *Corynebacterium diphtheriae*.

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Crystalline Bacterial Catalase*

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(*Received* 13 *January* 1948)

In recent years, the trend of enzyme research has shifted from the study of the reactions catalyzed to a study of the chemical nature of the enzymes themselves. This has led in the last fifteen years to the isolation of some forty enzymes in a crystalline or highly purified state, and in many cases to the identification of their prosthetic groups, resulting in a completely new outlook on enzyme chemistry.

Little progress, however, has been made in the study of bacterial enzymes from this standpoint, and up to the present there has not been recorded the isolation of a single bacterial enzyme in a pure state. This is the more regrettable since many interesting enzymes exist in bacteria which have not been found elsewhere, and some bacterial enzymes at least (e.g. lactic dehydrogenase, cytochromes a_1 and a_2) are known to differ greatly from their counterparts in animal tissues. This relative neglect of bacterial enzymes is in part due to purely technical reasons, namely, the difficulties involved in growing the large quantities of bacteria required, and the problem of liberating endo-enzymes from the bacterial cell. The first of these problems is well on its way to solution, but the second is more difficult. Most of the techniques hitherto used for destroying the cell wall and liberating intracellular enzymes (for example, autolysis, vacuum- or acetone-drying followed by extraction, shaking with glass beads (Curran & Evans,

* A preliminary account of part of this work appeared in *Nature, Lond.*, **160**, 125 (1947).

1942) or grinding with powdered glass (Kalnitsky, Utter & Werkman, 1945), the roller-crushing mill (Booth & Green, 1938), ultrasonic disintegration (Stumpf, Green & Smith, 1946, and others)) either tend to destroy labile enzymes, are difficult to employ on a large scale, or require specialized apparatus.

This paper describes the use of a relatively little-used method of liberating enzymes from bacterial cells, namely, lysis of the bacteria with lysozyme. This substance, which is now easily prepared in crystalline form from egg white, rapidly brings about smooth and complete lysis of susceptible bacteria; no specialized apparatus is required, and the method can be employed on any scale. Penrose & Quastel (1930), Quastel (1937) and Epps & Gale (1942) have used lysozyme to determine the true enzyme content of bacterial cells grown under different conditions, but it has not previously been used as an aid to enzyme purification.

The present paper describes the application of this method to the isolation of catalase from *Micrococcus lysodeikticus*. Catalase was one of the first enzymes to be described in bacteria (Gottstein, 1893) and much work has been done on it since, but no attempts have been made to isolate it in a pure state or determine its chemical nature. Using lysozyme to liberate the enzyme from the bacteria, we have been able to isolate *M. lysodeikticus* catalase as a pure, crystalline protein. As far as we are aware, this is the first bacterial enzyme to have been crystallized.

Bacterial catalase is on the whole very similar to the catalases that have been isolated from mammalian tissues, but there are certain differences. It is a conjugated protein with haematin as the prosthetic group, but unlike liver catalase it contains no verdohaematin. The protein part of the molecule has the same molecular weight as liver catalase protein, but differs in its resistance to organic solvents and low pH. The catalytic activity of the bacterial enzyme is considerably higher than that of blood or liver catalases; this difference also is to be attributed to the protein part of the molecule. Finally, it differs from other catalases in its crystalline form.

METHODS

Bacterial suspensions. The strain of *M. lysodeikticus* used (National Collection of Type Cultures no. 2665) was a descendant of that originally isolated by Fleming (1922). For large-scale production, the bacteria were grown on C.C.Y.-lactate agar (Gladstone & Fildes, 1940) in enamelled trays (photographic developing dishes) measuring 11 × 16 in., with overlapping lids, each containing 500 ml. of agar. Three ml. of inoculum, prepared by suspending the 24 hr. growth from a Roux bottle in 50 ml. of saline, was spread evenly over the surface of each tray with a glass spreader, and the trays incubated at 35°. Growth was almost complete in 24 hr., but the catalase activity of the bacteria continued to increase up to 40 hr., which was therefore adopted as the standard time for incubation. The bacterial growth was then removed from the agar surface with a scraper. The average yield was *c.* 1·5 g. bacteria (dry wt.)/tray.

Estimation of catalase activity. The activity of catalase preparations was measured essentially according to Euler & Josephson (1927). Suitably diluted catalase solution (1 ml., containing *c.* 0·3 μg. of pure enzyme) is added to 49 ml. of 0·015 N-H_2O_2 in 0·015 M-phosphate buffer of pH 6·8, kept at 0° in an ice-water bath. Five ml. are immediately withdrawn, pipetted into 5 ml. of 2 N-H_2SO_4, and titrated with 0·01 N-$KMnO_4$. Further samples are withdrawn and titrated after 3, 6, 9 and 12 min. For each time interval, the first-order velocity constant is calculated as $k = \frac{1}{t} \log_{10} \frac{a}{a-x}$, where a is the initial $KMnO_4$ titre and $a-x$ the titre after t min.

Under these conditions the reaction is approximately first order but, as with catalase of plant and animal origin, significant destruction of the enzyme by the H_2O_2 occurs, causing falling values of k. Following Sumner (1941), we plot k against t and determine k_0, the value of k at zero time, by extrapolation. The amount of catalase taken should be such that k_0 falls between 0·025 and 0·04 min.$^{-1}$; the value of k_0 is then directly proportional to the amount of enzyme taken.

Catalase solutions always have to be highly diluted for testing; the activity of the undiluted solution is calculated by multiplying the value of k found in the test by the overall dilution factor; e.g. if 1 ml. of a 1/10,000 dilution gives a k value of 0·03 in the test, then k for the undiluted solution is $0{\cdot}03 \times 50 \times 10{,}000 = 15{,}000$. The purity of an enzyme preparation is expressed according to Euler & Josephson (1927) as $Kat.\text{-}f. = \frac{k}{\text{g. enzyme in 50 ml.}}$, or under the above test conditions, $Kat.\text{-}f. = \frac{k \text{ in test}}{\text{g. enzyme in test}}$.

To obtain reliable results by this method, it is essential that all glassware should be cleaned with H_2SO_4-$K_2Cr_2O_7$, and that the very dilute enzyme solutions necessary for the test should be used immediately after diluting. Even so, we consider the error to be about 5%. All measurements reported in this paper are the means of duplicates.

Dry weights. Well-dialyzed enzyme solutions, or bacterial suspensions thoroughly washed in distilled water, were dried to constant weight at 105° and weighed on a micro-balance. The colorimetric method of Pressman (1943) was used as a rapid, rough method of determining the protein content of enzyme fractions; all measurements reported, however, are based on direct weighings. Dry weights of bacterial suspensions were as a routine measured turbidimetrically using a Hilger photoelectric absorptiometer previously calibrated with suspensions of known dry weight.

Haemin estimations. The method chiefly used was the pyridine-haemochromogen method of Keilin & Hartree (1936) and Rimington (1942). Results were occasionally checked by the cyanhaematin method of King & Gilchrist (1947); the two methods gave identical results.

Crystalline lysozyme. This was initially prepared from egg white by bentonite adsorption according to Alderton, Ward & Fevold (1945). Once a supply of seed crystals had been prepared, subsequent batches were made by direct crystallization from egg white (Alderton & Fevold, 1946); this method was found very simple and reliable.

Absorption spectra were observed with a Hartridge reversion spectroscope (calibrated with a neon lamp), and a Beckman spectrophotometer.

RESULTS

Action of lysozyme on Micrococcus lysodeikticus *suspensions*

When a dilute suspension (say 1 mg./ml.) of *M. lysodeikticus* in 0·5 % saline is treated with a small amount of lysozyme, the turbidity rapidly disappears leaving an almost water-clear solution in which no intact bacteria are observable microscopically; the phenomenon has been described in detail by Fleming (1922, 1929) and subsequent workers.

An interesting finding is that the catalase activity of *M. lysodeikticus* suspensions invariably increases after lysis with lysozyme, usually by about tenfold. For example, a typical suspension gave the following results (tested at 30°):

	Bacteria in test (mg.)	*k*	*Kat.-f.*
Before lysis	0·424	0·0382	89
After lysis	0·071	0·0606	854

The same effect is observed if the bacteria are disintegrated by shaking with glass beads. Similar results were observed by Penrose & Quastel (1930), and Krampitz & Werkman (1941) found that intact *M. lysodeikticus* suspensions had no action on oxaloacetic acid, whilst lyzed or acetone-dried bacteria rapidly decarboxylated this substance. The simplest explanation is that diffusion of substrate into the cell is a limiting factor.

Fleming (1929) noted that high salt concentrations inhibit lysis by lysozyme. We find also that in the total absence of salts (bacteria washed with distilled water and treated with dialyzed lysozyme solutions) lysis is almost completely inhibited for prolonged periods. The optimal salt concentration is about 0·5 % for NaCl and 0·02–0·05 M for phosphate buffers; under these conditions, 1 mg. of crystalline lysozyme is more than sufficient to bring about complete lysis of 1 g. of *M. lysodeikticus* in 1 hr. at 30°.

Previous workers on lysozyme have only studied its action on quite dilute bacterial suspensions. When large amounts of bacteria in thick (1–8 % dry weight) suspension are lyzed, interesting new phenomena are observed. When acted on by lysozyme, such suspensions of *M. lysodeikticus*, which are originally canary yellow and completely opaque, rapidly become greenish yellow and semi-transparent though not completely water-clear; the residual turbidity is due to the high concentration of 'ghosts'. As these changes take place the initially mobile suspension becomes highly viscous and slimy. The solution produced by lysis of a 1 % bacterial suspension has about the consistency of egg white, while an 8 % solution is a semi-solid gel which cannot be pipetted or poured out of a test-tube; a 4 % solution is the strongest that can conveniently be handled. Besides being highly viscous, such solutions have marked elastic properties. They are readily drawn out into threads, and on pouring from a measuring cylinder emerge as an elongated 'blob' which slowly descends while the cylinder is tilted and runs back into the cylinder if the latter is restored to an upright position. When caused to flow along a 2 mm. horizontal capillary under pressure (in an apparatus similar to that used by Scott Blair, Folley, Malpress & Coppen (1941) for testing the flow-elasticity of bovine cervical mucus), they show marked recoil when the pressure is suddenly released.

The chemical nature of this viscous substance or substances is unknown, nor is it known whether they are initially present inside the bacterial cell and released from it by lysis, or formed by the action of lysozyme; it is hoped to investigate these problems in the future. The phenomenon is not peculiar to *M. lysodeikticus*, similar viscous substances being formed when other sensitive bacteria are treated with lysozyme.

Isolation of crystalline catalase from lysed bacteria

The essentials of the purification method we have adopted are as follows. The solution of lyzed bacteria is treated with 0·5 vol. of ethanol at pH 5·6, which precipitates the viscous substances formed on lysis, and then shaken with chloroform, which denatures considerable quantities of inert proteins. The aqueous-ethanolic solution is then treated with solid ammonium sulphate which, with correct proportions of the three components, causes it to separate into two liquid phases, one containing all the catalase while the other contains most of the contaminating proteins. This 'partition' method is, as far as we know, a novel procedure in protein purification, and may well be applicable to other problems.

After repetition of the above process, two fractionations with ammonium sulphate bring the enzyme to 70–80 % purity, when it can be crystallized either by prolonged dialysis or by careful addition of ammonium sulphate.

The following are the details of a typical preparation:

Stage 1. The bacterial growth from 156 trays (78 l. of medium) was harvested, suspended without washing in *c.* 3 l. of 0·5 % NaCl, and strained through muslin to remove flakes of agar. Turbidimetric estimation showed that the total dry weight of bacteria was 203 g., and 0·5 % NaCl was added to make the final concentration of the suspension 4 % on a dry-weight basis (5085 ml.). Crystalline lysozyme was then added (1 mg./g. bacteria) and the suspension incubated 1 hr. at 30°, when lysis was complete. *Kat.-f.* of lyzed suspension = 910.

Stage 2. The highly viscous lyzed suspension was treated successively with 509 ml. of M-acetate buffer of pH 5·6, and 2800 ml. of ethanol (final ethanol concentration 33·3% v/v). A bulky yellow gelatinous precipitate was centrifuged off, washed with 2500 ml. of M/15 acetate (pH 5·6) containing 33·3% ethanol, and the washings added to the original supernatant fluid. This gave 8180 ml. of a pale yellow mobile liquid; *Kat.-f.* = 4020. (All the above operations were carried out at 0°; this is not absolutely essential, but gives better yields.)

Stage 3. The above liquid was treated with 1820 ml. of chloroform, shaken on a fast mechanical shaker for 15 min. and centrifuged, when two liquid layers formed with a thick layer of denatured protein at the interface. The top layer (7610 ml.) was siphoned off; *Kat.-f.* = 5200.

Stage 4. The top layer from the last stage was treated with $\frac{1}{20}$ vol. of M-sodium acetate and 2400 g. of solid $(NH_4)_2SO_4$ added (30 g. to each 100 ml.). This caused the separation on standing of two liquid layers, the lower containing most of the $(NH_4)_2SO_4$ and the upper most of the ethanol, some $(NH_4)_2SO_4$ and water. The smaller top layer (2760 ml.) was pale brown and contained all the catalase, whose characteristic absorption band at 631 mμ. could now be seen with a hand spectroscope; *Kat.-f.* = 15,650. (It is essential for the success of this step that the proportions of aqueous solution, ethanol and chloroform specified in stages 2 and 3 should be adhered to; otherwise two layers may not separate, or the catalase may be precipitated at the interface. The sodium acetate is added to keep the pH at about 5·6 on addition of the $(NH_4)_2SO_4$.)

Stage 5. The top layer from the above stage was shaken for 15 min. with an equal volume of chloroform, allowed to stand, and the top layer (1890 ml.) removed. (Comparatively little inert protein is removed by this step; the main purpose is to reduce the ethanol concentration.) Solid $(NH_4)_2SO_4$ was now added (23 g. to each 100 ml.) when two layers again separated. The top layer was much the smaller, and dark brown; it was removed in a separating funnel and dialyzed against running tap water overnight; final volume 600 ml., *Kat.-f.* = 20,200.

(Stages 4 and 5 bring about a fourfold purification and decrease the volume to $\frac{1}{15}$; this concentration is important for the subsequent $(NH_4)_2SO_4$ fractionation which is less effective if the protein concentration is too low.)

Stage 6. The dialyzed solution from stage 5 was brought to pH 5·6 by the addition of $\frac{1}{20}$ vol. of M-acetate buffer, and roughly fractionated by adding successive portions of solid $(NH_4)_2SO_4$ and centrifuging off the resulting precipitates. Fraction 6*a* (obtained with 1·96M-$(NH_4)_2SO_4$) which was greyish white and contained little catalase, was rejected. Fractions 6*b*, *c* and *d*, taken off at 2·27, 2·38 and 2·52M-$(NH_4)_2SO_4$, were brown and contained most of the catalase, little being left in the final supernatant. These three fractions were combined and dissolved in M/20 acetate pH 5·6 to give a volume of 61 ml., *Kat.-f.* = 40,000.

Stage 7. The combined fractions *b*, *c* and *d* were now carefully fractionated by dropwise addition of a 4M-$(NH_4)_2SO_4$ solution adjusted to pH 5·6 with NH_4OH. (It was found impossible to standardize these fractionations completely. The exact $(NH_4)_2SO_4$ concentration at which catalase begins to precipitate is markedly affected by the catalase concentration, the temperature and probably other factors. It is necessary to proceed empirically, adding the $(NH_4)_2SO_4$ drop by drop and with good stirring, taking off fractions at intervals. Fortunately, the colour of the catalase makes it easy to determine its distribution in the precipitates.) Three successive precipitates were taken off, after which little catalase remained in the supernatant fluid. The smaller 1st and 3rd precipitates were visibly paler than the 2nd, and obviously contained colourless protein impurities. The 2nd dark brown precipitate contained the bulk of the catalase and was dissolved in M/20-acetate pH 5·6 to give a volume of 63 ml., *Kat.-f.* = 62,900.

Stage 8 (*crystallization*). The solution resulting from the last stage was crystallized in three different ways. (i) A portion (10 ml.) of the solution was dialyzed against repeated changes of distilled water at 0°. After 5 days (longer than this may be necessary) it had mostly crystallized. (ii) Another 10 ml. portion of the solution was cautiously treated with 4M-$(NH_4)_2SO_4$ until the faintest turbidity appeared. The solution was allowed to stand at room temperature, and by the next day it had almost completely crystallized, leaving a nearly colourless mother liquor. (iii) The rest of the solution (43 ml.) was treated with just enough 4M-$(NH_4)_2SO_4$ to precipitate all the catalase. The precipitate was centrifuged down and redissolved by adding distilled water drop by drop, very slowly and with good stirring, until all but a small portion had dissolved; this was centrifuged off. The supernatant fluid was then saturated with amorphous catalase; it was allowed to stand at room temperature, and most had crystallized within 24 hr. (iii*a*). A smaller second crop of crystals (iii*b*) was obtained by adding a few drops of $(NH_4)_2SO_4$ to the mother liquor of the 1st crop. The four batches of crystals were separately dissolved in M/15-phosphate buffer pH 6·8, well dialyzed, and tested; *Kat.-f.* = 90,000–98,000, which is the highest value we have obtained. There is thus a considerable increase in purity on crystallization.

The yields and purities at each stage of the isolation procedure are shown in Table 1.

Table 1. *Purification of bacterial catalase*

Stage	Volume (ml.)	Total protein (g.)	Total catalase (g.)		*Kat.-f.*
1. Lyzed bacteria	5,085	203	1·94		910
2. Ethanol supernatant	8,180	40·2	1·69		4,020
3. Chloroform supernatant	7,610	29·5	1·60		5,200
4. First ethanol-$(NH_4)_2SO_4$ partition	2,760	8·9	1·46		15,650
5. Second ethanol-$(NH_4)_2SO_4$ partition	600	5·4	1·14		20,200
6. First $(NH_4)_2SO_4$ fractionation	61	2·1	0·90		40,000
7. Second $(NH_4)_2SO_4$ fractionation	63	0·735	0·485		62,900
8. Crystals: (i)	—	0·050	0·050		93,300
(ii)	—	0·065	0·065	0·376	98,000
(iii*a*)	—	0·206	0·206		99,000
(iii*b*)	—	0·055	0·055		90,000

Properties of the crystalline enzyme

General properties. Solutions of the pure enzyme have a red-brown colour resembling that of methaemoglobin. Strong solutions (1 % or over) are stable for many weeks at 0°, or even at room temperature, if bacterial contamination is avoided, but very dilute solutions such as are used in *Kat.-f.* determinations lose their activity fairly rapidly, and must be tested immediately. In this they resemble liver catalase (Sumner & Dounce, 1937).

Crystalline form. The pure enzyme crystallizes as regular octahedra, which are isotropic when viewed between crossed polaroids; they have the same form, however the enzyme is crystallized (Pl. 3). In this respect bacterial catalase differs from beef-liver catalase, which according to Sumner & Dounce (1937) may crystallize as needles, plates or prisms, according to the conditions of crystallization.

Prosthetic group. Bacterial catalase has a higher activity (*Kat.-f.*) than catalases from other sources (Tables 1 and 3). It is important to discover whether this is to be attributed to differences in the protein

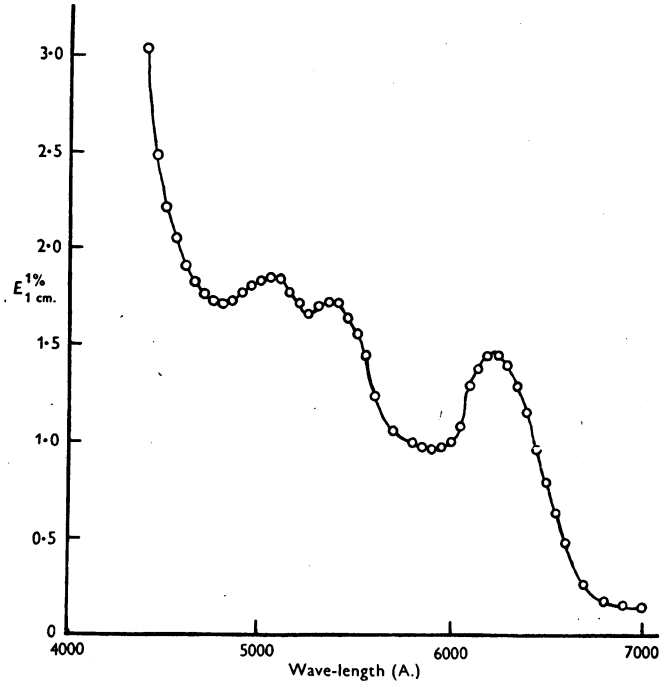


Fig. 1. Absorption spectrum of bacterial catalase. Crystalline enzyme (*Kat.-f.* = 99,000) in M/15 phosphate pH 6·8.

Bacterial catalase is fairly stable at high pH values (e.g. in 0·1N-ammonia) but much less stable to acid pH. Horse liver and erythrocyte catalase are stable down to pH 3·2 (Agner & Theorell, 1946), but bacterial catalase is instantly denatured and precipitated at pH 4·0, and fairly rapidly at pH 4·6. Hence, treatment with acetate buffer pH 3·8, which Bonnichsen (1947) used effectively for purifying mammalian catalases, cannot be employed for purifying the bacterial enzyme. Dioxan, which Sumner & Dounce (1937, 1939) used to purify liver catalase, denatures bacterial catalase very rapidly at room temperature and still rapidly at 0°; we were able to obtain fairly pure preparations by Sumner's method working at 0°, but only in very low yields.

Strong solutions of bacterial catalase have a characteristic absorption spectrum (Fig. 1), showing three bands centred at 506, 545 and 631 mμ. (in M/15 phosphate pH 6·8), which are close to the values reported for other catalases. The exact positions and intensities of the bands are affected both by the pH and the nature of the buffer anion (cf. Agner, 1942). These data are not always recorded in the literature; it is uncertain, therefore, whether the slight spectroscopic differences observed between different catalases are apparent or real.

On treatment with pyridine and sodium hyposulphite ($Na_2S_2O_4$) in 0·1N-NaOH bacterial catalase

gives a typical haemochromogen spectrum, and it forms characteristic compounds with cyanide and azide. Neither azide-catalase nor cyanide-catalase, nor catalase itself, is reduced by hyposulphite. Azide-catalase forms a characteristic pink compound with hydrogen peroxide, which gradually reverts to the original spectrum as the hydrogen peroxide decomposes. This behaviour is exactly similar to that of liver catalase as described by Keilin & Hartree (1937), and the absorption bands of the compounds have similar positions (Table 2).

at 502, 547 and 644 mμ., identical with those of haemin in this solvent. It contained no detectable trace of the 'blue pigment' (biliverdin) that is produced when liver catalases are treated in a similar way (Sumner & Dounce, 1939). On evaporating off the acetone *in vacuo*, the haemin precipitated, leaving a colourless supernatant fluid. The haemin was centrifuged off, washed with water, and dissolved in 10 ml. of dilute Na_2CO_3. Part of this alkaline haematin solution was reserved for conversion to the cyanide- and pyridine-haemochromogen derivatives; the remainder was immediately coupled with a *c.* 1·5% solution of globin prepared from human-blood haemoglobin by the method of

Table 2. *Absorption bands of catalase and catalase-haematin derivatives*

(Positions of the absorption bands, determined with the Hartridge reversion spectroscope, are given in mμ.)

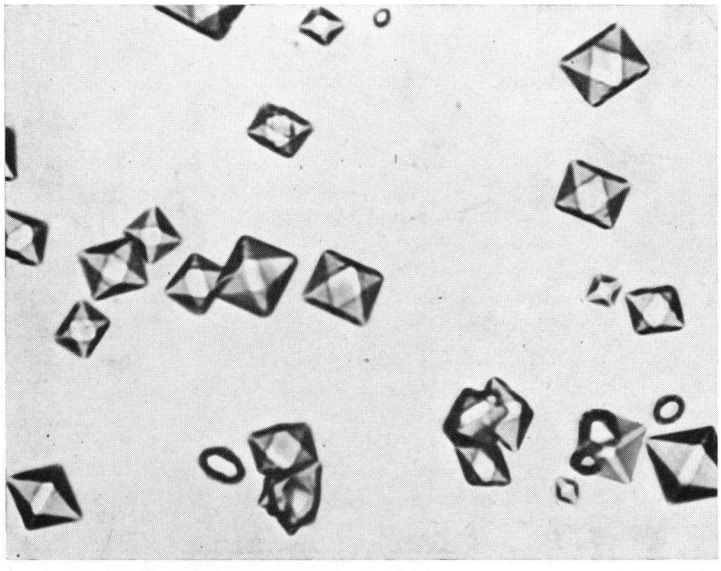
	Catalase derivatives	
	M. lysodeikticus catalase (this paper)	Horse-liver catalase (Keilin & Hartree, 1937)
Catalase	506, 545, 631·5	506·5, 544, 629·5
Azide-catalase	504, 541, 623	506·5, 544, 624·5
Azide-catalase—H_2O_2	548, 588	547, 588
Cyanide-catalase	555·5, 592	556·5, 595·5
	Haematin derivatives	
	Bacterial catalase haematin	Blood haematin
Haematin (in ether-acetic acid)	505, 545, (584), 638	506, 544·5, (585), 637
Cyanhaematin	547	547
Reduced cyanhaematin	537, 578·5	536·5, 578
Pyridine haemochromogen	525, 557	526, 557
	'Resynthesized haemoglobin' derivatives	
	From globin + bacterial catalase haematin	From globin + blood haematin
Methaemoglobin (alkaline)	542, 575·5, 603·5	541·5, 575·5, 603
Haemoglobin	557	560
Oxyhaemoglobin	540, 577·5	540·5, 577
CO-haemoglobin	536·5, 572	537, 572

The above facts strongly suggest that the prosthetic group of bacterial catalase is haematin, but further evidence is needed. This was obtained by splitting off the prosthetic group with acetone-hydrochloric acid and coupling the isolated pigment to globin, when it formed methaemoglobin which could be converted to reduced haemoglobin, oxyhaemoglobin and carboxyhaemoglobin, identical with the corresponding derivatives formed from globin and pure haemin. This is proof that the prosthetic group of bacterial catalase actually is haematin; the same experiment also showed that it contains no verdohaematin or similar substance giving rise to biliverdin on treatment with acetone-hydrochloric acid. Details are given below:

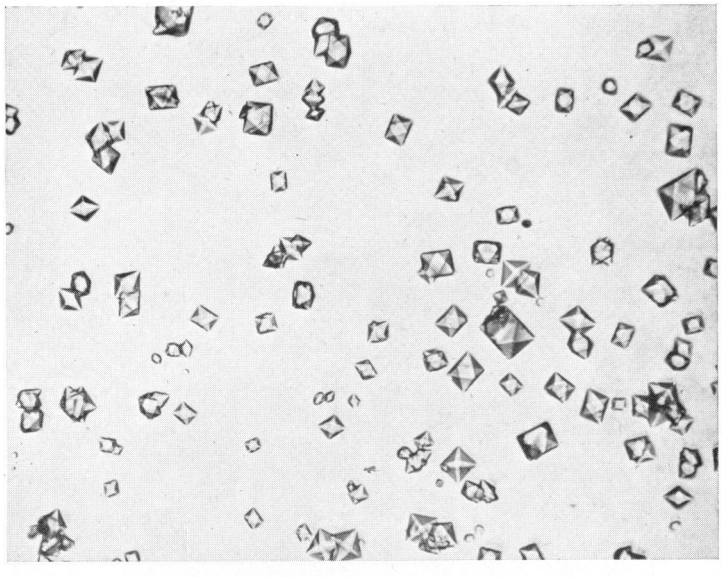
Bacterial catalase (15 ml. of 1·5%) was run into 300 ml. of pure acetone containing 5 ml. of conc. HCl. The flocculent protein precipitate was filtered off and washed with acetone-HCl, the washings being added to the original filtrate. The washed protein precipitate was pure white. The acetone-hydrochloric acid filtrate was brown, showing diffuse bands

Anson & Mirsky (1929–30). The globin solution (which was free from denatured globin) was added drop by drop to the solution of catalase haematin until visual and spectroscopic observation showed that all of the latter had coupled with the globin; the solution then showed the absorption bands of methaemoglobin. This was reduced with the minimum quantity of Stokes's reagent (1% $FeSO_4$ in 2% tartaric acid) required to convert it to reduced haemoglobin. On shaking vigorously with air this was converted to oxyhaemoglobin, which was further converted to CO-haemoglobin by saturation with CO. The same series of compounds was formed from the same globin solution by coupling with a freshly prepared solution of recrystallized blood haemin; the absorption bands of both series, and of the other catalase haematin derivatives, are shown in Table 2. The spectra of the two series of haemoglobin derivatives were identical within the errors of reading (Table 2).

For comparison, samples of purified horse-liver catalase and crystalline human-blood catalase were treated with acetone-hydrochloric acid in the same way. The blood catalase behaved exactly like the bacterial catalase, giving a brown acetone solution



(*a*) Crystallized from $(NH_4)_2SO_4$; ×500.



(*b*) Crystallized by dialysis; ×200.

Crystalline catalase from *Micrococcus lysodeikticus*.

containing, as far as could be ascertained, only haematin. The liver catalase, however, gave a bright blue acetone solution, containing both haematin and biliverdin, as Sumner & Dounce (1939) have described. This could be detected with even a few drops of a 1·5% liver catalase solution; bacterial catalase contains none of the substance (probably verdohaematin, see Lemberg & Legge, 1943) that gives biliverdin on treatment with acetone-hydrochloric acid.

Solutions of bacterial catalase and blood catalase are identical in colour to the naked eye, while horse-liver catalase is distinctly greener in colour, and can be seen spectroscopically to have a higher end-absorption in the red. This difference in colour is almost certainly due to the presence of verdohaematin in the liver catalase, and its absence in the other two catalases.

Catalytic activity, haematin content and molecular weight

Table 3 records the *Kat.-f.* and haematin content of crystalline bacterial catalase preparations compared with those of other catalases, and also the ratio *Kat.-f.*/percentage of haematin. Evidently this ratio should be independent of the purity of the catalase preparation provided that impurities present (*a*) contain no haematin, (*b*) have no effect on the activity of the enzyme. Fig. 2 shows *Kat.-f.* plotted against the percentage of haematin for bacterial catalase preparations of varying degrees of purity, the lowest

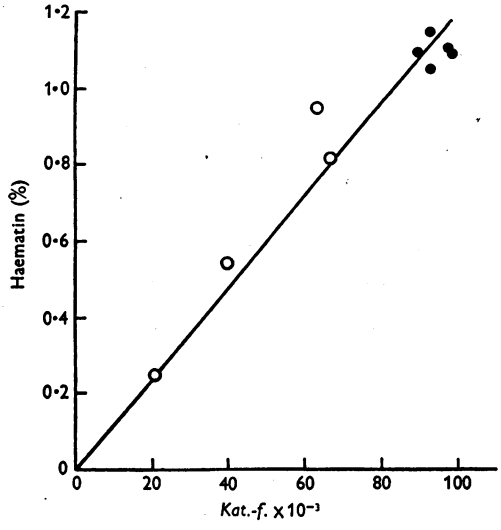


Fig. 2. Relation between haematin content and *Kat.-f.* for bacterial catalase at different stages of purification. Crystalline preparations, ●; amorphous, ○.

only 20% pure. The points are well fitted (within the limits of error) by a straight line passing through the

Table 3. *Catalytic activity and haematin content of bacterial catalase compared with liver and erythrocyte catalases*

Source	*Kat.-f.*	Haematin (%)	Bile pigment (%)	*Kat.-f.*/% of haematin	Sedimentation constant	References
			Liver catalases			
Ox	30,000–35,000	0·46–0·54	0·49–0·55*	57,000–67,000	$11·2 \times 10^{-13}$	Sumner & Dounce (1939); Sumner & Gralén (1938)
Horse	25,000–40,000	0·41–0·68	0·37–0·62*	58,000–61,000	—	Sumner, Dounce & Frampton (1940)
	34,000–48,000	0·67–0·92	+	50,000–53,000	—	Keilin & Hartree (1945*a*)
	50,000	0·89–0·91	+	55,000–56,000	$11·3 \times 10^{-13}$	Bonnichsen (1947), Agner (1938)
			Erythrocyte catalases			
Ox	48,000	?	Nil	?	—	Laskowski & Sumner (1941)
Horse	65,000–70,000	1·042	Nil	62,000–67,000	—	Agner & Theorell (1946)
	65,000	1·08	Nil	60,000	—	Bonnichsen (1947)
Human	50,000	0·83	Nil	60,300	—	Bonnichsen (1947)
	63,000	1·15	Nil	54,700	$11·26 \times 10^{-13}$	Herbert & Pinsent (1948); Cecil & Ogston (1948)
			Bacterial catalase			
M. lysodeikticus	93,300†	1·042	Nil	89,500	—	This paper
	98,000‡	1·103	Nil	89,000	—	This paper
	99,000§	1·096	Nil	90,000	$11·0 \times 10^{-13}$	This paper; Cecil & Ogston (1948)
	90,000‖	1·096	Nil	82,200	—	This paper

* From 'bile pigment Fe' analyses.
† Crystallized by dialysis, stage 8 (i).
‡ Crystallized by $(NH_4)_2SO_4$, stage 8 (ii).
§ Crystallized by $(NH_4)_2SO_4$, stage 8 (iii*a*).
‖ Crystallized by $(NH_4)_2SO_4$, stage 8 (iii*b*).

origin. This shows that our preparations are not contaminated with other iron-porphyrin proteins.

Table 3 shows that the haematin content of our preparations is the same as those recorded by other workers for blood catalases and higher than those reported for liver catalases; the *Kat.-f.*, however, is *c.* 50% higher than the most active catalases of animal tissues, the average value being *Kat.-f.* = 95,000. Similarly, the ratio *Kat.-f.*/percentage of haematin is higher, being *c.* 88,000. To make certain that this is not due to any error in our analytical methods, we prepared crystalline blood catalase by a new method (Herbert & Pinsent, 1948),

Cecil & Ogston (1948) have examined our crystalline preparations in the ultracentrifuge and found them to consist essentially of a single homogeneous protein with sedimentation constant $11{\cdot}0 \times 10^{-13}$ (Table 3). In the best preparation examined in the ultracentrifuge, the catalase accounted for 85% of the sedimenting material. As the error of this determination is *c.* 5%, we may conclude that our preparations were essentially homogeneous. The haematin content and the agreement between the chemical and ultracentrifugal calculations of the molecular weight lead us to suspect that the small amount of impurity apparently present is probably denatured catalase. If this is so, then the activity

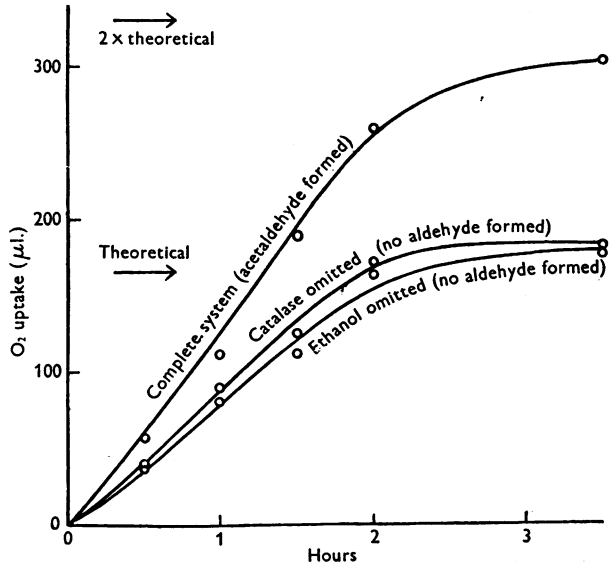


Fig. 3. Coupled oxidation of ethanol. Warburg manometers contained xanthine oxidase 8 mg., hypoxanthine 1 mg., ethanol 0·1 ml., crystalline bacterial catalase 0·8 mg., M/20 phosphate pH 7·2, total vol. 3 ml. Temp. 30°, gas phase air.

and determined its activity and haematin content in parallel with determinations on bacterial catalase. Any systematic errors in our *Kat.-f.* and haematin determinations would apply equally to both enzymes. In fact our values for blood catalase (Table 3) are in good agreement with those of other workers. We concluded, therefore, that bacterial catalase actually has a considerably higher activity, whether measured on a dry weight or on a haematin basis, than the catalases of mammalian tissues. Since the prosthetic groups are the same, the difference must be attributed to the protein component of the enzyme.

Assuming our crystalline preparations to be pure, the mean haematin content of 1·09% corresponds to a molecular weight of $58{,}000 \times n$, where n is the number of haematin groups/mol. Assuming $n = 4$, as for catalases of mammalian tissues, this gives a value for the molecular weight of 232,000.

of the completely pure enzyme may be some 10% higher than the values given above.

Once the molecular weight and *Kat.-f.* of the pure enzyme are known, it is possible to calculate the 'Turnover Number', defined by Warburg & Christian (1933) as the number of molecules of substrate decomposed by one molecule in 1 min. The relation between Turnover no. and *Kat.-f.* is obtained as follows. The initial velocity is obtained from the velocity constant k_0 by the first-order reaction equation $\left(\frac{-dS}{dt}\right)_{t=0} = 2{\cdot}303\ k_0 S_0$. By definition, $Kat.\text{-}f. = \frac{k_0}{\text{g. catalase/50 ml.}} = 20\,k_0/EM$, where S_0 and S are the concentrations of hydrogen peroxide at 0 and t min., and E is the concentration of catalase (all in mol./l.), and M is the molecular weight of catalase.

Hence,

$$\text{Turnover no.} = \frac{1}{E}\left(\frac{-dS}{dt}\right)_{t=0} = Kat.\text{-}f. \times 0{\cdot}115\, MS_0.$$

For bacterial catalase of mol. wt. = 232,000 and $Kat.\text{-}f.$ = 95,000, under the test conditions (0·0075 M-H_2O_2 at 0°), this gives a Turnover no. of 19×10^6, the highest recorded for any enzyme.

By similar calculations, the relation between $Kat.\text{-}f.$ and the Q_{O_2} (μl. O_2 evolved/mg. of enzyme/hr.) is given by

$$Q_{O_2} = Kat.\text{-}f. \times 77{,}500\, S_0.$$

Under the test conditions, bacterial catalase has a Q_{O_2} of 55×10^6. (The activity of catalase is usually measured at low hydrogen peroxide concentrations, when the percentage of total enzyme combined with substrate is small and proportional to the substrate concentration, so that the reaction is first order. Under these conditions k and $Kat.\text{-}f.$ are independent of the initial substrate concentration, but the Turnover no. and Q_{O_2} are directly proportional to the hydrogen peroxide concentration, which should always be stated.)

Coupled oxidation of alcohols

Keilin & Hartree (1936, 1945*b*) have shown that if catalase and ethanol are added to any enzyme system which produces hydrogen peroxide, the latter is used by the catalase to bring about a 'coupled oxidation' of the ethanol to acetaldehyde. In other words, under these special conditions (continuous slow supply of hydrogen peroxide at a very low concentration), the catalase acts as a peroxidase.

Fig. 3 shows that bacterial catalase behaves exactly like liver catalase in this respect. Xanthine oxidase-hypoxanthine was used as the source of hydrogen peroxide; the coupled oxidation is shown by a doubling of the theoretical oxygen uptake and the formation of acetaldehyde, recognized by its smell and the brown coloration imparted to the potassium hydroxide papers in the manometer cups. Acetaldehyde is only formed when catalase, ethanol, xanthine oxidase and hypoxanthine are all present.

DISCUSSION

The objects of this work were twofold: to investigate the use of lysozyme for the liberation of enzymes from the bacterial cell, and to apply this technique to the isolation of bacterial catalase.

Our present results indicate that the lysozyme technique is an excellent one, though not entirely without drawbacks. It is, of course, only applicable to lysozyme-sensitive micro-organisms. The viscous substances released from the bacteria by lysozyme (or possibly formed by its action) are a decided hindrance in the initial stages of enzyme purification. Nevertheless, the simplicity of the technique, the fact that it is unlikely to destroy labile enzymes, and its ready applicability on either a large or a small scale, should make it a useful tool in studying the intracellular components of bacteria.

The isolation of the catalase of *M. lysodeikticus* was undertaken partly in the hope that interesting differences might be revealed between the bacterial enzyme and the catalases of mammalian tissues. In fact, the differences observed are less striking than the similarities. The prosthetic group is the same as that of mammalian catalases, the molecular weight is the same, and there are the same number of haematin groups in the molecule. The main difference is that the catalytic activity of the bacterial enzyme is considerably higher than that of mammalian catalases; this difference must be attributed to the protein component of the enzyme.

The protein components of sheep, ox, horse and human catalases have all been shown to be different by immunological methods (Tria, 1939; Campbell & Fourt, 1939; Bonnichsen, 1947), and there is little doubt that the protein of bacterial catalase is different again. All these proteins, however, when combined with haematin, have the common property of catalyzing specifically the decomposition of hydrogen peroxide. It seems reasonable to suppose that this common property is related to some common structural element of the molecule—possibly some particular grouping of certain amino-acids—on which the catalytic activity depends. A careful study of the same enzyme isolated from several different sources might throw considerable light on the nature of enzymic catalysis. Catalase, which has now been isolated pure from so many different sources, should be a suitable enzyme to choose for such an investigation.

Another point of interest arising from this work is the remarkably high concentration of catalase found in *M. lysodeikticus*. This can be calculated from the $Kat.\text{-}f.$ values of the pure enzyme (95,000) and the lyzed bacteria (800–1800 in different batches), giving a catalase content for this organism of 1–2 % of its dry weight. This is about ten times the catalase content of human red blood corpuscles (Herbert & Pinsent, 1948). It can also be calculated that a single bacterial cell (dry weight taken as $2{\cdot}5 \times 10^{-13}$ g.) contains $10\text{–}20 \times 10^3$ enzyme molecules.

Such calculations raise the question of the function of catalase in *M. lysodeikticus*. The above concentrations of catalase would enable each bacterial cell to decompose *c.* 35–70 times its own weight of hydrogen peroxide/min. (at 0° and hydrogen peroxide concentration of 0·01 M). Such large amounts of peroxide could scarcely be formed as a result of cell metabolism, and it is difficult to devise any teleological explanation for this high enzyme concentration unless, as the discovery of 'coupled

oxidation' by Keilin & Hartree (1936, 1945*b*) suggests, catalase has other functions besides the simple decomposition of hydrogen peroxide.

SUMMARY

1. The use of lysozyme in the study of intracellular bacterial enzymes and some phenomena noticed when lysozyme acts on concentrated bacterial suspensions are discussed.

2. Using lysozyme to liberate the enzyme from the bacterial cell, catalase has been isolated from *Micrococcus lysodeikticus* in crystalline form.

3. Bacterial catalase is in many respects similar to the catalases of animal tissues. The crystalline enzyme contains *c.* 1·1 % of haematin, identical with ordinary blood haematin.

4. It differs from liver catalases, but resembles erythrocyte catalases, in containing no verdohaematin.

5. Haematin content and ultracentrifugal data indicate a molecular weight of *c.* 230,000, with four haematin groups/molecule.

6. The catalytic activity is considerably higher than that of mammalian tissue catalases; the difference is to be attributed to the protein component of the enzyme.

Our thanks are due to Dr D. W. Henderson, Chief Superintendent of the Microbiological Research Department, Porton, and his staff, for their invaluable assistance in growing the large quantities of bacteria necessary for this work.

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