catalase haematin is bound as complex II and at pH 3.6 nearly all are, under the particular experimental conditions.

8. The formation of complex II is responsible for the decrease of catalase activity during the usual Kat. f. determination and for the rapid decrease of catalase activity in acid solutions.

9. The steady-state concentration of hydrogen peroxide in the presence of catalase and the notatin system is very small. Under particular experimental conditions, the hydrogen peroxide concentration is calculated to be of the order of 10^{-9} M.

10. The very rapid reaction of catalase-bound methyl hydrogen peroxide I with hydrogen peroxide

can be studied in greater detail when hydrogen peroxide is continuously generated by the notatin system. The velocity constant for this reaction is of the same order as that of catalase-hydrogen peroxide I with hydrogen peroxide ($3.5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$). In this reaction, hydrogen peroxide acts as an acceptor as in peroxidatic reactions of complex I with ethanol, but is about 30,000 times as active as ethanol.

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The Enzyme-substrate Compounds of Bacterial Catalase and Peroxides*

BY B. CHANCE AND D. HERBERT

The Johnson Research Foundation, University of Pennsylvania, U.S.A., and Medical Research Council Unit for Bacterial Chemistry, Lister Institute, London S.W. 1

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One of the approaches to the relation between enzyme activity and the nature of the protein component is the study of enzymes with dissimilar protein components and identical prosthetic groups. A comparative study of the peroxidatic activity of horse radish peroxidase and ferrimyoglobin affords a gross example of the potent influence of the protein component. But here the effect is too great; ferrimyoglobin is practically completely inactive. A more sensitive investigation of the differences between the activities of erythrocyte catalase and the recently

crystallized bacterial catalase (Herbert & Pinsent, 1948) would seem to be more fruitful, and is the subject of this paper.

Both erythrocyte and bacterial catalases apparently have four protohaematin prosthetic groups attached to protein molecules of very nearly the same molecular weight. However, Herbert & Pinsent (1948) obtained definite evidence that differences exist between the two protein components, especially as indicated by their relative stability in organic solvents and at low values of pH. There is also good evidence that the activity of bacterial catalase is different from that of erythro-

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cyte catalase (the values of 'Katalasefähigkeit' (Kat. f.) found by the above authors were 95,000 and 61,000 respectively). Thus these two catalases are suitable for a quantitative study of the effect of the protein component upon the activity of the two enzymes.

This paper gives detailed data on the spectroscopic and kinetic properties of the compounds of bacterial catalase with hydrogen peroxide and with alkyl hydrogen peroxides. The data show that the mechanism of action of this enzyme is identical with that of erythrocyte catalase. However, the reaction velocity constants for the combination of bacterial catalase with peroxides and for the reactions of the bacterial catalase-peroxide complexes with alcohols and related substances show significant differences from those of erythrocyte catalase; bacterial catalase reacts more rapidly with small molecules and more slowly with larger molecules than erythrocyte catalase. The difference between the activities of these two enzymes may thus be attributed to a difference of protein structure. It is suggested that the haematin of bacterial catalase are more deeply 'buried' in the protein molecule than are those of erythrocyte catalase, and reactions with larger molecules are thus impeded. But the activity of the haematin of bacterial catalase towards the small hydrogen peroxide molecule is enhanced by the proximity of the protein component and the prosthetic groups.

EXPERIMENTAL

Light absorption measurements. Unless otherwise stated, a Beckman quartz spectrophotometer was used, and all results are given in terms of the millimolecular extinction coefficient $\epsilon_{\text{mm}} = E/cl$ (l.mmol.⁻¹ cm.⁻¹), where E is the extinction ($\log I_0/I$), c the mm-concentration and l the optical depth in cm. Wavelengths (in m μ .) are indicated by superscripts, e.g. $\epsilon_{\text{mm}}^{405}$. In the case of catalase, c is expressed in the units mol. catalase/l. and not mol. catalase-haematin/l. as employed by some workers.

Preparations. Bacterial catalase was prepared from *Micrococcus lysodeikticus* by the method previously described (Herbert & Pinsent, 1948). To conserve material, the preparations were not carried through to crystallization, and represented the material obtained after stage 7 of the above procedure; they were only about 60–70% pure, but were free from other haematin compounds.

Enzyme concentrations. The content of pure catalase in solutions of the approx. 60% pure enzyme was determined by (a) haematin determinations by the pyridine haemochromogen method, (b) spectrophotometric measurement of the Soret absorption band of catalase at 405 m μ .

(a) Pyridine haemochromogen was determined by spectrophotometric measurements at two wavelengths, the peak of the α absorption band and the minimum between the α and β bands. According to recent measurements by Biörck (1949) with the Beckman spectrophotometer, these lie at 556 and 540 m μ . respectively, and the difference between the molecular extinction coefficients is

$$(\epsilon^{556} - \epsilon^{540}) = 22.0 \times 10^3.$$

Hence, when measurements are made in an optical depth of 1 cm.,

$$\text{Haematin Fe concentration (mm)} = (E^{556} - E^{540})/22.0.$$

These values are slightly different from those obtained by Drabkin (1941), using a different type of instrument; since our measurements were made with a Beckman spectrophotometer, Biörck's data were used.

(b) The millimolecular extinction coefficient of bacterial catalase at the peak of the Soret band was obtained by measuring the absorption at 405 m μ . of solutions standardized by method (a), and is $\epsilon_{\text{mm}}^{405} = 405 \pm 5$. This value was thereafter used for routine determinations of catalase concentration. It is 6–7% higher than the corresponding values for erythrocyte catalase given by Bonnichsen (1949), and Bonnichsen, Chance & Theorell (1947). For these bacterial catalase preparations, the value of ϵ^{280} is 1.5 to 2 times that of ϵ^{405} .

RESULTS

Activity towards hydrogen peroxide

In previous work on bacterial catalase the activity was expressed as Kat. f., and measured by the titration method of Sumner (1941), using catalase concentrations of approximately 0.006 $\mu\text{g./ml.}$ which gave a halftime for the reaction of about 10 min. By this method, the value found for Kat. f. was 95,000 (Herbert & Pinsent, 1948). Bonnichsen *et al.* (1947) pointed out the disadvantages of this technique, the main one being that considerable destruction of enzyme by the hydrogen peroxide occurs, giving falling values of the velocity constant k'_1 , which must be extrapolated to zero time to obtain the true value, an unsatisfactory procedure. The activity of bacterial catalase was therefore redetermined by the rapid titration method of the above authors, using considerably higher enzyme concentrations (approx. 0.12 $\mu\text{g./ml.}$) which gave a halftime of approx. 30 sec. Under these conditions, no appreciable destruction of enzyme occurred and the value of k'_1 remained satisfactorily constant throughout the reaction.

Under such conditions, the destruction of hydrogen peroxide by catalase follows exactly the equation (Bonnichsen *et al.* 1947)

$$-\frac{dx}{dt} = k'_1 ex, \quad (1a)$$

and k'_1 is calculated from the titration results as

$$k'_1 = \frac{1}{et} \ln \frac{x_0}{x_t}, \quad (1b)$$

where e and x are the molar concentrations of catalase and hydrogen peroxide, and t is the time in sec. Kat. f. is related to k'_1 by the equation:

$$\text{Kat. f.} = \frac{520 k'_1}{M}, \quad (1c)$$

where M is the molecular weight of bacterial catalase (230,000).

Table 1. Determinations of k'_1 by spectrophotometric measurements at 230 m μ .(1.06 $\times 10^{-9}$ M-bacterial catalase, pH 7.0, 0.01 M-phosphate. 330 μ M-Hydrogen peroxide added.)

Initial recorder deflexion	...	...	21	29	29	30	26	23
Halftime of the reaction (sec.)	...	...	12.6	12.4	12.4	10.3	14.8	11.8
$k'_1 = \frac{\ln 2}{(\text{Average } t_{0.5}) \times 1.06 \times 10^{-9}} \text{ (M}^{-1} \text{ sec.}^{-1}\text{)}$								5.3×10^7

A rather careful series of measurements by the rapid titration method gave, at 0°,

$$k'_1 = 4.30 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1},$$

or Kat. f. = 97,300, a slight increase on the previous value.*

Since measurements in the rapid-flow apparatus cannot conveniently be made at 0°, the change in k'_1 with temperature was measured by the rapid titration method over the range 0–30°; the results are given in Fig. 1. The unusually low temperature co-

Fig. 1. Temperature coefficient of bacterial catalase in the range 0–30°. Determinations by the rapid titration method. Final concentrations: hydrogen peroxide, 0.005 M; catalase, 4.76×10^{-10} M; phosphate (pH 6.8), 0.01 M. Reaction halftime approx. 30 sec. Two experiments with different enzyme solutions, each at three temperatures. Each point is the mean of quadruplicate determinations. Temp. in °C., T in °K.

efficient taxes the accuracy of the titration method, but within the experimental error the results appear to follow the Arrhenius equation, giving a linear plot of $\log k'_1$ against $1/T$, and a value for the apparent activation energy (μ) of 1410 cal. This is appreciably lower than the value obtained for erythrocyte catalase by Bonnichsen *et al.* (1947), who found $\mu = 1700 \pm 100$ cal.

The activity of bacterial catalase has also been determined by a new method in which the disappearance of hydrogen peroxide is followed spectrophotometrically by measurement of its absorption

* $4.30 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$ corresponds to $4.30 \times 10^7 \text{ l.mol.}^{-1} \text{ sec.}^{-1}$ in the alternative terminology.

at 230 m μ . By appropriate modification of the electrical circuits of the Beckman spectrophotometer adequate stability may be obtained in the measurement of small changes of optical density $\log I_0/I = 0.01$ approx. (Chance, unpublished data). Thus it is possible to record directly, and on a linear scale, the decrease of hydrogen peroxide concentration during the catalase reaction. Furthermore, a special form of the 'stopped-flow' method has recently been developed which is suitable for use in the ultraviolet region of the spectrum. The observation chamber of this apparatus has a depth of 1 cm.

Fig. 2. Illustrating the determination of the velocity constant for the disappearance of hydrogen peroxide in the presence of bacterial catalase. The measurement of hydrogen peroxide concentration is made spectrophotometrically at 230 m μ . Modified Beckman spectrophotometer, stopped-flow method ($d_0 = 1$ cm.). Final concentrations: 2.8×10^{-9} M-bacterial catalase, 0.01 M-phosphate, pH 7.0. Temp. 25°.

A typical example of such a measurement at 230 m μ is given in Fig. 2. Initially the observation chamber is filled with the end products of a previous identical experiment in which all the hydrogen peroxide has been consumed. On initiating the flow of reactants, the observation chamber is filled with mixed bacterial catalase (2.8×10^{-9} M)† and hydrogen peroxide (160 μ M)† and the increment in the concentration of the latter is indicated by the rapid upward deflexion of the trace. The disappearance of hydrogen peroxide proceeds and causes the exponential fall of

† Here and throughout this paper, concentrations referred to are the final concentrations in the reaction mixture.

the trace. The halftime of the reaction is measured (4.7 sec.) and k'_1 is computed:

$$k'_1 = \frac{\ln 2}{4.7 \times 2.8 \times 10^{-9}} = 5.3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1} \text{ (at } 25^\circ \text{)}.$$

Similar experiments with $1.06 \times 10^{-9} \text{ M}$ -enzyme give the same value for k'_1 (Table 1). In comparison, the value of k'_1 at 25° determined by the rapid titration method is $5.35 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ (equivalent to a Kat. f. of 121,000).

Fig. 3. The spectra of the primary and secondary catalase-hydrogen peroxide complexes in the region of the Soret band. Curve A, free bacterial catalase, $0.5 \mu\text{M}$, in phosphate (pH 7.0), 0.01 M . Curve I, bacterial catalase-hydrogen peroxide complex I. Catalase concentration as above, hydrogen peroxide supplied by the notatin system (approx. 10^{-9} M -notatin, 0.7 mM -glucose). Curve II, bacterial catalase-hydrogen peroxide complex II, measured 60 min. after mixing bacterial catalase and the notatin system.

The excellent agreement between these values, obtained by two different observers using quite different methods, speaks for the fundamental soundness of both methods, and verifies that the activity of bacterial catalase is considerably greater than that of erythrocyte catalase, where the values of k'_1 are 3.5×10^7 at 25° (determined by both methods) and 2.63×10^7 at 0° .

Spectroscopic studies of the bacterial catalase-peroxide complexes

An intermediate compound of enzyme and substrate was discovered previously in the reaction of erythrocyte catalase and hydrogen peroxide by the use of the rapid-flow method (Chance, 1947), and these studies show that a very similar compound exists in the reaction of bacterial catalase and hydrogen peroxide. Such a complex may be stab-

ilized for a considerable time if hydrogen peroxide is continuously generated by an oxidase system which produces hydrogen peroxide, for example, the system notatin, glucose and oxygen (Keilin & Hartree, 1948). In the presence of this enzyme system (and in the absence of ethanol) the spectrum of the primary bacterial catalase-hydrogen peroxide complex (complex I) is measured in the Beckman spectrophotometer in the region $370\text{--}455 \text{ m}\mu$, and the values of the extinction coefficients are plotted in Fig. 3.

Fig. 4. The spectra of the primary and secondary bacterial catalase-methyl hydrogen peroxide complexes in the region of the Soret band. Curve A, free bacterial catalase. Curve I, bacterial catalase-methyl hydrogen peroxide complex I. Curve II, bacterial catalase-methyl hydrogen peroxide complex II. Final concentrations, $0.5 \mu\text{M}$ -bacterial catalase in 0.01 M -phosphate, pH 7.0. Curve I, rapid-flow apparatus; circles, $600 \mu\text{M}$ -methyl hydrogen peroxide; points, $5 \mu\text{M}$ -methyl hydrogen peroxide. (Exp. 414.) Curve II, Beckman spectrophotometer, $200 \mu\text{M}$ -methyl hydrogen peroxide.

The Soret band of complex I of bacterial catalase is in many respects very similar to that found for erythrocyte catalase (Chance, 1949*g*); it is similar to, but less intense than, that of the free enzyme. But there is a quantitative difference: the change of ϵ_{405}^{405} from the free enzyme to complex I is considerably larger, 82 compared with 45 for erythrocyte catalase (Chance, 1950*a*). This difference is discussed further below.

After 1 hr. in the presence of hydrogen peroxide, the spectrum II of Fig. 3 is obtained. This represents the conversion of most of the bacterial catalase into the secondary complex, in accordance with studies of erythrocyte catalase (Chance, 1950*a*).

Bacterial catalase also forms primary and secondary compounds with methyl hydrogen peroxide as

shown by the curves I and II of Fig. 4. The spectrum of the secondary complex is readily measured in the ordinary spectrophotometer upon the addition of an excess of methyl hydrogen peroxide (approx. $200\ \mu\text{M}$) to bacterial catalase (approx. $0.5\ \mu\text{M}$). The primary complex is, however, unstable, and either spontaneously decomposes into free catalase or is converted into the secondary complex in a short time. The spectrum of complex I is therefore measured in the rapid-flow apparatus. The changes of extinction coefficient from free catalase to complex I and from complex I to complex II are measured according to the method described previously (Chance, 1949c). In the region $370\text{--}420\ \text{m}\mu$, the extinction coefficients based upon the conversion of complex I to complex II are considered the more accurate, while those based upon the conversion of catalase to complex I are considered more accurate in the region above $420\ \text{m}\mu$. In this way the spectrum of Fig. 4 is constructed. It was noted that the values of the change of extinction coefficient from free catalase to complex I in the region $380\text{--}410\ \text{m}\mu$ lie somewhat above the curve based upon the change from complex I to complex II given in Fig. 4. This discrepancy was observed previously (Chance, 1949c), and is again attributed to the large spectral interval required of the monochromator employed with the rapid-flow apparatus.

The composition of the bacterial catalase-hydrogen peroxide complex: spectrophotometric data

The change of ϵ_{405}^{405} from free catalase to the primary methyl hydrogen peroxide complex (200) is much larger than that shown in Fig. 3 for the hydrogen peroxide complex (82). Since this change is identical for the primary peroxidase hydrogen peroxide and methyl hydrogen peroxide complexes (Chance, 1949g), it is here assumed that the change of extinction coefficient from the free enzyme to the primary peroxide complex is the same per haematin group for hydrogen peroxide and methyl hydrogen peroxide. Thus the smaller value for the bacterial catalase-hydrogen peroxide complex may be attributed to the fact that not all the catalase haematin groups are bound to hydrogen peroxide. Quantitatively, only $82/200 = 1.6$ of the four catalase haematin groups are so bound.

A similar effect obtains in erythrocyte catalases, but here 1.0 ± 0.1 haematin groups are bound according to studies by a similar method (Chance, 1950a). Thus there is a higher steady-state concentration of the catalase-hydrogen peroxide complex in the reaction of bacterial catalase than in the reaction of erythrocyte catalase with hydrogen peroxide.

A graphic illustration of the difference of the extinction coefficients of the hydrogen peroxide and methyl hydrogen peroxide complexes is given in Fig. 5. The cuvette ($d = 1\ \text{cm.}$) of a recording spectrophotometer is initially filled with a dilute solution

of bacterial catalase. As a control the solution is initially stirred, and, except for the momentary increase of optical density caused by stirring, the base-line is unaffected. Upon addition of hydrogen peroxide to a concentration of $67\ \mu\text{M}$, an abrupt decrease of extinction is recorded and is caused by the formation of the primary catalase-hydrogen peroxide complex. The addition of hydrogen peroxide is repeated and causes no further decrease of extinction. This indicates that the saturation value of the catalase-hydrogen peroxide complex

Fig. 5. Spectrophotometric recording of the reactions of bacterial catalase with hydrogen peroxide and with methyl hydrogen peroxide. Modified Beckman spectrophotometer, 1 cm. cuvette; $0.01\ \text{M}$ -phosphate, pH 7.0; $\lambda = 405\ \text{m}\mu$.

has been measured. The next addition is of methyl hydrogen peroxide which combines with all the catalase haematin groups and thereby causes a large decrease of extinction. Further addition of methyl hydrogen peroxide causes no increase in the concentration of catalase-methyl hydrogen peroxide. Since saturation with peroxide has been achieved in these two cases, and since it is assumed that the extinction coefficients of the two peroxide compounds are the same on a haematin-iron basis, the fraction of catalase haematin groups bound in the hydrogen peroxide complex is $7.5/20.5$ scale divisions of Fig. 5. For a four-haematin catalase this corresponds to 1.5 haematin groups bound. This is in fair agreement with the value of 1.6 measured in Fig. 3.

Cyanide results

The composition of the mammalian catalase-hydrogen peroxide complexes has been determined by measuring spectrophotometrically the relative amounts of catalase-cyanide formed by the free catalase or by the catalase-hydrogen peroxide complex (Chance, 1949b). In those tests, conditions were specified under which a successful experiment could be performed. These conditions involved the dissociation constant of catalase-cyanide. For this reason, the properties of bacterial catalase-cyanide are given. The spectrum of bacterial catalase-cyanide in the region $370\text{--}460\ \text{m}\mu$ is shown in Fig. 6, and is seen to be very similar to that of erythrocyte

catalase-cyanide (Chance, 1949c). $\epsilon_{\text{mm}}^{425}$ is 360 and is 13 % greater than the corresponding value for erythrocyte catalase-cyanide.

The titration of bacterial catalase with cyanide is illustrated in Fig. 7, and the values of the apparent dissociation constant are computed by the usual formula. The average is $21.8 \times 10^{-6} \text{M}$. This is over five times the value found for the equilibrium constant of erythrocyte catalase and cyanide (Chance, 1949a). This change is apparently not caused by a difference in the rates of formation of the cyanide compounds; measurements of this value for bacterial catalase give about $2 \times 10^6 \text{M}^{-1} \text{sec}^{-1}$ compared with 0.9×10^6 for mammalian catalases (Chance, 1949a).

Fig. 6. The spectrum of bacterial catalase-cyanide in the region of the Soret band (Curve B). Final concentrations: $0.4 \mu\text{M}$ -bacterial catalase, $400 \mu\text{M}$ -hydrocyanic acid, 0.01M -phosphate, pH 7.0. Curve A, free bacterial catalase for comparison.

Thus it is the velocity constant for the dissociation of bacterial catalase-cyanide that is increased greatly: from the product of the dissociation constant and the velocity constant for the formation of catalase-cyanide a value of 44sec^{-1} is computed for the velocity constant for the dissociation of bacterial catalase-cyanide. This is about eleven times that for erythrocyte catalase-cyanide and is comparable to the dissociation velocity of oxyhaemoglobin.

With higher concentrations of hydrocyanic acid (approx. $20 \mu\text{M}$), the combination of cyanide with the free haematin groups of the bacterial catalase-hydrogen peroxide complex is clearly shown in Fig. 8A on a slow-time scale and in Fig. 8B on a fast-time scale. These measurements were made in the rapid-flow apparatus. On initiation of the flow, the catalase-cyanide remaining from the previous ex-

periment is flushed out of the observation tube and is replaced by fresh catalase, hydrogen peroxide, and hydrocyanic acid as indicated by the abrupt decrease in optical density. The catalase-hydrogen peroxide complex is not measured at this wavelength because it forms very rapidly, and because $425 \text{m}\mu$ is near the isobestic point for catalase and catalase-hydrogen peroxide (Fig. 3). Thus the extinction in the observation tube is nearly the same as that of free catalase. The rapid upward deflexion (D_2) as the flow stops is caused by the combination of catalase and cyanide, and this reaction, according to Fig. 8B, is complete in about 0.1 sec. But Fig. 8A clearly shows that the upward deflexion continues at a slower rate until a constant value E_1 is reached. The slow reaction is caused by the decomposition of catalase-hydrogen peroxide which liberates catalase haematin

Fig. 7. Titration of bacterial catalase with hydrocyanic acid. The values of the dissociation constant K_1 are computed for the various points. Final concentrations: $0.4 \mu\text{M}$ -bacterial catalase, 0.01M -phosphate, pH 7.0; $\lambda = 435 \text{m}\mu$.

to combine with cyanide. Thus E_2/E_1 is a measure of the catalase haematin not bound to hydrogen peroxide in this complex, and $1 - E_2/E_1$ a measure of the catalase haematin bound to hydrogen peroxide. Fig. 8 is, therefore, clearly in agreement with the previous tests which show that not all the catalase haematin are bound to hydrogen peroxide in this complex.

Fig. 9 represents the effect of cyanide concentration upon the value of $1 - E_2/E_1$ and clearly shows a decrease in this quantity with increasing cyanide concentration. This demonstrates that cyanide may compete with peroxide for catalase haematin. However, this competition interferes with the accurate measurement of the maximum value of $1 - E_2/E_1$, which would be obtained at very low cyanide concentrations. Such data are difficult to obtain with bacterial catalase because of the large dissociation constant of the cyanide complex ($22 \times 10^{-6} \text{M}$) which necessitates a relatively large excess of hydro-

cyanic acid to give readily measurable amounts of catalase cyanide. Therefore the maximum value of $1 - E_2/E_1$ is obtained by extrapolation of the data of Fig. 9 to zero hydrocyanic acid concentration, as indicated by the dotted portion of the curve. A value of about 0.39 is obtained, which corresponds to about 1.6 haematin bound to hydrogen peroxide for a four-haematin catalase.

Fig. 8. The simultaneous reactions of hydrogen peroxide and hydrocyanic acid with bacterial catalase on a slow-time scale (A) and on a fast-time scale (B). Final concentrations: $0.045 \mu\text{M}$ -bacterial catalase, $200 \mu\text{M}$ -hydrogen peroxide, $20 \mu\text{M}$ -hydrocyanic acid, 4 mM -methanol, 0.01 M -phosphate, pH 7.0; $\lambda = 435 \text{ m}\mu$.

Fig. 9. The relation between the quantity $1 - E_2/E_1$ (see Fig. 8) and the initial hydrocyanic acid concentration. The decrease of $1 - E_2/E_1$ with increasing hydrocyanic acid concentration illustrates competition between peroxide and cyanide for catalase haematin. Conditions as in Fig. 7.

The fact that it is necessary to obtain the value of $1 - E_2/E_1$ by extrapolation in the case of bacterial catalase shows that the definition of the experimental conditions for a successful experiment for erythrocyte catalase by this method (Chance, 1949c) is inadequate for bacterial catalase. We can point to one inadequacy of the previous analysis: the criterion of whether cyanide would displace hydrogen peroxide from complex I was based upon the initial hydrogen peroxide concentration. However, the

initial hydrogen peroxide concentration will have been greatly reduced by the catalytic reaction by the time the cyanide begins to combine with the catalase, and competition may indeed occur with more dilute cyanide than would otherwise be expected. This situation is aggravated by the fact that bacterial catalase has a higher activity towards hydrogen peroxide, and a larger dissociation constant of the cyanide complex, than has erythrocyte catalase. It is therefore recommended that the relation between $1 - E_2/E_1$ and cyanide concentration be investigated for each new catalase for which this test is used.

The relation between the kinetics of disappearance of hydrogen peroxide and the kinetics of the catalase-hydrogen peroxide complex

Numerous attempts have previously been made to devise methods for the measurement of the kinetics of the catalase-hydrogen peroxide complex at the same time as the kinetics of the disappearance of hydrogen peroxide (Chance, 1947). By the development of suitable electronic circuits and a stopped-

Fig. 10. Illustrating the relationship between the kinetics of disappearance of hydrogen peroxide measured at $230 \text{ m}\mu$, and the kinetics of the bacterial catalase-hydrogen peroxide complex measured at $405 \text{ m}\mu$. Modified Beckman spectrophotometer, stopped-flow method, cell depth 1 cm . Time markers are at 1 sec . intervals. Final concentrations: $0.07 \mu\text{M}$ -bacterial catalase, $80 \mu\text{M}$ -hydrogen peroxide, 8 mM -methanol, 0.01 M -phosphate, pH 7.0. (Exp. 416b.)

flow method suitable for measurements in the ultra-violet region, the rapid disappearance of hydrogen peroxide can be measured with a fairly rapid response. This stopped-flow apparatus has an observation chamber with an optical path of 1.0 cm , and permits the measurement of the kinetics of the catalase-hydrogen peroxide complex in very dilute solutions ($0.07 \mu\text{M}$).

A preliminary record of this type is shown in Fig. 10 and represents the performance obtainable with the present technique operating near the limit of its sensitivity. At $230 \text{ m}\mu$ is registered the kinetics of the disappearance of hydrogen peroxide, and the description given for Fig. 2 applies here except that the time scale is more rapid. At $405 \text{ m}\mu$, the abrupt

downward deflexion is caused by the rapid combination of catalase and hydrogen peroxide. The complex remains at its saturation value for over 2 sec. and decomposes on reaction with methanol.

This record clearly shows that the hydrogen peroxide concentration must fall to a very small value (too small to be measured accurately) before the reaction of the catalase-hydrogen peroxide complex with methanol begins. Thus the previous statements based on less direct evidence are verified (Chance, 1947).

It is of considerable interest to compare this record with that of the reaction kinetics of a conventional Michaelis intermediate complex (Chance, 1943, Fig. 3; Chance, 1949*f*, Fig. 1), where the concentration of the peroxidase-hydrogen peroxide complex determines the rate of disappearance of hydrogen peroxide. With catalases, the rate of disappearance of hydrogen peroxide and the concentration of the enzyme-substrate compound are related in a much less obvious manner.

The combination of bacterial catalase with peroxides

Fig. 11 illustrates the measurement of the velocity of formation of the bacterial catalase-hydrogen peroxide complex by the rapid-flow method. The capillary observation tube is initially filled with free catalase, the end product of the previous identical experiment. On initiation of the flow of fresh reactants into the capillary ($0.36 \mu\text{M}$ -catalase mixed with $10 \mu\text{M}$ -hydrogen peroxide and 8 mM -methanol), the rapid combination of catalase and hydrogen peroxide causes the rapid decrease in optical density. The reaction proceeds to over half completion in the time indicated by the top trace: the flow velocity attained corresponds to a time after mixing of about 3 msec. As the flow stops, longer times are measured and the reaction goes rapidly to completion. The catalase-hydrogen peroxide complex is not stable and its decomposition is indicated by the upward slope of the trace. After about 0.8 sec., the flow of reactants is re-initiated and there is an upward deflexion because catalase and hydrogen peroxide have not time to react completely at the time measured (approx. 2 msec.). As the flow stops, the reaction goes to completion. The catalase-hydrogen peroxide complex is then allowed to decompose completely, and the final extinction is indicated by the final mark on the record. This record illustrates the most satisfactory manner of measuring on one record the initial and final values of the reaction, as well as a few values of the extent of the reaction at various times after mixing. It is pointed out that initial and final values are especially difficult to measure in the catalase-hydrogen peroxide reactions, in which the total change in extinction is only a small portion of the extinction of the enzyme solution.

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The velocity constant k_1 computed from this record is about $5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. The results of a series of similar measurements of the velocity of

Fig. 11. Illustrating the measurement of the combination of bacterial catalase and hydrogen peroxide. Rapid-flow apparatus, $\lambda = 405 \text{ m}\mu$. Final concentrations: $0.34 \mu\text{M}$ -bacterial catalase, $10 \mu\text{M}$ -hydrogen peroxide, 8 mM -methanol, 0.01 M -phosphate, pH 7.0. Temp. 20° .

Fig. 12. The second-order combination of bacterial catalase and hydrogen peroxide, methyl hydrogen peroxide, and ethyl hydrogen peroxide, demonstrated by the 45° straight lines through the experimental points. Substrate concentrations are given in terms of molarity and the units of the reaction velocity constants are (shown as 6×10^7 etc.) $\text{M}^{-1} \text{ sec}^{-1}$. Rapid-flow apparatus, $\lambda = 405 \text{ m}\mu$. Final concentrations: $0.34 \mu\text{M}$ -bacterial catalase, 0.01 M -phosphate, pH 7.0. Temp. 20° for H_2O_2 , 25° for CH_3OOH and $\text{C}_2\text{H}_5\text{OOH}$.

combination of bacterial catalase and hydrogen peroxide are given in Fig. 12 where the graph of k_1 [substrate] versus [substrate] is plotted in log-

arithmic co-ordinates. A 45° straight line indicates a constant value of k_1 and a second-order reaction for the combination of bacterial catalase and hydrogen peroxide. The average value of the reaction-velocity constant k_1 is $6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. It is not possible, with present techniques, to demonstrate the validity of this second-order reaction over a wider range of hydrogen peroxide concentrations because of the extreme rapidity of the reaction; these data only cover the range $1\text{--}20 \mu\text{M}$ -hydrogen peroxide. Above $20 \mu\text{M}$ -hydrogen peroxide, the reaction proceeds too rapidly for accurate measurement; it is over 90% complete at the shortest time measured. Nevertheless, the values of k_1 measured under these conditions are less than $6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ and may indicate a deviation from the second-order reaction.

The data obtained in this experiment are more consistent than are those obtained from studies of mammalian catalase where the accuracy was inadequate to prove that the reaction is of the second order (Chance, 1947). These data are more accurate because the reaction of bacterial catalase and hydrogen peroxide gives a larger change of extinction than that of an equal concentration of mammalian catalase.

The values of the reaction velocity constants (k_1) for bacterial and mammalian catalases may be compared at similar hydrogen peroxide concentrations ($4\text{--}10 \mu\text{M}$), and are 6×10^7 and $3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ respectively. The velocity of combination of bacterial catalase with hydrogen peroxide is thus roughly twice that of mammalian catalase. This is in accord with the larger activity of bacterial catalase.

Fig. 12 indicates that bacterial catalase combines with methyl or ethyl hydrogen peroxide in true second-order reactions of velocity constants 2×10^8 and $2.8 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ respectively. These reactions are considerably slower than the corresponding reactions of mammalian catalase (Chance, 1949d).

The titration of bacterial catalase with hydrogen peroxide

The amount of the bacterial catalase-hydrogen peroxide complex decreases with decreasing hydrogen peroxide concentration, as shown by the relation between the initial hydrogen peroxide concentration and the oscillograph deflexion at $405 \text{ m}\mu$, corresponding to the change in extinction from free catalase to the complex. The latter is measured as in Fig. 11, and the results are plotted in Fig. 13. A 'dissociation constant' may be computed on the basis that the saturation value of the complex corresponds to 1.6 out of 4 haematins or $0.45 \times 1.6 = 0.72 \mu\text{M}$, and computations based on the data at higher peroxide concentrations give a value of about 0.4×10^{-6} . As pointed out previously (Chance, 1947), the true dissociation constant is considerably smaller because

a large portion of the initial hydrogen peroxide is decomposed during the formation of the complex.

Fig. 13. The titration of bacterial catalase with hydrogen peroxide. Rapid-flow apparatus, $\lambda = 405 \text{ m}\mu$. Final concentrations: $0.45 \mu\text{M}$ -bacterial catalase, 4 mM -methanol, 0.01 M -phosphate, pH 7.0.

The spontaneous decomposition of the primary bacterial catalase-hydrogen peroxide complex

The velocity of this reaction is considerably less than that measured for mammalian catalases (Chance, 1947), and halftimes of roughly 200 sec. are measured, corresponding to a velocity constant of 0.0035 sec^{-1} . Assuming that only the rate of formation and spontaneous decomposition are involved, the true dissociation constant of the bacterial catalase-hydrogen peroxide complex I may be as small as 6×10^{-10} .

The reaction of bacterial catalase peroxides with acceptors

The data of Fig. 14 give the results of measurements of the rate at which the catalase-hydrogen peroxide complex decomposes in the presence of various acceptors at various concentrations. Again the data are plotted on a logarithmic scale in order to cover the extreme range. The 45° slope of the straight lines indicates that the reaction of catalase hydrogen peroxide with the acceptors is of the second order and the position of the lines gives the values of the reaction velocity constants. The values of k_4 are, in general, smaller than the corresponding values for mammalian catalase (Chance, 1947; 1950b). However, the striking effect is the great decrease in reaction velocity with the size of the acceptor molecule; the velocity constant for ethanol is much less than that for methanol, and strong propanol gives a scarcely measurable reaction ($k_4 = 0.025 \text{ M}^{-1} \text{ sec}^{-1}$ approx.).

The primary bacterial catalase-methyl hydrogen peroxide complex also reacts with acceptors and, in this case, the values of k_4 are computed from measurements of the 'cycle' of the enzyme-substrate complex (Chance, 1949d). The measured values of k_4

for formate, methanol and ethanol are 150, 54 and 11 $\text{M}^{-1} \text{sec}^{-1}$ respectively. The agreement with the data of Fig. 13 is fair except in the case of methanol where the value is smaller for the alkyl hydrogen peroxide complex.

Fig. 14. The second-order reaction of bacterial catalase-hydrogen peroxide complex I with various acceptors. The 45° straight lines through the experimental points demonstrate the second-order reaction mechanism. Acceptor concentrations are given in terms of molarity and the units of the velocity constants are $\text{M}^{-1} \text{sec}^{-1}$. Final concentrations: 0.185 μM -bacterial catalase, 80 μM -hydrogen peroxide, 0.01 M -phosphate, pH 7.0; $\lambda = 405 \text{ m}\mu$.

The reaction of the primary catalase-alkyl hydrogen peroxide complex with formaldehyde (probably as methylene glycol) proceeds satisfactorily and $k_4 = 270 \text{ M}^{-1} \text{sec}^{-1}$. The difficulties in the measurement of the reaction of erythrocyte catalase-hydrogen peroxide with formaldehyde and erythrocyte catalase-methyl hydrogen peroxide with nitrite are recited elsewhere (Chance, 1950b) and are verified in these experiments.

DISCUSSION

The great similarity of erythrocyte and bacterial catalase indicated by the work of Herbert & Pinsent (1948) is apparently restricted to those properties of the enzymes which are principally determined by the chemical nature of their prosthetic groups and by the size of their protein components: haematin content, light absorption, sedimentation constant, etc. In addition, their enzymic functions and specificities are found to be generally the same.

Generally speaking, these studies extend the similarity of the two enzymes to the properties of their enzyme-substrate compounds. Bacterial catalase forms with hydrogen peroxide and with methyl or ethyl hydrogen peroxide primary and secondary compounds which have absorption spectra that are similar to those of the primary erythrocyte catalase-peroxide compounds. In addition, these compounds form rapidly and oxidize alcohols and related com-

pounds. It is of special interest to note that the oxidation of acceptors by bacterial catalase and peroxides apparently involves only the green primary complex, in accord with previous studies of mammalian catalases. Superficially, the enzymes might be said to be practically identical. But Herbert & Pinsent (1948) found the value of activity (Kat. f.) towards hydrogen peroxide is larger for bacterial catalase than for erythrocyte catalase. This difference has been studied in some detail and is verified by these data which show that the velocity constant (k'_1) for the disappearance of hydrogen peroxide in the presence of bacterial catalase is $5.3 \times 10^7 \text{ M}^{-1} \text{sec}^{-1}$ compared with 3.5×10^7 for erythrocyte catalase. This difference is related to two other differences revealed by these experiments: (1) The number of hydrogen peroxide molecules per bacterial catalase molecule in the primary complex is 1.6 ± 0.1 , compared with 1.0 ± 0.1 found for erythrocyte catalase by similar methods. (2) The reaction-velocity constant (k_1) for the combination of bacterial catalase and hydrogen peroxide is about $6 \times 10^7 \text{ M}^{-1} \text{sec}^{-1}$ compared with about 3×10^7 for erythrocyte catalase under similar conditions. Thus the greater activity of bacterial catalase may be attributed to the more rapid combination with hydrogen peroxide which in turn causes a higher steady-state concentration of the catalase-hydrogen peroxide complex. Since the rate of disappearance of hydrogen peroxide is proportional to the steady-state concentration of the intermediate complex, the activity towards hydrogen peroxide, as measured in terms of Kat. f. or k'_1 , would be expected to be larger than the value for erythrocyte catalase which has a smaller steady-state concentration of the intermediate complex.

The relation between these factors is expressed quantitatively as follows. The mechanism for catalase action is generally represented:

The lower-case letters under the symbols of equations (2) and (3) represent their concentrations at any time t . The kinetic equations are:

$$\frac{dp}{dt} = k_1 x(e-p) - k_4 xp, \quad (4)$$

$$\frac{dx}{dt} = -k_1 x(e-p) - k_4 xp. \quad (5)$$

In the steady state $dp/dt = 0$, and equation (4) is solved for p/e :

$$\frac{p}{e} = \frac{1}{1 + k_4/k_1}. \quad (6)$$

This quantity (p/e) is the fraction of the enzyme bound as enzyme-substrate complex, and is 1.0/4.0 for erythrocyte catalase and 1.6/4.0 for bacterial catalase. Equation (6) shows that the quantity p/e is independent of x but dependent upon k_4/k_1 . By substitution of these values of p/e , equation (6) also shows that the ratio of the values of k_4/k_1 for erythrocyte and bacterial catalases must be 3.0:1.5. Assuming that the values of k_4 are the same for bacterial and erythrocyte catalases, then the ratio of the values of k_1 is 2:1, in agreement with the experimentally determined ratio ($6 \times 10^7 : 3 \times 10^7$).

The effect of p/e upon the activity of the two catalases as measured in terms of the rate of disappearance of hydrogen peroxide (dx/dt) is shown by adding equations (4) and (5)

$$\frac{dp}{dt} + \frac{dx}{dt} = -2k_4xp. \quad (7)$$

For the steady state $dp/dt = 0$ and

$$\frac{dx}{dt} = -2k_4xp. \quad (8)$$

The velocity of disappearance of hydrogen peroxide is directly proportional to p , the molar amount of catalase combined as enzyme-substrate complex. Since in the previous discussion it is assumed that k_4 is constant, and the value of p is found experimentally to be 1.6 times as great for bacterial catalase as for erythrocyte catalase, the relative values of their activities (dx/dt), computed as Kat. f. or k'_1 (equation 1) would be expected to differ by a factor of 1.6, as indeed they very nearly do ($\frac{5.3 \times 10^7}{3.5 \times 10^7} = 1.5$).

Thus the consecutive reaction mechanism affords a satisfactory basis for the observed differences of the erythrocyte and bacterial catalase kinetics. However, we are not yet able to obtain satisfactory

agreement between the absolute values of p/e , k_1 , k_4 and k'_1 on the basis of the simple mechanism.

It is of considerable importance in the theoretical analysis of catalase action to discover a catalase with significantly more than one hydrogen peroxide molecule bound per catalase molecule. Bacterial catalase, with 1.6 hydrogen peroxide molecules so bound, provides direct evidence against a particular theory of catalase action and substantially eliminates it from consideration. This theory postulates that catalases, on combination with one molecule of hydrogen peroxide, acquire 'special properties' which prevent the combination of further molecules of hydrogen peroxide (Chance, 1949*e*). This theory actually fits most of the data on erythrocyte catalase, but may now be rejected since with bacterial catalase 1.6 molecules of hydrogen peroxide are bound. The alternative theory that the binding of hydrogen peroxide molecules is purely a statistical effect in the consecutive reactions of hydrogen peroxide with free catalase and catalase-hydrogen peroxide is therefore retained as the most promising explanation for catalase action.

Whereas bacterial catalase is relatively more active than erythrocyte catalase towards hydrogen peroxide, it is relatively less active with larger substrate and acceptor molecules. The decrease in the reactivity of erythrocyte catalase towards substrates and acceptors of increasing size has already been commented upon (Chance, 1949*d*). However, the effect is much more striking with bacterial catalase. The velocity constants for its reaction with methyl and ethyl hydrogen peroxide are each about 10 times smaller than the values for erythrocyte catalase. And in the reactions with acceptors, nitrite reacts more rapidly, while propanol, which reacts at an appreciable rate with erythrocyte catalase-peroxides, gives a scarcely measurable rate

Table 2. *A comparison of the velocity constants for the reactions of bacterial and erythrocyte catalases with substrates and acceptors*

(Velocity constants for the combination of enzyme and substrate. Units $M^{-1} \text{ sec.}^{-1}$)

Substrate	...	...	Hydrogen peroxide	Methyl hydrogen peroxide	Ethyl hydrogen peroxide
Erythrocyte catalase			3×10^7	8×10^5	20×10^3
Bacterial catalase			6×10^7	2.2×10^5	2.8×10^3

(Velocity constants for the reaction of the primary enzyme-substrate complexes with acceptors. Units $M^{-1} \text{ sec.}^{-1}$)

Acceptor	...	...	Nitrite*	Formaldehyde	Methanol	Ethanol	Formate*	Propanol
Erythrocyte catalase-hydrogen peroxide, I			2000	—	1000	1000	470	17
Bacterial catalase-hydrogen peroxide, I			2800	—	91	13	175	0.025
Erythrocyte catalase-methyl hydrogen peroxide, I			—	1400	910	910	460	—
Bacterial catalase-methyl hydrogen peroxide, I			—	270	54	11	150	—

* pH = 7.0.

with bacterial catalase-peroxides. These various velocity constants are summarized in Table 2 and amplify the comparison considerably. Reasoning along the same lines as with erythrocyte catalase, it is concluded that the haematoms of bacterial catalase are even less accessible than those of erythrocyte catalase, and only very small molecules are suitable as acceptors. Following this idea further, it is proposed that the greater proximity of protein and prosthetic group of bacterial catalase causes interactions which result in a higher activity towards hydrogen peroxide. In other words, the proximity of protein and prosthetic group in bacterial catalase enhances its activity towards small molecules but results in steric effects which abruptly decrease the activity towards larger molecules. Thus rather indirect evidence is presented for the spatial interaction of protein and prosthetic group resulting in increased activity and a higher specificity of the enzyme. Further studies of related haemoproteins are required to extend this theory.

SUMMARY

1. Bacterial catalase forms primary and secondary enzyme-substrate complexes with hydrogen peroxide, methyl hydrogen peroxide and ethyl hydrogen peroxide.
2. The spectra of the bacterial and erythrocyte catalase-methyl hydrogen peroxide complexes in the region of the Soret band are nearly identical. However, a quantitative difference exists between the Soret bands of the primary hydrogen peroxide complex of bacterial and erythrocyte catalases (see below).
3. The velocity constant* for the decomposition of hydrogen peroxide in the presence of bacterial catalase is $5.3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ for a four-haematin catalase, and may be compared with the value of 3.5×10^7 for erythrocyte catalases; i.e. the ratio of the velocity constants (or of the Kat. f. values) for the two enzymes is 1.5:1.
4. The bacterial catalase-hydrogen peroxide complex consists of 1.6 ± 0.1 hydrogen peroxide molecules per bacterial catalase molecule, as determined by spectrophotometric measurements of catalase-methyl hydrogen peroxide and cyanide compounds. This value may be compared with that of 1.0 ± 0.1 found for erythrocyte catalase on the basis of methyl hydrogen peroxide data.
5. The velocity constant for the combination of bacterial catalase with hydrogen peroxide is about $6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ and the reaction is shown to be of the second order up to $20 \mu\text{M}$ -hydrogen peroxide. This value may be compared with that of about 3×10^7 for erythrocyte catalase.
6. On the basis of the mechanism for catalase action which involves consecutive reactions of hydrogen peroxide with catalase and with the catalase-hydrogen peroxide complex, the greater activity of bacterial catalase compared with erythrocyte catalase may be quantitatively attributed to the more rapid combination of bacterial catalase with hydrogen peroxide to form the bacterial catalase-hydrogen peroxide complex. The consequence of this more rapid reaction is a larger steady-state concentration of the bacterial catalase-hydrogen peroxide complex and a higher velocity constant for the disappearance of hydrogen peroxide.
7. The velocity constants for the combination of bacterial catalase with methyl or ethyl hydrogen peroxide are 2.2×10^5 and $2.8 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ respectively. These values are about 10 times slower than the corresponding values for erythrocyte catalases.
8. The primary bacterial catalase-peroxide complexes react with acceptors according to the mechanisms found to obtain for the corresponding erythrocyte catalase-peroxide complexes; only the green primary complexes appear to be involved in these reactions. The specificity appears to follow an identical pattern; velocity constants are evaluated for nitrite, formaldehyde, formate, methanol, ethanol and propanol.
9. Bacterial catalase and hydrogen peroxide oxidizes nitrite more rapidly than does erythrocyte catalase. Also bacterial catalase is relatively more active towards formate and formaldehyde with respect to methanol and ethanol than is erythrocyte catalase.
10. In the reactions of bacterial catalase with substrates or acceptors, the values of the reaction velocity constants are dependent on the size of the substrate or acceptor molecule to a much greater extent than was found to be the case with erythrocyte catalase; large molecules react much more slowly than small ones. This greater dependence is interpreted to indicate that the haematoms of bacterial catalase are 'buried' in the protein molecule to a greater extent than are the haematoms of erythrocyte catalase. Nevertheless, the haematoms of bacterial catalase react with hydrogen peroxide more rapidly than do the haematoms of erythrocyte catalase. It is suggested that the proximity of the protein groups to the haematoms of bacterial catalase enhances their activity towards molecules to which the haematoms are accessible.

* Velocity constants are measured at 20–25°.

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The Site of Hydrochloric Acid Production in the Stomach as Determined by Indicators*

By NORAH M. BRADFORD AND R. E. DAVIES

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

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In order to correlate the biochemical function and the morphological structure of acid-secreting gastric mucosa, it is necessary to know exactly where the hydrochloric acid is formed. Bernard (1858) first used microchemical methods in an attempt to locate the site of formation of the hydrochloric acid, and Babkin (1944) reviewed the work on this topic. Although it is generally held that the oxyntic cells secrete hydrochloric acid at concentrations about isotonic with blood (Hollander, 1943), some workers (Hoerr & Bensley, 1936) have stated that a precursor of the acid occurs within the gastric tubules and is hydrolysed to form hydrochloric acid in the foveolae or even the lumen of the stomach.

Frog gastric mucosa can secrete some dyestuffs *in vivo* (Henning, 1932) and hydrochloric acid *in vitro* (Delrue, 1930). Tied bags of isolated frog gastric mucosa were used in this study to find out which dyestuffs are transported across the tissue without interfering with respiration or acid secretion. Observations were made of dye- and acid-secreting oxyntic cells in both amphibian and mammalian gastric mucosa. These established the fact that the contents of the canaliculae of these cells have a pH < 1.4. This shows that acid is formed in the pericanalicular zone of oxyntic cells and is incompatible with the hypothesis that some hypothetical precursor of the hydrochloric acid is secreted by oxyntic cells.

* A part of this work was communicated to the Biochemical Society (Bradford & Davies, 1948).

EXPERIMENTAL

Saline media. Mammalian tissue was incubated in the physiological salt solution (bicarbonate saline) of Krebs & Henseleit (1932), gassed with 5% CO₂ + 95% O₂. For experiments on frog gastric mucosa this solution was diluted with 0.29 vol. of water, when the balance of the O₂ uptake and the CO₂ output was to be measured. The phosphate saline of Krebs (1933) diluted with 0.29 vol. of water was used for measurements of the O₂ uptake of frog gastric mucosa.

The solution used during the preparation of the mucosa was a basal saline similar to amphibian bicarbonate saline but had a HCO₃⁻ content of 0.004M and, under working conditions, pH 7.4. All the salines contained 0.2% (w/v) glucose, and histamine was added at the start of all experiments to a final concentration of 5 × 10⁻⁵M.

Dyestuffs. The following dyestuffs were used. *Basic dyes:* acridine, acriflavine, azur I, azur II, benzene-azo- α -naphthylamine, Bismarck brown, brilliant cresyl blue, brilliant green, chrysoidin, dimethyl yellow, malachite green, methyl violet R.S., methylene blue, neutral red, rhodamine B, rhodamine-6G, safranin-O, thionine, toluene-azo-amino-toluene 2:1:1':4':3' and toluidine blue. *Acid dyes:* brom-thymol blue, congo red, cresol red, fluorescein, methyl orange and phenolphthalein.

Material. Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo bufo* L.) were captured locally and housed in an outdoor froggery under natural conditions. Six-month old polecats and cats were obtained from a local breeder.

Preparation of the tissue. Frog and toad gastric mucosa was washed, isolated and closed with silk thread at both ends according to the method of Davies (1948a). In all cases the pyloric antrum was completely removed and the