split peptide linkages, but acts in rendering the elastin soluble. It is our belief that possibly the long rod-shaped molecules of elastin are transformed into globular ones without the liberation of amino-acids. Verification of this hypothesis requires further investigation.

The occurrence of an elastolytic enzyme in the human or animal organism has not been shown before. Eijkman (1904) studied the elastin-dissolving activity of bacteria. He prepared, from the lungs of calves, from ligamenta nuchae and from arteries, culture media with which he demonstrated the lysis of elastin as the result of bacterial action. *Bacillus pyocyaneus* showed the highest activity.

SUMMARY

1. Pancreatic extract contains an enzyme which dissolves the elastic fibres of the aorta wall and which is called elastolytic enzyme or elastase.

2. Trypsin prepared according to Kunitz & Northrop (1935–6) has little effect on arteries.

3. The elastase dissolves only the elastin in the aorta wall and leaves the collagen unchanged.

4. Elastase does not liberate amino-acids from the aorta wall, but insoluble elastin is changed into a soluble protein.

5. The pH optimum of the elastase (10·3) is more alkaline than that of trypsin.

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The Reactions of Catalase in the Presence of the Notatin System

By B. CHANCE* (John Simon Guggenheim Memorial Fellow)
Molteno Institute, University of Cambridge

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If hydrogen peroxide (often termed 'bottle hydrogen peroxide' in order to distinguish it from that generated continuously by an oxidizing system) is added to a catalase solution which is concentrated enough for spectroscopic measurements the hydrogen peroxide is all decomposed into oxygen and water in a fraction of a second, thus leaving no time to observe any change in the absorption spectrum of the mixture by ordinary methods. However, by applying the rapid spectrophotometric technique to this reaction it has been possible to detect and to record the absorption spectrum of the catalase-hydrogen peroxide complex which will be referred to as the primary complex (Chance, 1947*a*). If hydrogen peroxide is continuously generated by an oxidizing system, catalase is maintained saturated with hydrogen peroxide for several minutes, and the spectrum of catalase-hydrogen peroxide can be studied by an ordinary spectrophotometric method.

Under these conditions, it has been found that the primary catalase-hydrogen peroxide complex was converted into a new catalase-hydrogen peroxide complex, and that the persistent absorption spectrum which was thus observed belongs to a secondary complex. This complex is catalytically inactive and is analogous to the secondary complex formed by catalase in the presence of alkyl hydrogen peroxides (Chance, 1949*d*). The secondary complex which is also formed in the presence of a solution of 'bottle hydrogen peroxide', is the principal cause of the inactivation of catalase which takes place during the determination of its activity, 'Katalasefähigkeit' (Kat. f.).

If an alcohol and catalase are added to a system composed of an enzyme, catalysing the oxidation of its substrate by means of molecular oxygen which is reduced to hydrogen peroxide, the catalase, instead of decomposing hydrogen peroxide to molecular oxygen and water, uses the peroxide for the oxidation of the alcohol to aldehyde (Keilin & Hartree, 1936). Thus hydrogen peroxide, which is continuously generated when glucose oxidase (or notatin) catalyses the oxidation of glucose to gluconic acid, can be used by catalase for the coupled oxidation of alcohols. A remarkable feature of this reaction is that all the hydrogen peroxide formed in the primary

* Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania.

oxidation reaction is used by catalase in the coupled or peroxidatic oxidation of the alcohol and none is decomposed catalytically to oxygen and water (Keilin & Hartree, 1945).

It has already been shown that the primary catalase-hydrogen peroxide complex reacts with alcohols (Chance, 1947*a*) and causes their oxidation: but the catalase-hydrogen peroxide complex also participates in the decomposition of hydrogen peroxide into water and oxygen. The latter reaction is shown here to be suppressed in coupled oxidations because of the extremely low steady-state concentration of hydrogen peroxide (approx. 10^{-9} M).

The rapid reaction between the primary catalase-alkyl hydrogen peroxide complexes and hydrogen peroxide (Chance, 1949*e*) has been further studied in the presence of the notatin system, and the turnover of a large amount of catalase-bound alkyl hydrogen peroxide has been measured quantitatively. In this reaction, hydrogen peroxide reacts about 30,000 times as rapidly as ethanol.

METHODS

Preparations. The notatin preparation was dissolved in water and, after centrifuging off some insoluble material, was found to have essentially the same absorption spectrum as the pure material; the ratio of extinctions at 380 and 450 mμ. is 1·16, while the ratio for the pure material is given as 1·04 (Keilin & Hartree, 1948). The concentration was determined by measuring the extinction of the solution at a wavelength of 450 mμ. From this the millimolecular extinction coefficient $\epsilon_{\text{mM}}^{450}$ was found to be 11 from the expression

$$\epsilon_{\text{mM}} = \frac{1}{cl} \log \frac{I_0}{I},$$

where c = mM-concentration of notatin (assuming the sample to be pure notatin), l = depth of liquid layer in cm., and I_0 and I = intensities of incident and transmitted light respectively.

One of the horse-liver catalase preparations used here was kindly supplied by Dr E. F. Hartree, the others by Dr R. K. Bonnichsen.

Method. Some of the reactions studied in this paper are relatively rapid and it has been necessary to use a modified form of the flow method of Roughton & Millikan (1936). In order to conserve material, the speed of response and the sensitivity of the photoelectric spectrophotometer have been increased several 100-fold over that used by Millikan (1936). Thus the extinction of the solutions may be recorded in about 0·01 sec., and thereby the duration of the flow of liquid through the apparatus may be very brief and the total expenditure of reactants is proportionately reduced. The greatly increased sensitivity of the apparatus permits the use of very dilute enzyme solutions (approx. 1×10^{-6} M-haematin iron in a 1 mm. bore capillary observation tube) and further contributes to the fluid economy. A great practical advantage of using such dilute solutions is that the halftimes of second-order reactions, which are usually found in enzyme systems, are proportionately longer in these dilute solutions and the measurement of extremely short time intervals (< 3 msec.) is unnecessary. In fact some of the reactions described in this paper fall in the time range above 50 msec., and can be measured spectrophotometrically after the flow of reactants through the observation tube has been abruptly stopped. In this case the fluid economy is much higher than in the flow method.

In a current model of the apparatus shown in Fig. 1 two 1 ml. tuberculin-type glass syringes (*A*) are fused into a stopcock (*B*) to permit their filling with the two reactants to be mixed, or their manual discharge into a four-jet (0·4 mm. bore) mixing chamber (*C*) and 1 mm. bore observation tube (*D*) which are fused to the barrel of the stopcock. Optical measurements are made usually between 8 and 13 mm. beyond the point of mixing. Mixing is complete in less than 1 msec. with this type of apparatus.

Light from the exit slit of a double-grating monochromator (*E*) illuminates the observation tube and the light transmission of the reactants is measured by an RCA-type 929 photocell (*F*). The wavelength range is 370–630 mμ., although the most effective range for the Soret bands of haematins is 370–450 mμ.

Rapid recording and high sensitivity of the optical measurement are obtained only by accurate stabilization of the intensity of the incident light from the light source (*M*) and by careful design of the electronic circuits (*L*) for such stabilization and for the photoelectric measurement of the transmitted light. Currently available circuits measure extinctions with an error of $\log I_0/I \approx 10^{-4}$. Thus it is possible to record the spectroscopic shift at 405 mμ. from catalase to the primary catalase alkyl hydrogen peroxide complex in solutions as dilute as 10^{-7} M (4×10^{-7} M-haematin iron), but such high sensitivity is rarely necessary.

The design principles of such circuits are well known, and their fundamental limitations of the performance can be reasonably well specified mathematically (Chance, 1947*b*). The output of the amplifying circuits is recorded automatically by pen and moving paper for slow reactions (*G*, Fig. 1) or by mirror galvanometer (*H*) and moving photographic paper for rapid reactions (see Figs. 12–14).

Since the flow velocity is not maintained constant, but is manually raised rapidly from zero to some maximum value and then decreased to zero again in order to conserve enzyme solution, the instantaneous values of the flow velocity are measured and are recorded photographically by a second mirror galvanometer on the same record as the optical density trace. A convenient method for measuring flow velocity electrically is to connect the syringe plungers to a potentiometer (*I*) so that a voltage (or current) proportional to displacement is measured. Such a current is readily differentiated to give flow velocity by various electrical circuits, for example, by a series-connected resistance and capacitance (*J*). The output of the latter circuit is amplified (*K*) and recorded (*H*).

The actual value of time after mixing is computed from the quotient of a constant obtained in a separate calibration involving the various mechanical, electrical and hydraulic factors of the apparatus and the magnitude of the oscillograph deflexion.

A great advantage of recording both the flow velocity and extinction traces on moving film (*H*) is that both 'flow' and 'stop' data are recorded, and the single record covers a time range from about 3 msec. to 10 or more sec.; and as much longer as is desired on the ink recorder (*G*). There is, however, a gap in the data from the stop and flow methods between about 10 and 50 msec., but this causes no practical difficulties in these studies.

In certain experiments it is desirable to record the concentration of O_2 or hydrogen peroxide in the observation tube. Spectrophotometric measurements at these wavelengths are not practicable and a polarographic method based on the platinum micro-electrode is employed, since the electrode is small enough to be inserted directly into the capillary observation tube. For O_2 determinations, the electrode (*N*) is connected to a negative potential and the diffusion current may be measured as shown by Davies & Brink (1942). For hydrogen peroxide, the electrode is connected to a positive potential (approx. 0·4 V.) (Chance, unpublished data; Bonnichsen, Chance & Theorell, 1947). The reference electrode (*Q*) may be calomel, although a brass cup has often been used. The current is amplified (*O*) and recorded on a separate trace of the galvanometer oscillograph (*H*) or on a separate pen and ink recorder (*P*). This amplifier

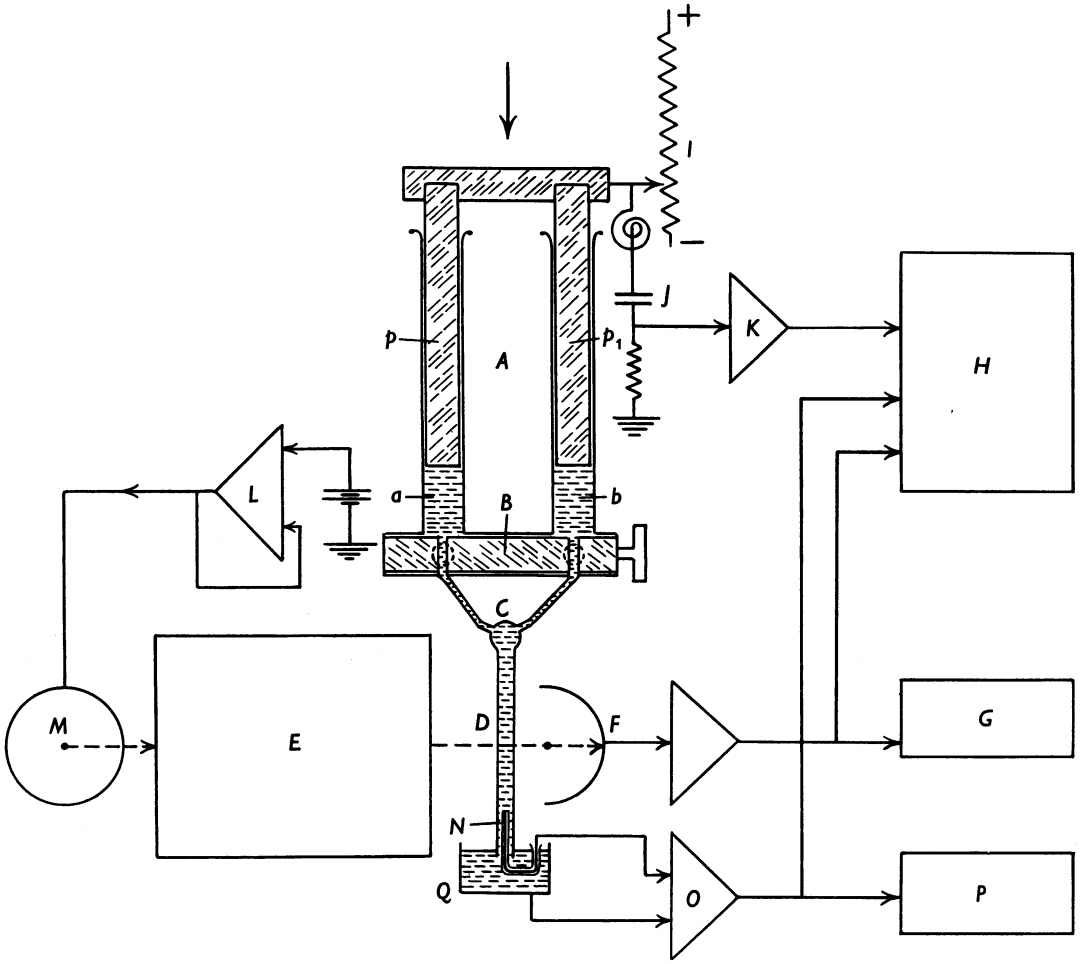


Fig. 1. Schematic diagram of apparatus for the 'accelerated' and 'stopped' flow methods. *A*, two 1 ml. tuberculin-type glass syringes. Plungers *p* and p_1 are connected at the top by a bar and are discharged manually by pushing on this bar. 0·2 ml. suffices for a complete record. *B*, two-way stopcock for filling syringes with reactants or for discharging reactants into mixing chamber and observation tube. *C*, four-jet mixing chamber (0·4 mm. jets). *D*, bore observation tube (1·0 mm.). *E*, double grating monochromator, 370–1000 mμ. (Coleman Electric Co.). *F*, RCA-type 929 photocell and high gain amplifier. Gain approx. 10^5, band width 0–0·1 or 100 cyc./sec., noise approx. 2 μV. *G*, *P*, pen and ink recorders, 0–0·5 cyc./sec. (Esterline-Angus Co.). *H*, three-channel galvanometer oscillograph and moving paper camera, 0–750 cyc./sec. (General Electric Co.). *I*, displacement-measuring potentiometer; linearity 0·2 %. *J*, resistance-capacitance differentiating circuit connected by a flexible wire to displacement potentiometer. *K*, flow-velocity amplifier, 0–100 cyc./sec., gain 100. *L*, lamp voltage-control amplifier; stabilization ratio approx. 7×10^4 amp./V. *M*, tungsten lamp (8·5 V., 4·0 amp.). *N*, platinum micro-electrode, approx. 0·1 mm. wire (see detail). *O*, amplifier for micro-electrode including polarizing circuit, 0–10 cyc./sec., gain approx. 10^5, noise approx. 2 μV. *Q*, reference electrode; here a brass cup is used.

contains special circuits which reduce the effect of flow velocity through the observation tube upon the electrode current (Chance, unpublished data). These circuits cause some error in the recording of rapid change of O_2 tension, but give satisfactory results for the studies of this paper.

RESULTS

The general characteristics of the reactions of catalase in the presence of the notatin system. Fig. 2 (column *A*) shows the reaction kinetics of oxygen, notatin, and catalase as measured in the apparatus shown in Fig. 1. The records of catalase kinetics at 405 mμ. and notatin kinetics at 460 mμ. were measured

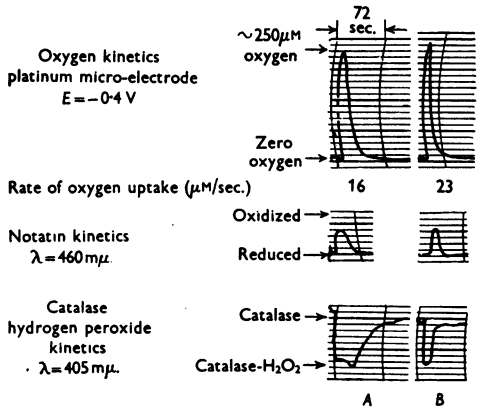


Fig. 2. The kinetics of oxygen, notatin and catalase in the presence of glucose (column *A*) or glucose and ethanol (column *B*); 0·95 μM-notatin, 0·54 μM-horse-blood catalase, 2 mM-glucose, 100 mM-ethanol (column *B* only); pH 6·5, 0·01 M-phosphate. The time interval between the vertical lines in the records is 72 sec. The horizontal lines on the records are not related to the calibration marks at the margins of the records.

spectrophotometrically. At these two wavelengths, there is little mutual interference between the extinction changes caused by the formation of catalase-hydrogen peroxide from catalase or oxidized notatin from reduced notatin. The oxygen kinetics were measured by means of a platinum micro-electrode. In the first column, *A*, the abrupt rise of the top trace indicates the moment at which the deoxygenated solution, remaining in the observation tube of the flow apparatus from the preceding experiment, is flushed out and replaced by a freshly oxygenated solution. At the same time, reduced notatin is replaced by the oxidized form as shown by the rise of the spectrophotometric record at 460 mμ. Since an excess of glucose is present and the observation tube of the flow apparatus is closed to the air, the oxygen uptake of the notatin system begins and causes the decrease of oxygen concentration indicated by the fall of the trace in the platinum micro-electrode record. The oxidase reaction follows this equation:

$$C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O_2. \quad (1)$$

The hydrogen peroxide formed immediately combines with catalase as shown by the spectrophotometric record at 405 mμ. In the identical experiment preceding the one shown, the reaction product is free catalase so that the observation tube is initially filled with free catalase. The abrupt drop of the trace indicates the rapid formation of the catalase-hydrogen peroxide complex.

Contrary to the experiments of the previous paper, where a solution of hydrogen peroxide was added to catalase and the complex began to disappear immediately (Chance, 1947*a*), the continuous production of hydrogen peroxide by the oxidase system (indicated by the continuous fall of oxygen tension in the platinum micro-electrode trace) gives a constant concentration of the catalase-hydrogen peroxide complex for roughly 25 sec., as indicated by the steady deflexion of the trace at 405 mμ.

As the oxygen concentration decreases, more notatin becomes reduced, and the production of hydrogen peroxide from the notatin system finally stops. Catalase breaks down the remaining hydrogen peroxide, and then the catalase-hydrogen peroxide complex spontaneously decomposes into free catalase as described previously (Chance, 1947*a*).

In this experiment, all the hydrogen peroxide is decomposed into water and oxygen by catalase so that the complete equation is

$$2C_6H_{12}O_6 + O_2 \rightarrow 2C_6H_{12}O_7. \quad (2)$$

As Keilin & Hartree (1945) have shown, the addition of an alcohol to this system causes catalase and hydrogen peroxide to oxidize the alcohol to aldehyde instead of decomposing the hydrogen peroxide into oxygen and water. The records of column *B* (Fig. 2) show the effect of 100 mM-ethanol upon this system. First the decrease of oxygen concentration is more rapid since scarcely any oxygen is being supplied from the decomposition of hydrogen peroxide by catalase. Thus the time for the exhaustion of all the oxygen in the capillary observation tube is much less. The catalase-hydrogen peroxide complex forms as before, but in this case the steady-state concentration of the complex is slightly less than the saturation value obtained previously. When the generation of hydrogen peroxide stops due to the exhaustion of the oxygen, the catalase-hydrogen peroxide complex decomposes very rapidly because of its reaction with the ethanol present.

The equation for the reaction occurring when all of the hydrogen peroxide is used in the oxidation of ethanol by catalase is

$$C_6H_{12}O_6 + O_2 + C_2H_5OH \rightarrow C_6H_{12}O_7 + H_2O + CH_3CHO. \quad (3)$$

The Soret bands of the primary and secondary catalase-hydrogen peroxide complexes. The above data show that the catalase-hydrogen peroxide complex is stable when hydrogen peroxide is being generated by the notatin system. If an ordinary spectrophotometer is used, it is necessary to stabilize complex I for several minutes, and therefore the generation of hydrogen peroxide from the dissolved oxygen is prolonged by using more dilute notatin than in Fig. 2. As shown later, ethanol present in the catalase preparation must be removed by a 24 hr. dialysis or the saturation value of complex I may not be obtained.

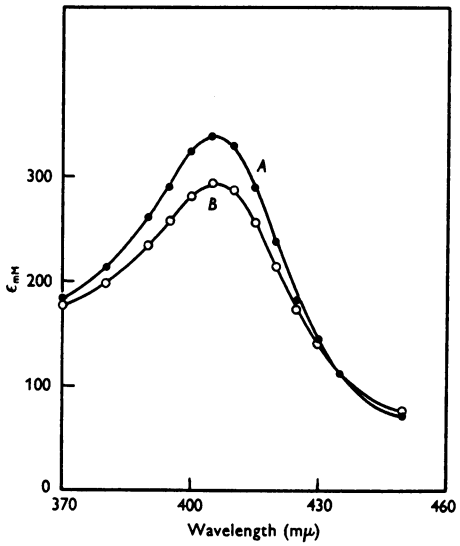


Fig. 3. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide I (*B*) obtained in the presence of notatin, glucose and oxygen; 1·4 μM-horse-liver catalase, $6{\cdot}4 \times 10^{-9}$ M-notatin and 3·3 mM-glucose; pH 6·5, 0·01 M-phosphate.

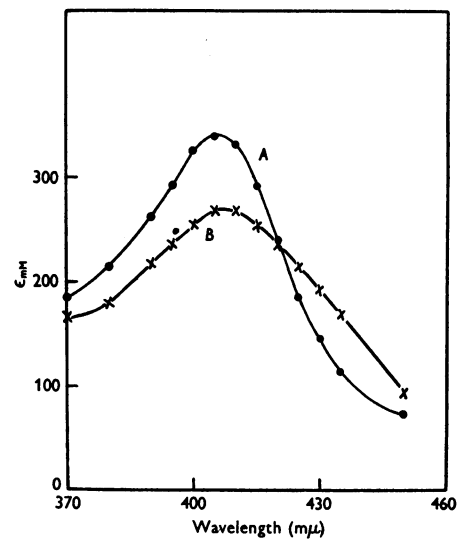


Fig. 4. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide II (*B*) at pH 6·5. The conditions are similar to those of Fig. 3 except that 45 min. elapsed since mixing the solutions. Also the solution was occasionally stirred; 1·3 μM-horse-liver catalase, $1{\cdot}9 \times 10^{-8}$ M-notatin, 6·7 mM-glucose, pH 6·5, 0·01 M-phosphate.

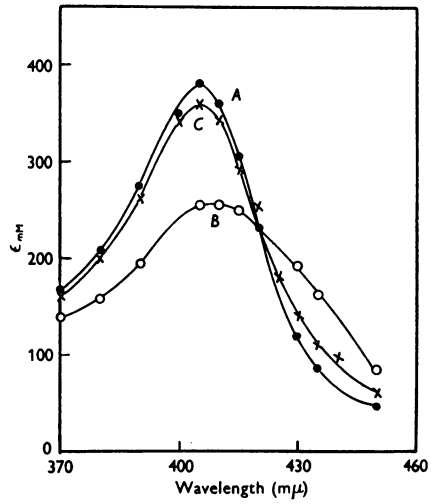


Fig. 5. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide II obtained from horse-erythrocyte catalase (*B*) and the nearly complete recovery of catalase from complex II by stopping hydrogen peroxide generation (*C*); 1 μM-horse-blood catalase, 1×10^{-8} M-notatin, 6 mM-glucose, pH 6·5, 0·01 M-phosphate. Curve *B*, 30 min. after mixing; curve *C*, 1 hr. after covering solution with paraffin oil.

The spectrum of complex I obtained in this way is shown in Fig. 3 (*B*), and is in remarkably good agreement with that obtained by the use of the flow method and a solution of hydrogen peroxide. Here the change of ϵ_{mM}^{405} is 45, and the isobestic point between catalase and complex I is found at 435 mμ. In another experiment, using horse-blood catalase instead of horse-liver catalase, the change of ϵ_{mM}^{405} is 48. Thus the number of catalase haematins bound to peroxide is again shown to be independent of a reduction in the number of intact haematins from 4 to 3 (Chance, 1949*b*).

If a solution of catalase and the notatin system, similar to that used for obtaining Fig. 3, stands exposed to air so that the generation of hydrogen peroxide continues for about 0·5 hr., a very definite change in the spectrum of complex I is obtained as shown in Fig. 4 (*B*); increased absorption appears in the region of 430 mμ. as has been observed upon the formation of the secondary catalase-alkyl hydrogen peroxide compounds. Thus it appears that the

Table 1. *A comparison of the Soret bands of complex II formed in the presence of either the notatin system or ascorbic acid*

(0·91 μM-horse-erythrocyte catalase. Exp. 326.)

Experimental conditions	Wavelength (mμ.) ...	370	380	390	400	405	410	415	420	425	430	435	440	450
4 mM-Glucose, approx. 10^{-8} M-notatin, 0·001 M-phosphate buffer acidified with HCl to give pH 3·8	ϵ_{mM}	134	150	183	225	241	244	233	220	211	196	172	138	76
1 mM-Ascorbic acid, 0·001 M-acetate buffer, pH 4·2	ϵ_{mM}	127	145	174	217	233	239	244	237	220	208	182	149	93

primary catalase-hydrogen peroxide complex has been slowly converted into a secondary and presumably inactive complex. The number of catalase haematins converted into the secondary complex and the kinetics of this reaction are given later.

In a similar experiment with horse-blood catalase, the spectrum of complex II is also seen as shown by Fig. 5 (*B*). If the production of hydrogen peroxide is stopped by covering the solution with a layer of paraffin oil, the secondary complex slowly decomposes, and, after 1 hr., the spectrum of free catalase is obtained. The destruction of catalase during the entire experiment amounts only to less than 5 % as shown by curve *C* (Fig. 5).

A nearly identical Soret band is obtained when ascorbic acid is added to catalase. The data of Table 1 give the values of the extinction coefficient of the complexes formed from the notatin system or from ascorbic acid at pH ≈ 4. The data show the close similarity of the two Soret bands.

Spectroscopy of the primary and secondary catalase-hydrogen peroxide compounds in the visible region. As measured in the microspectroscope (Prof. Keilin made a number of these spectroscopic observations), the addition of 2 mM-glucose and 4×10^{-9} M-notatin to 10 μM-horse-liver catalase caused only a slight diminution of the catalase band at 629 mμ.; the combination of one of the catalase haematins with hydrogen peroxide to form the primary compound caused no other observable spectral shift (but see Fig. 6). After about 0·4 hr., the formation of the secondary catalase-hydrogen peroxide complex was complete, and the red colour and absorption bands of complex II at 536 and 572 mμ. were observed, the band at 629 mμ. having disappeared. Upon addition to catalase of ascorbic acid, which on autoxidation forms hydrogen peroxide, a nearly identical spectrum is observed; the absorption bands lie at 535 and 570 mμ. These bands are the same as those of the secondary compounds of catalase and alkyl hydrogen peroxides (Chance, 1949*c*), and thus this hydrogen peroxide complex has the same covalent iron-peroxide bonding and, as later data show, also lacks catalytic activity.

The proof that complex I has an absorption band beyond 653 mμ. As mentioned above, no shift of the absorption bands of catalase upon formation of complex I could be observed by visual spectroscopy. A definite shift of the catalase spectrum is indicated by Fig. 6. A sensitive spectrophotometer (Chance, 1947*b*) and a 1·3 cm. cuvette containing 8·2 μM-horse-liver catalase were used to record the relatively rapid formation of the primary catalase-hydrogen peroxide complex at 653 mμ., an isobestic point between the catalase and catalase-hydrogen peroxide-II spectra. Fig. 6 clearly shows the formation

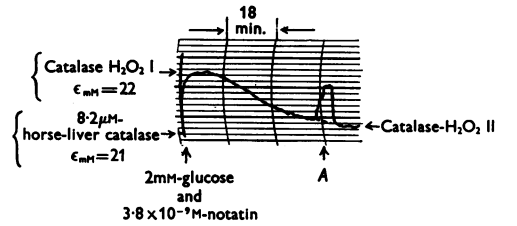


Fig. 6. The rapid formation of complex I of horse-liver catalase and its slow conversion into complex II registered at 653 mμ., an isobestic point between the spectra of catalase and complex II. The artifact at *A* is due to a temporary cut off of the electricity supply; pH 6·5, 0·01 M-phosphate.

of complex I on addition of notatin and glucose. A steady state ensues for some minutes, and then complex I disappears to form complex II. This record demonstrates for the first time that catalase-hydrogen peroxide has an absorption band in the red portion of the visible spectrum as has previously been observed with the primary peroxidase-hydrogen peroxide complex (Theorell, 1941).

The nature of the secondary catalase-hydrogen peroxide complex. In an experiment similar to that described above (Fig. 6), the formation of complex II is recorded in Fig. 7 at 435 mμ. by using appropriately diluted catalase. Upon addition of notatin to the mixture of glucose and catalase, there is an induction period during which the primary complex forms. No density change is, however, measured at 435 mμ. since catalase and complex I are isobestic at that wavelength. After this induction period, the reaction proceeds. The halftime for the formation of complex II is 780 sec., and its concentration reaches a maximum after about 0·5 hr.

To determine whether the reaction is reversible, 170 mM-ethanol was added and reduced the concentration of complex I instantly to very nearly zero because of the peroxidatic reaction between complex I and ethanol. This reduction in the concentration of complex I caused complex II to decompose slowly

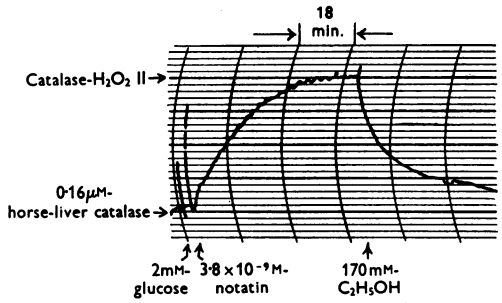


Fig. 7. The slow formation of complex II of horse-liver catalase at 435 mμ., an isobestic point between the spectra of catalase and complex I. The addition of ethanol causes the complex to disappear; pH 6·5, 0·01 M-phosphate.

with a halftime of 600 sec., corresponding to a first-order velocity constant of $1{\cdot}2 \times 10^{-3}$ sec.$^{-1}$ or a value of $k_4 = 7 \times 10^{-2}$ M^{-1} sec.$^{-1}$ which is of the same order of magnitude as obtained with the secondary catalase-alkyl hydrogen peroxide complexes (Chance, 1949*d*). The secondary catalase-hydrogen peroxide complex is therefore nearly completely inactive towards ethanol in the peroxidatic reaction. (The value of k_4 for the active primary complex is 10^3 M^{-1} sec.$^{-1}$ (Chance, 1947*a*).)

The addition of a large excess of hydrogen peroxide to the secondary complex has been found to cause no change in its concentration. Thus the secondary complex is stable in the presence of an excess of hydrogen peroxide and is, therefore, an inhibitor of catalase activity. The addition of ethanol to the complex formed in the presence of ascorbic acid causes the complex to decompose exactly as in Fig. 7.

The Soret band of complex II given in Fig. 4 (*B*), is not so high as that of catalase-methyl hydrogen peroxide II at 435 mμ. $\epsilon_{mM}^{435} = 210$ and the shape of the band and the position of its maximum indicates that not all catalase haematins were converted into the secondary complex under these conditions. This is demonstrated by reactions which were carried out in an open cuvette and which are summarized in Fig. 8. Record *A* shows that catalase cyanide forms very rapidly on adding cyanide to catalase. At 435 mμ., the optical density change is 26 scale divisions. In Record *B*, the secondary catalase-hydrogen peroxide complex is formed as in Fig. 7 with a smaller amplifier gain. Upon addition of cyanide to this complex, the density increment is 15·5 scale divisions which corresponds to the free catalase haematins. Thus $\frac{26-15{\cdot}5}{26} \times 3{\cdot}3 = 1{\cdot}3$ haematin groups are bound to peroxide. In contrast to the previous experiments where hydrogen peroxide bound to catalase in complex I prevents cyanide from attaching to one catalase haematin (Chance, 1949*b*), cyanide can replace peroxide from complex II here: the velocity

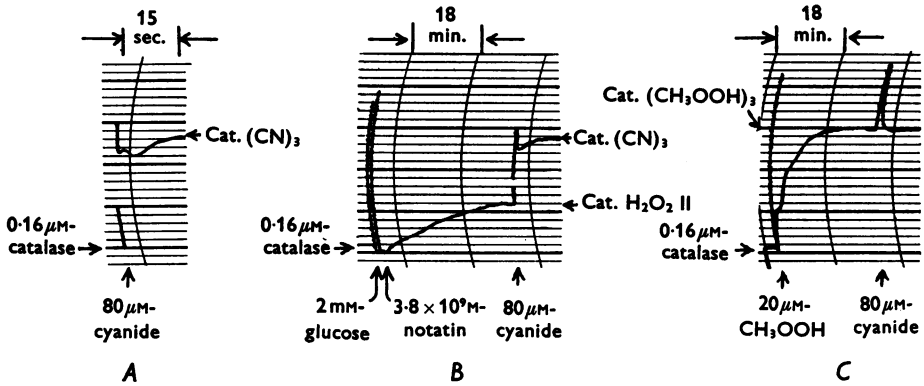


Fig. 8. A demonstration that not all catalase haematins are combined as complex II. Record *A*, the optical density change on addition of cyanide to free catalase. Record *B*, the formation of catalase-hydrogen peroxide II followed by the addition of cyanide. Record *C*, the formation of catalase-methyl hydrogen peroxide followed by the addition of cyanide. This horse-liver catalase was found to have 3·3 intact haematin groups. pH 6·5; 0·01 M-phosphate; $\lambda = 435$ mμ.

of combination of the rather large concentration of cyanide (80×10^{-6} M) with catalase

$$(80 \times 10^{-6} \times 9 \times 10^5 = 72 \text{ sec.}^{-1})$$

greatly exceeds the velocity of combination of the very dilute peroxide (approx. 10^{-9} M) (see p. 397) with

catalase (approx. $10^{-9} \times 3 \times 10^{7} = 0{\cdot}03$ sec.$^{-1}$). Thus these 1·3 haematins are bound as complex II. Record *C* (Fig. 8) shows a control experiment in which the secondary catalase-methyl hydrogen peroxide complex is formed, and, as the data indicate, the spectrophotometric change is larger and no haematin groups are free to combine with cyanide. Since the extinction coefficient of the catalase-alkyl hydrogen peroxide complex and the secondary catalase-hydrogen peroxide complexes are probably the same per haematin-group bound to peroxide (Chance, 1949*c*), $\frac{12}{29} \times 3{\cdot}3 = 1{\cdot}4$ haematins are bound to peroxide in the secondary catalase-hydrogen peroxide complex. Thus only 1·4 catalase haematins have been converted into the secondary catalase-hydrogen peroxide complex at this value of pH.

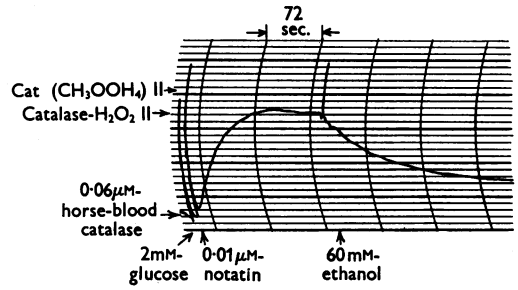


Fig. 9. The formation of the secondary catalase-hydrogen peroxide complex at pH 3·5. Note that time scale is fifteen times faster than in Fig. 7. The phosphate buffer was acidified with phosphoric acid to give pH 3·5 (final phosphate concentration 0·001 M); $\lambda = 435$ mμ.

However, Fig. 9 shows that at pH 3·5, the formation of the secondary catalase-hydrogen peroxide complex occurs much more rapidly than at pH 7·0 and involves nearly all of the catalase haematins. The halftime for formation of the complex is 29 sec. compared with 780 sec. at pH 7·0. Its decomposition time upon addition of ethanol is also smaller; 65 compared with 600 sec. In this case, the addition of ethanol does not liberate catalase to the extent obtained in Fig. 7. Also, the density change on formation of the secondary complex is very nearly as large as that shown for the formation of the secondary catalase-methyl hydrogen peroxide complex: $\frac{32}{38} \times 4 = 3{\cdot}4$ of the 4 erythrocyte catalase haematins are bound to peroxide.

In acid solutions, the more rapid formation of the secondary complex and the greater extent of the reaction is in excellent agreement with the rapid and large decrease of catalase activity as measured by the rate of decomposition of hydrogen peroxide (Bonnichsen *et al.* 1947).

The inhibition of catalase activity by the secondary catalase-peroxide complexes. In order to demonstrate that the formation of the secondary complex inhibits the destruction of hydrogen peroxide by catalase experiments similar to that shown in Fig. 7 were carried out and samples were withdrawn, and, after suitable dilution (to about 1×10^{-9} M), were tested for catalase activity by the method described previously (Chance, 1949*a*). At pH 6·5, complex II is stable for a long interval (see Fig. 7) and is here assumed to be unaffected by the 100-fold dilution.

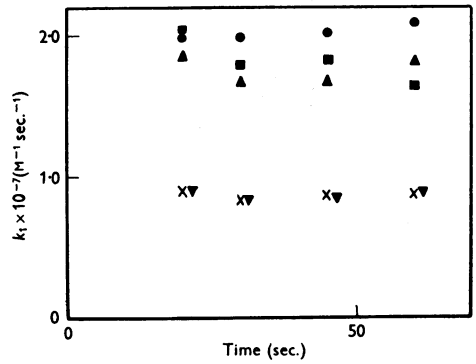


Fig. 10. The inhibition of catalase activity by the formation of the secondary complex at pH 6·5; 1·6 μM-horse-blood catalase mixed with 6×10^{-9} M-notatin and 2 mM-glucose. After the formation of complex II the solution is diluted to $5{\cdot}4 \times 10^{-10}$ M-catalase, 5 mM-hydrogen peroxide being added. Determination of k_1 gives the values represented by ×▼, k_1 being calculated from the equation $k_1 = (2{\cdot}3/et) \log x_0/x$, where e = M-concentration of catalase, t = time (sec.), x_0 and x = initial concentration of hydrogen peroxide and concentrations of hydrogen peroxide at subsequent times t. The untreated control solution gave points ■. Twelve min. after the addition of 0·25 mM-ethanol to the solution containing complex II, the activity test is repeated as shown by the points ▲. The corresponding effect upon the control is shown by the points ●.

The lower set of points, ×▼ (Fig. 10), shows that formation of complex II clearly inhibits the destruction of hydrogen peroxide by catalase. The spectrophotometric data show that about 1·7 haematins of the 4 horse-erythrocyte catalase haematins are converted into complex II at pH 6·5 (Fig. 8), and Fig. 10 shows that the relative decrease of activity is about 50 %. The reduction in activity over the range covered by these data is roughly proportional to the decrease in the number of haematins. Fig. 10 also shows that the inhibition caused by complex II can be largely reversed by the addition of ethanol in accordance with the spectrophotometric data of Fig. 7. In this case, several hours elapsed between the complete formation of complex II and the activity test. Thus the catalase solutions have lost considerable activity due to an irreversible reaction occurring on standing as indicated by the control experiments.

Table 2. *Relation between the inhibition of catalase activity and the amount of catalase-hydrogen peroxide II*

(Spectrophotometric test at $\lambda = 435$ mμ.: 0·17 μM-catalase, 4 mM-glucose, 5×10^{-9} M-notatin. Complete conversion to the complexes gives extinction increments corresponding to 42 and 40 scale divisions on the record respectively in the two experiments below. Activity test: $1{\cdot}7 \times 10^{-9}$ M-catalase, 6 mM-hydrogen peroxide, pH 6·2, $\lambda = 215$ mμ., Exp. 254. k_1 is the bimolecular velocity constant for peroxide decomposition in the equation: rate of decomposition $= k_1$ [catalase] $[H_2O_2]$. Cat. = catalase.)

Time after adding notatin (min.)	0	8	17	23	30	40
Horse-liver catalase						
Extinction increment at 435 mμ. (scale divisions) (Cat. $(CH_3OOH)_3$ II = 42 scale divisions)	0	11	13	23	27	29
Activity k_1 (M^{-1} sec.$^{-1}$ $\times 10^{-7}$)	1·1	0·95	0·94	0·74	0·68	0·55
Horse-blood catalase						
Extinction increment at 435 mμ. (catalase $(CH_3OOH)_4$ II = 40 scale divisions)	0	—	—	—	—	28
Activity k_1 (M^{-1} sec.$^{-1}$ $\times 10^{-7}$)	4·15	—	—	—	—	2·05

The progressive decrease of activity during the formation of complex II followed by withdrawing samples from the spectrophotometric test is shown in Table 2. This particular liver catalase had a rather low initial value of k_1.

In the case of horse-blood catalase, the activity is also reduced to about half the initial value owing to the binding of somewhat more than half the catalase haematins as complex II. It is very interesting that a decrease of the number of free haematins by about half causes a reduction of the activity by about half for both the horse-blood and horse-liver catalase containing four and less than four intact haematins respectively.

In acid solutions, the initial rapid decrease of catalase activity has already been demonstrated (Chance, 1948) and according to the data of Fig. 11, this loss of activity is correlated with the formation of the inactive secondary complex. In a spectrophotometric test at pH 4·0, the halftime for the formation of complex II is 3 min. Curve *A* (Fig. 11) shows that the decrease in the activity of catalase during the activity test by a solution of hydrogen peroxide occurs at about the same speed. The extent of conversion of catalase into complex II is in the spectrophotometric test $\frac{22}{46} \times 4 = 2$ haematins. The activity which was then measured in presence of notatin and glucose, as shown in curve *B* (Fig. 11), has a smaller initial value than the final value of curve *A*. Thus hydrogen peroxide from the notatin system produces a somewhat larger concentration of complex II than does a solution of hydrogen peroxide. Since the dissociation velocity of complex II is more rapid at this pH, the activity increases as shown in curve *B* and approaches the final values of curve *A*.

The addition of ascorbic acid to catalase has already been shown to produce a compound very similar to complex II, and curve *C* shows that the resulting inhibition of catalase activity is slightly greater than that obtained with the notatin system, in accord with a somewhat greater optical density change measured spectrophotometrically upon addition of ascorbic acid to catalase at this pH.

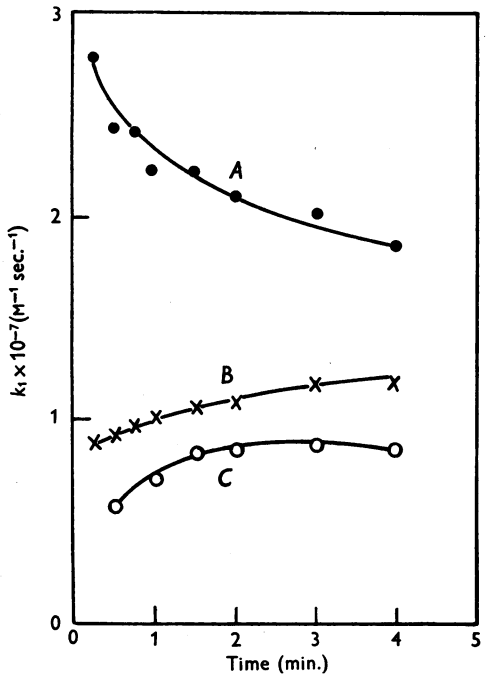


Fig. 11. The inhibition of catalase activity by the formation of complex II at pH 4·0. Curve *A*, activity of untreated horse-blood catalase (1×10^{-9} M) in the presence of 5 mM-hydrogen peroxide. Curve *B*, activity of horse-blood catalase after the formation of complex II in the presence of 4 mM-glucose and 5×10^{-9} M-notatin. Curve *C*, activity of horse-blood catalase after the formation of complex II in the presence of 1 mM-ascorbic acid. All curves in 0·001 M-phosphate buffer acidified with phosphoric acid to give pH 4·0.

At pH 3·6, the formation of complex II is nearly complete in a minute with either a solution of

Table 3. *Inhibition of catalase activity by the secondary complexes at* pH 3·6

(Spectrophotometric test at 435 mμ.: 0·056 μM-horse-blood catalase. Activity test: $1–2 \times 10^{-9}$ M-catalase, 5 mM-hydrogen peroxide; $\lambda = 215$ mμ.; k_1 is the bimolecular velocity constant for peroxide decomposition in the equation: Rate of decomposition $= k_1$ [catalase] $[H_2O_2]$. Activity of catalase at pH 6·2; $k_1 = 3·5 \times 10^{-7}$ M^{-1} $sec.^{-1}$.)

	k_1 at 60 sec. after starting the activity test (M^{-1} $sec.^{-1} \times 10^{-7}$)	Extinction increment at 435 mμ. (scale divisions)	Halftime for formation of the complex (sec.)
Free catalase	1·24	—	—
Catalase + notatin, glucose, and oxygen (as in Table 2)	0·48	27	38
Catalase + methyl hydrogen peroxide (40 μM)	0·15	29	38
Catalase + ascorbic acid and oxygen (1 mM)	0·22	34	220

hydrogen peroxide in the activity test or with the notatin system or methyl hydrogen peroxide in the spectrophotometric test. The extinctions of complex II and the values of k_1, as determined in the activity test, are given in Table 3. The activity of untreated catalase is only one-third of the initial value after 60 sec. exposure to a solution of hydrogen peroxide in the activity test in accordance with previous data (Chance, 1948). The activity of the catalase solutions previously exposed to the notatin system or to methyl hydrogen peroxide is very low.

Since ascorbic acid is very slowly autoxidizable at this pH, the halftime for the formation of complex II is rather large because of the slow rate of production of hydrogen peroxide. Nevertheless, the conversion of catalase into complex II and the inhibition of catalase activity are very large.

In these experiments, as in those summarized in Fig. 11, the activity of catalase treated with the notatin system, methyl hydrogen peroxide or ascorbic acid increases during the activity test indicating a partial dissociation of complex II under the conditions of the activity test.

At pH 3·6, it is possible to form spectroscopically measurable amounts of complex II by direct addition of a solution of (bottle) hydrogen peroxide to catalase, several additions of dilute hydrogen peroxide give about 20 % of the amount formed in Table 3 by using the notatin system.

The reaction kinetics of catalase-hydrogen peroxide I in the presence of the notatin system. Not only has the notatin system led to the discovery of the secondary complex just described, but this system has been very useful in the study of the primary complex of catalase and hydrogen peroxide, not only for the determination of its spectra, but also in studies of the mechanism of catalase action.

In the rapid-flow apparatus (Fig. 12), the formation of the primary complex when a solution of hydrogen peroxide is added (left) is compared with that from hydrogen peroxide formed by the notatin system (right). The record with the solution of hydrogen peroxide begins with the observation tube filled with free catalase. On initiating the flow of reactants, the primary complex is formed completely at the shortest time measured due to the rapidity of the combination of catalase and 200 μM-hydrogen

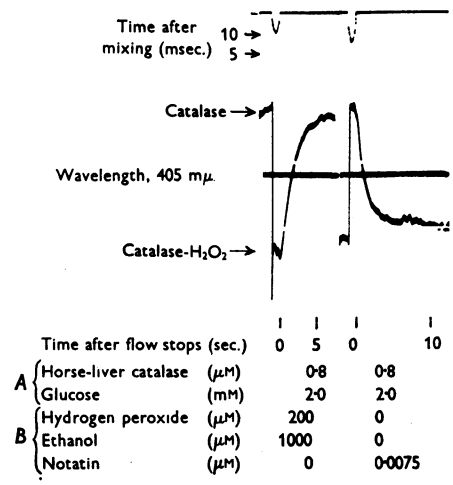
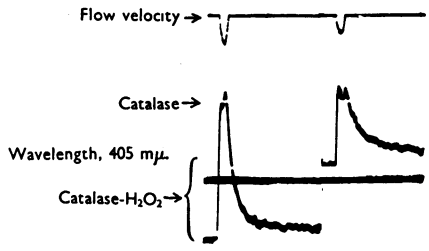


Fig. 12. A comparison of the formation of catalase-hydrogen peroxide I in the presence of a solution of hydrogen peroxide and ethanol (left) and hydrogen peroxide produced from notatin, glucose and oxygen (right). Ethanol causes the rapid disappearance of the primary complex in the left-hand record. Solutions *A* were mixed with solutions *B* in the rapid-flow apparatus; pH 6·5, 0·01 M-phosphate.

peroxide. The complex remains at a constant concentration until the flow has stopped. Then the reaction of the primary complex and ethanol gives free catalase after several seconds. With the notatin system, the record begins with the observation tube filled with the primary complex. On initiating the flow, the observation tube is filled with free catalase, and no formation of the primary complex is seen until the flow has stopped. Then the primary complex

forms in several seconds and remains saturated with hydrogen peroxide produced by the notatin system. The great difference in the speed of formation of the primary complex in these two records is caused by the great difference in the hydrogen peroxide concentration. Not only does the notatin system furnish a small amount of hydrogen peroxide, but the catalase also combines with the peroxide and decomposes it. Nevertheless, the formation of the primary complex goes very nearly to completion because the hydrogen peroxide is continuously generated.

The steady-state concentration of complex I is very sensitive to the presence of ethanol as shown by Fig. 13, where the addition of only 20 μM-ethanol reduces the steady-state concentration of complex I to about one-half the value obtained without



Time after flow stops (sec.)			0	10	0 10
A	Horse-liver catalase	(μM)	0·8		0·8
	Glucose	(mM)	2·0		2·0
B	Notatin	(μM)	0·0075		0·0075
	Ethanol	(μM)	0		20

Fig. 13. The reduction in the steady-state concentration of catalase-hydrogen peroxide I upon the addition of 20 μM-ethanol (right-hand record). Solutions *A* were mixed with solutions *B* in the rapid-flow apparatus; pH 6·5, 0·01 M-phosphate.

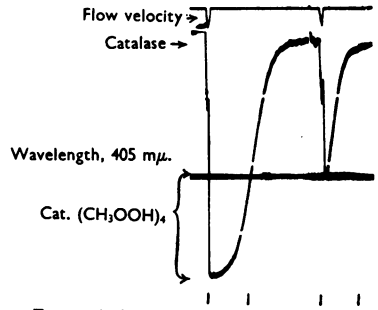
ethanol. For these conditions, the rate of reaction of complex I with ethanol is roughly equal to the rate of formation of complex I. Numerically, the rates of these reactions are equal to the products, pk_4a and $\frac{1}{2}ek_1x$ respectively, where k_4 is the velocity constant for the reaction of the complex I (p) with ethanol (a), and k_1 is the velocity constant for the reaction of catalase (e) with hydrogen peroxide (x) (see Chance, 1948, equations 3 and 4). If these are the only two reactions involved, their rates are equal in the steady state and $x=k_4a/k_1$, since $\frac{1}{2}e=p$. Since $k_1=3\times10$ M^{-7} sec.$^{-1}$ and $k_4=1\times10^3$ M^{-1} sec.$^{-1}$ (Chance, 1947*a*), the value of x is about

$$\frac{10^3\times20\times10^{-6}}{3\times10^7}=7\times10^{-10}\text{M}.$$

This is, however, a minimum value since the 'catalatic' reaction, the reaction of complex I with a second molecule of peroxide (Chance, 1948), is neglected. A mathematical analysis on the basis of two possible mechanisms for these reactions shows that the error caused by this rough approximation is not very large. Complete details will be given later.

The reaction of hydrogen peroxide with catalase-methyl hydrogen peroxide I. In another paper (Chance, 1949*e*), it was shown that methyl hydrogen peroxide groups bound to catalase haematin react with a solution of hydrogen peroxide. It is now possible to study this interesting reaction in more detail by using hydrogen peroxide generated continuously by the notatin system.

In Fig. 14, the formation and decomposition of the catalase-methyl hydrogen peroxide complex requires more than 10 sec. in the presence of ethanol; but if



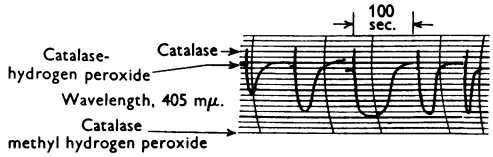
Time scale (sec.)			0 10	0 10
Horse-liver catalase		(μM)	1·6	1·6
Methylhydrogen peroxide		(μM)	26	26
Ethanol		(μM)	400	400
Notatin		(μM)	0	0·42
Glucose		(μM)	2·0	2·0
$-dO_2/dt$		(μM/sec.)	0	5·0
$k_3=x_0/(P_{max}\ t_{\frac{1}{2}})$		(sec.$^{-1}$)	0·4	8·2

Fig. 14. The reaction of catalase-methyl hydrogen peroxide I with hydrogen peroxide continuously generated by the notatin system. The left-hand record shows the kinetics of catalase-methyl hydrogen peroxide I in the presence of ethanol only; in the right-hand record the notatin system is present. Catalase, glucose and ethanol are mixed with methyl hydrogen peroxide and notatin. The oxygen consumption was measured by the platinum micro-electrode; pH 6·5, 0·01 M-phosphate.

hydrogen peroxide is generated at the rate of 5 μM/sec. by the notatin system, there is a dramatic decrease in the saturation value and in the halftime for decomposition of the complex. The value for k_3 calculated by the formula used previously (Chance, 1949*d*), has increased to 8·2 sec.$^{-1}$, equivalent to the addition of about 10 mM-ethanol. Thus this record demonstrates the action of hydrogen peroxide as an acceptor according to the mechanism discussed previously (Chance, 1949*e*).

In Fig. 15, the methyl hydrogen peroxide and notatin concentrations are varied in order to study the reaction more closely. Since ethanol is omitted, the observation tube is initially filled with the catalase-hydrogen peroxide complex as in Fig. 12.

On initiating the flow of reactants, the extinction of free catalase is obtained, and as the flow stops, the methyl hydrogen peroxide combines with the catalase haematins and then reacts with the hydrogen peroxide giving values of k_3 which clearly increase with the notatin concentration and with the rate of oxygen uptake ($-dO_2/dt$), and decrease slightly with



Notatin ($M \times 10^9$)	0·76	0·76	0·76	1·9	7·6
Methyl hydrogen peroxide (μM)	1·2	2·4	4·8	4·8	4·8
dO_2/dt (μM/sec.)	0·028	0·028	0·028	0·07	0·20
$k_3 = X_0/(P_{max}\ t_{\frac{1}{2}})$ (sec.$^{-1}$)	0·076	0·048	0·045	0·13	0·39
$\frac{k_3}{dO_2/dt} \times 10^6$ (M^{-1})	2·7	1·7	1·6	1·9	1·4

Fig. 15. The effect of notatin and methyl hydrogen peroxide concentrations upon the reaction of catalase-methyl hydrogen peroxide I and hydrogen peroxide; 0·8 μM-horse-liver catalase and 2·0 mM-glucose are mixed with varying amounts of notatin and methyl hydrogen peroxide. The blank rate is $k_3 = 0{\cdot}008$ sec.$^{-1}$. The oxygen uptake was measured by the platinum micro-electrode; pH 6·5, 0·01 M-phosphate.

the methyl hydrogen peroxide concentration. The proportionality between the values of k_3 and the rate of oxygen uptake is indicated by the constancy of their quotient given on the bottom row of the Table in Fig. 15.

By using the value of free hydrogen peroxide concentration estimated from the data of Fig. 13 (approx. 10^{-9} M) for $0{\cdot}76 \times 10^{-9}$ M-notatin, the value of the velocity constant for the reaction of catalase-methyl hydrogen peroxide I and hydrogen peroxide is $\frac{k_3'}{[H_2O_2]} = \frac{0{\cdot}048}{1 \times 10^{-9}} = 5 \times 10^7\ M^{-1}$ sec.$^{-1}$. As already stated, the value of the hydrogen peroxide concentration is not very accurate. Nevertheless, the magnitude of the velocity constant strongly suggests that hydrogen peroxide reacts with catalase-bound methyl hydrogen peroxide as rapidly as with catalase-bound hydrogen peroxide.

DISCUSSION

The use of the notatin system for studying catalase has verified and extended the earlier studies of the properties of the primary catalase-hydrogen peroxide complex in which a solution of (bottle) hydrogen peroxide was used. The Soret band of complex I has been accurately measured and verifies the earlier data obtained in the 1 mm. circular capillary observation tube of the rapid-flow apparatus which employs a wider spectral interval (approx. 7·5 mμ., Chance, 1947*a*). The values of the change of millimolecular extinction coefficient ($\Delta\epsilon_{mM}^{405}$) at 405 mμ. on formation of complex I are 45 and 48 cm. for a horse-liver catalase (about 3·3 haematins) and for a horse-blood catalase (4 haematins) respectively. These values agree to within the accuracy with which the catalase concentrations are known. These values are about 10 % larger than those obtained previously, as would be expected from the smaller spectral interval which can be used in these experiments (approx. 0·5 mμ.).

In a previous paper, the change of ϵ_{mM}^{405} per catalase haematin bound to methyl hydrogen peroxide was given as 45 (Chance, 1949*c*). Reasoning as in that paper, the composition of catalase-hydrogen peroxide I is, to within the experimental error, one catalase haematin bound to peroxide. The experimental error is at least 1 % of the total extinction measured ($\epsilon_{mM}^{405} = 380$, error = 4) which gives the value of $1{\cdot}0 \pm 0{\cdot}1$ peroxide-bound haematins. This apparently conflicts with the value, $1{\cdot}2 \pm 0{\cdot}1$ peroxide-bound haematins, previously obtained (Chance, 1949*b*). The accuracy of the two methods is about the same, and the cause of the discrepancy is not known.

Since the applicability of a particular catalase mechanism depends upon whether this value differs from exactly one, the accuracy of these determinations is still inadequate for a final decision.

The existence of an absorption band of catalase-hydrogen peroxide I beyond 653 mμ. has finally been demonstrated spectrophotometrically. Attempts to observe this band visually were, however, unsuccessful since the change of extinction coefficient is very small. This type of absorption band which was first observed in the primary peroxidase-hydrogen peroxide complex (Theorell, 1941) is clearly seen in the primary catalase-alkyl hydrogen peroxide complexes (Chance, 1949*c*) where all of the intact catalase haematins are bound and is now established as being characteristic of the primary complexes.

The sensitivity of the available apparatus in the region above 660 mμ. was not sufficient to locate the peak of the band of catalase-hydrogen peroxide I with accuracy. The absorption band of catalase-alkyl hydrogen peroxide I lies at 670 mμ. Since the isobestic points between catalase-hydrogen peroxide I and catalase, and catalase-alkyl hydrogen peroxide II and catalase lie at about the same wavelengths, 653 and 650 mμ. respectively, the peak of the band of catalase-hydrogen peroxide I probably also lies at 670 mμ. (Chance, 1949*c*).

When a solution of hydrogen peroxide is added to catalase, no formation of the secondary catalase-hydrogen peroxide complex has been observed spectroscopically in any previous study. The formation of this secondary complex, like that of the alkyl hydrogen peroxides, is slow; the probability of the conversion from the primary to the secondary com-

plex is small, and the formation of significant amounts of the latter requires that the steady-state concentration of the primary complex be maintained for some time. As previous records clearly show, a steady concentration of catalase-hydrogen peroxide I occurs for only a few tenths of a second when a solution of hydrogen peroxide is used, and this explains why complex II has not previously been detected spectroscopically. However, with any system which produces hydrogen peroxide continuously at a rate sufficient to saturate catalase with hydrogen peroxide, the formation of complex II is readily observed spectroscopically. Thus complex II, which was first identified using the notatin system, has also been formed in the presence of ascorbic acid and could probably be demonstrated using any of the other hydrogen peroxide-producing oxidase systems studied by Keilin & Hartree (1936, 1945).

The kinetics of formation of the secondary complex have not been studied in detail. However, the rate increases with an increase in the rate of hydrogen peroxide production and with a decrease in the pH. The quantitative evaluation of a reaction velocity constant is difficult because the concentration of free hydrogen peroxide can at present only be measured from the kinetics of complex I in the presence of ethanol as described above. The rate of formation of the secondary catalase-alkyl hydrogen peroxide complexes was found to decrease with decreasing peroxide concentration. Therefore, the comparatively slow formation of the secondary catalase-hydrogen peroxide complex shown in Fig. 7 is in accord with the very small value of the free hydrogen peroxide concentration estimated from the results of Fig. 13 (approx. 10^{-9} M). One may venture to calculate a velocity constant on the basis of the halftime for formation of complex II given in Fig. 7 and this hydrogen peroxide concentration

$$\frac{0{\cdot}7}{730 \times 1 \times 10^{-9}} = \sim 10^{6}\,\text{M}^{-1}\,\text{sec.}^{-1}.$$

The approximate value thus obtained is larger than the values found with the alkyl hydrogen peroxides.

The number of catalase haematins which are bound as complex II varies with the experimental conditions. Using the notatin system, about half the catalase haematins are bound at pH 6·5 and nearly all at pH 3·6 under the same experimental conditions. On the other hand, with ascorbic acid nearly all the catalase haematins are bound at pH 6·5 and 3·6. Furthermore, the conditions for the tests of catalase activity give, in acid solutions, less of the complex II than is formed from the notatin system or ascorbic acid. There is, therefore, an equilibrium between the primary and secondary complexes which depends upon the pH and the manner in which the hydrogen peroxide is produced.

That the secondary complex is in equilibrium with the primary complex has already been suggested by studies of the catalase-alkyl hydrogen peroxides, and is supported by these studies where the removal of the primary complex by the addition of ethanol causes a slow dissociation of the secondary complex. Keilin & Hartree (1945) give excellent confirmation of the fact that complex II will not form when complex I can react peroxidatically with ethanol; in their cyclic oxidations (see their Fig. 2), the activity of catalase continues with no measurable decrease for several hours.

The mechanism by which catalase haematins are progressively converted from complex I to complex II is not yet known. It is suggested that, as soon as a particular haematin-peroxide bond changes from complex I to complex II, one of the remaining free catalase haematins combines with peroxide to form complex I and so on until equilibrium conditions are reached.

There are several reasons for concluding that Lemberg & Foulkes's (1948) catalase-ascorbic acid compound is actually catalase-hydrogen peroxide complex II. The complex I has already been shown to be formed from hydrogen peroxide produced from ascorbic acid oxidation. These studies show that complex I is converted into complex II in the presence of a continuous supply of hydrogen peroxide which can be obtained by oxidation of ascorbic acid. The visible absorption bands and the Soret bands of the complexes formed from ascorbic acid or from the notatin system show great similarity. Both complexes decompose in the presence of ethanol. In both cases, the activity of catalase is inhibited and is here shown to be inhibited in proportion to the amount of complex formed as determined spectrophotometrically at 435 mμ.

There are, however, two discrepancies: (1) the position of the β bands differ by 2 mμ. (Lemberg & Foulkes (1948) find the β band to lie at 565 mμ., a discrepancy of 7 mμ.); and (2), the amount of complex II formed in the presence of ascorbic acid considerably exceeds that formed in the presence of the notatin system at pH 7, but not at pH 3·5–4·0. It is possible that the equilibrium between the primary and secondary complexes is affected by the ascorbic acid molecule itself and thereby both the β-band position and the amount of complex II would be affected.

The discovery of complex II is a most fortunate event for the study of the destruction of hydrogen peroxide by catalase. For the first time, a direct explanation is provided for the inactivation of catalase in the determination of Kat. f. The exponential decrease of reaction velocity constant during the course of the activity determinations by the method of von Euler & Josephson (1927) is in accord with the measured first-order kinetics of

formation of complex II. It has been possible to make measurements of the concentration of complex II and of the activity of this catalase solution containing complex II. These studies clearly demonstrate that complex II is an inhibitor of catalase activity, and permit the explanation of a large number of the phenomena associated with catalase activity which have not previously been fully understood.

At neutral pH, the halftime for the formation of complex II is shown in Fig. 7 to be about 10 min. When the activity of complex I is measured over the first minute only, then a negligible amount of complex II forms, and constant activity is obtained and affords a better method for the measurement of catalase activity (Bonnichsen *et al.* 1947). The success of this method depends only upon measuring the activity before complex II forms; the increase of catalase concentration to about 10^{-9} M is needed only to increase the accuracy of measuring the reaction-velocity constant titrimetrically. This method fails, however, at pH 3·5 where complex II has nearly completely formed in the first minute of the reaction.

In the use of this method, the velocity of the destruction of hydrogen peroxide by catalase increases linearly with the peroxide concentration, at least up to 0·3 M-hydrogen peroxide, and the 'Michaelis constant' found by earlier workers can now be attributed to the increase in the formation of complex II at the higher peroxide concentrations. This effect not only counterbalances the increase of reaction velocity with peroxide concentrations and gives a plateau, but it also causes a decrease in reaction velocity in very high hydrogen peroxide concentrations. It is very likely that complex II is not the only factor causing these effects, probably some irreversible enzyme inactivation occurs.

In their analysis of the effect of hydrogen peroxide concentration upon catalase activity, Lineweaver & Burke (1934) have postulated an inactive enzyme-substrate complex of catalase and hydrogen peroxide; but their mechanism was based upon the supposed Michaelis constant for catalase action of 0·025 M and, therefore, does not represent a formulation of these reactions.

George (1947, 1948, 1949) has studied 'rapid' and 'slow' phases of catalase activity in the presence of very high hydrogen peroxide concentrations. As measured by a rapid manometric technique, the activity of catalase is found to decrease rapidly for about 2 min. at pH 5·85. Then the activity of catalase is relatively constant. The experiments of this paper suggest that the initial decrease of catalase activity is caused by the formation of complex II and that the constant activity thereafter is caused by the catalase haematins not bound as complex II. In fact, a 'peroxide complex having a lower catalytic activity' was postulated by George to explain his results. A number of the effects found by George can be explained qualitatively on the basis of the lack of activity of complex II, and the changes in equilibrium between complex I and complex II caused by hydrogen peroxide concentration and by inhibitors. However, this explanation is not yet applied quantitatively to his experiments for the following reasons: (1) the initial activity of catalase measured by George is only 1·0–2·5 % of that found in these tests; (2) the rate of decrease of activity in George's tests is more rapid than the rate of formation of complex II measured spectrophotometrically in the presence of the notatin system; (3) a quantitative study of the equilibrium of complex I and complex II has not yet been made.

Bonnichsen (1948) has recently studied the relation between the number of intact haematin groups in bile pigment-containing catalases and their activity. The formation of complex II affords a much simpler method of decreasing the number of active catalase haematins of a given catalase.

Since the number of catalase haematins bound to peroxide as complex II increases from about half the total number at pH 6·5 to nearly all at pH 3·6, these studies can be made over a wider range than was possible in Bonnichsen's (1948) work. The decrease of activity during the formation of complex II at pH 5–6·5 can also be studied by withdrawing samples from the solution in which complex II is forming and testing their activity. These exploratory experiments clearly show the decrease of activity caused by the formation of complex II and show rough proportionality between activity and number of free haematins. At pH 6·5, about half the catalase haematins are bound as complex II, and a 50 % decrease of the activity is obtained for horse-blood and horse-liver catalase preparations; a reduction from 4 to 2 active catalase haematins causes about the same decrease in activity as a reduction from 3 to 1 active haematin. This result does not agree with that found by Bonnichsen (1948), where it was concluded that more than 1 free catalase haematin was required for catalase activity. There are, however, large differences in the experimental method; Bonnichsen reduced the number of active haematins by bile pigment formation. Here the bile pigment content is not increased; the haematins are only bound in an inactive peroxide complex.

Highly accurate studies of this relationship between the number of active haematin groups and the catalase activity require an apparatus in which catalase activity and the concentration of complexes I and II can be measured simultaneously and in the same solution. Such experiments require a method about 100 times more sensitive or more rapid than that used in the present studies.

Continuously generated hydrogen peroxide has been very useful in studies of the mechanism of catalase action. With a solution of hydrogen peroxide, it is possible to obtain a steady state only in the concentration of the primary catalase-hydrogen peroxide complex for a few tenths of a second; the hydrogen peroxide concentration itself is decreasing rapidly. With continuously generated hydrogen peroxide, a steady state in both the concentration of complex I and the hydrogen peroxide concentration is obtained for several minutes. Under these conditions, the complicated differential equations for the various catalase mechanisms can be readily solved. This 'double' steady state has been achieved by continuous flow devices (Stead, Page & Denbigh, 1948), but it has not been previously recognized that it could be accomplished enzymically. The solutions of these equations, which will be presented in a later paper, indicate that the approximations used here to calculate the free hydrogen peroxide concentration cannot be far from correct values. By observing that the saturation of the primary catalase-hydrogen peroxide complex, formed in the presence of the notatin system, could be halved by the initiation of the peroxidatic reaction on the addition of a very small amount of ethanol under the particular experimental conditions of this coupled oxidation, it has been possible to estimate the free hydrogen peroxide concentration to be about 10^{-9} M. This low hydrogen peroxide concentration is characteristic of coupled oxidations.

Calculations based on this value give the velocity constant for the reaction of hydrogen peroxide with catalase-methyl hydrogen peroxide I of the same order of magnitude as that required for the reaction with catalase-hydrogen peroxide I in the usual 'catalatic' reaction. The similarity of these two reaction velocity constants is in accord with the suggestion that hydrogen peroxide is here acting as an acceptor; other data show that the velocity of reaction of the acceptor molecule with the enzyme-substrate compound is usually unaffected by whether hydrogen peroxide or alkyl hydrogen peroxides are bound to catalase haematin. The theory of catalase action which postulates a peroxidatic reaction between the primary catalase-hydrogen peroxide complex and hydrogen peroxide, receives support from these experiments. It is interesting that such a small hydrogen peroxide concentration causes such a rapid reaction; in Fig. 13 approx. 10^{-9} M-hydrogen peroxide caused the same reaction velocity as would have 5×10^{-5} M-ethanol.

In this reaction, hydrogen peroxide is acting as a reducing agent, whereas it acts as an oxidizing agent in the oxidation of alcohols by catalase hydrogen peroxide. The range of possible modes of action of catalase peroxides in biological systems are: (1) the breakdown of hydrogen peroxide into water and oxygen in which hydrogen peroxide molecules are both oxidant and reductant; (2) the oxidation of substances similar to the lower alcohols, methylene glycol and formic acid with (*a*) hydrogen peroxide as substrate, (*b*) alkyl hydrogen peroxides as substrates; (3) the oxidation of alkyl hydrogen peroxides with hydrogen peroxide as reductant.

SUMMARY

1. With hydrogen peroxide continuously generated by the notatin system, an absorption band beyond 653 mμ. in the spectrum of the primary catalase-hydrogen peroxide complex (complex I) has been found and this completes the spectroscopic analogy between catalase-peroxide and peroxidase-peroxide complexes in which this type of absorption band has previously been observed by visual spectroscopy to lie at 670 mμ.

2. In the presence of the notatin system, the Soret band of the primary catalase-hydrogen peroxide complex has been measured in the ordinary spectrophotometer and agrees substantially with that obtained previously by using the flow method and a solution of (bottle) hydrogen peroxide.

3. The change of millimolecular extinction coefficient from catalase to catalase-hydrogen peroxide I at 405 mμ. *i.e.* $\Delta\epsilon_{mM}^{405}$ is 48 for horse-erythrocyte catalase which agrees to within the experimental error with the value for a horse-liver catalase. On comparison with the value for catalase-methyl hydrogen peroxide I, the composition of catalase hydrogen peroxide I is $1{\cdot}0 \pm 0{\cdot}1$ catalase haematins bound to hydrogen peroxide in the primary complex.

4. With the notatin system, the conversion of the primary catalase-hydrogen peroxide complex into an inactive form (complex II) has been observed. This complex has visible absorption bands at 536 and 572 mμ., and is analogous to the inactive secondary catalase-alkyl hydrogen peroxide complexes except that the number of catalase haematins bound as complex II varies with the experimental conditions.

5. Complex II is formed in the presence of hydrogen peroxide produced by the autoxidation of ascorbic acid or by the notatin system. The catalase-ascorbic acid compound of Lemberg & Foulkes (1948) is probably complex II. A small amount of complex II has been observed spectroscopically by direct addition of a solution of hydrogen peroxide to dilute catalase.

6. The rate of formation of complex II increases with an increase in the steady-state hydrogen peroxide concentration and with a decrease in pH.

7. As determined by the rate of destruction of hydrogen peroxide, the activity of catalase partially converted into complex II is proportional to the number of free haematins. At pH 6·5, about half the

catalase haematins are 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 ($3{\cdot}5 \times 10^{7}$ M^{-1} 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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