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On the Hematin and the Bile Pigments in Catalase

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The protein part of blood and liver catalase from horse has previously been found to be identical [1], but there is a great divergence of opinion about the relation between the hematin and the bile pigments in liver catalase in the literature.

Sumner, Dounce and Frampton [2] found that the activity and the protohematin iron content varied much in their liver catalase preparations.

Lemberg and Legge [3] also found a great variation in the ratio bile pigment iron/total iron, from 0.3—0.5. They were of the opinion that this large amount of bile pigment could not be formed during the preparation and considered it more likely that bile pigment is preformed *in vivo* in liver catalases.

In a previous publication we reported bile pigment corresponding to only 10 % of the total hematin iron in our liver catalase preparations [4].

The object of this work was to study this discrepancy in the ratio bile pigment/total hematin.

Ox blood catalase [5], and horse blood catalase [6] contain only protohematins, so bile pigments are not a constituent of these catalases.

In our experiments, the properties of blood catalase, which contains 4 protohematins/molecule, were compared with those of liver catalase, which has a varying number of protohematins/molecule. In each case our improved method of determining the activity was used [4].

As a result of these experiments, the conclusion is drawn that the bile pigment in liver catalase preparations is an artifact, or in any case only small amounts can be present in the liver catalase *in vivo*.

Additional investigations of blood and liver catalases from different species of animals have been made. Besides information about the bile pigments in liver catalases other conclusions have been drawn in the discussion from the results obtained.

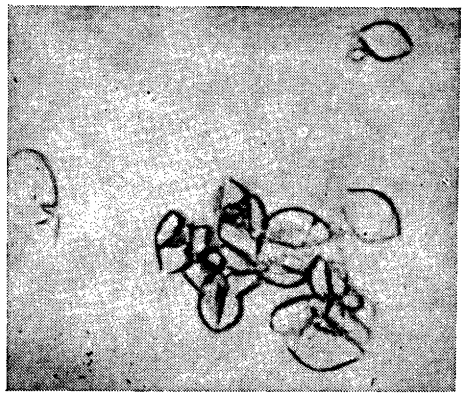

Fig. 1. Crystalline guinea pig liver catalase, crystallized from ammonium sulphate (× 200).

EXPERIMENTAL PART

Preparations

The catalases referred to later as pure are prepared according to earlier descriptions [1]; they were crystallized from ammonium sulphate solution.

The guinea pig liver catalase was prepared by a simplified method. Other liver catalases can be prepared in this way and the method allows a very rapid preparation. One hour is enough to achieve a preparation with a ratio of the extinction at 280 mμ to 405 mμ, 2 : 1; then the dialysis takes some time corresponding to a percity of 50 %.

1. The livers are ground in a »Turmix» with a double amount of water for a couple of minutes.

2. Immediately afterwards the extract is shaken with one tenth of its volume of an alcohol-chloroform mixture 5 : 1 and centrifuged.

3. The alcohol concentration is then increased to 70 vol %. The enzyme then precipitated together with some inactive protein.

4. The enzyme is dissolved in distilled water and dialyzed, whereby some inactive protein precipitates and is discarded. Crystallisation from ammonium sulphate can now be carried out as usual [1]. Other liver catalases at this step contain large amounts of ferritin, that can be removed using acetate buffer and alcohol as previously described [1].

In Table 1 are shown some figures from two guinea pig liver catalase preparations. No. 1 is prepared from healthy animals. No. 2 from animals used for experiments with infective material.

The yield is 700—800 mg enzyme from 500 g of liver. Fig. 1 shows the crystalline enzyme. Guinea pig livers weighing from 12—20 g contain about 50 mg catalase, and it is possible to prepare the pure enzyme from a single liver.

Certain precautions should be taken in all liver catalase preparations as will be shown later:

1. The livers should be used as soon after the animal is killed as possible, and the extraction time only last a couple of minutes.

2. The alcohol-chloroform added should be just enough to denature the hemoglobin.

3. The enzyme must never be left standing in the alcohol-chloroform solution, but immediately either be precipitated with alcohol or the alcohol-chloroform removed by evaporation *in vacuo*.

Bile pigment determination

The bile pigment was determined in some cases after the method of Lemberg, Lockwood and Legge (Addition of glacial acetic acid and extraction with ether)[7]. By this method Lemberg and Legge[3] recovered 50—70 % of the protohematin; the rest was bound to the denatured protein. In the determination of bile pigment they assumed the same loss as found in the protohematin determinations. Evidence given below indicates that this calculation gives too high a figure for the bile pigment in our preparations.

In our experiments the method of Lemberg and Legge[3] also gave about the same loss. When the enzyme was split in HCl-acetone we found that the loss of hematins was much less from 10—30 %, depending upon the amount of HCl used, but the recovery of bile pigment was found to be constant and independent of the strength of the acid.

Even small amounts of HCl (*e. g.* 0.2 ml *N*/1 HCl to 20 ml acetone) split off all the bile pigments, in agreement with earlier determinations by Agner[8].

As a control the denatured protein from catalase that was split in either acetic acid or in HCl-acetone was dissolved in water, and strong HCl was added. If the protein did not dissolve in water the HCl acetone was added with stirring. After this treatment only a trace of bile pigment was found in the aqueous phase. While almost all the protohematin was recovered.

When a blood catalase is split either in strong acetic acid or in HCl-acetone (*e. g.* 0.05 ml *N*/1 HCl to 20 ml acetone) the hematins are completely split off leaving a white protein which does not give any pyridine hemochromogen spectrum.

If the hematins are determined in the blood catalase before it is split by the standard method of Lemberg and Legge or by Agner[8], and the amount of recovered hematins determined, there is always a constant loss in hematins ranging from about 7 % when the enzyme is split in HCl-acetone to 10—12 % when split in acetic acid.

Both these methods require many manipulations, and the mentioned loss is probably due to minor losses adding up during the procedure in the standard method to the figures above. When calculating the loss of bile pigment during the splitting of liver catalase with the same methods, these same losses were assumed.

Some figures from bile pigment determinations in different liver catalases are given in Table 1.

Table 1. Analytic data for different catalase preparations.

Catalase	$\beta \times 10^7 \cdot cm^2 \cdot mole^{-1}$ CN⁻			k_1^4 22°C	Bile pigment % of total hematin	Protohematin % of dry weight	Ratio $^{630}/_{570}$ mμ of the hemochromogen spectrum	Iron % of dry weight	Calc. ratio bile pigm. / total hematin
	280 mμ	405 mμ	425 mμ						
Human blood	70	86	73	3.5	—	1.08	0.12	0.09	—
Horse blood	68	86	73	3.5	—	1.08	0.11	0.098	—
Horse liver 1	69	76	62.9	3.0	14	0.92	0.23	0.097	0.10
Horse kidney	78*	84	—	3.4	—	1.01	0.20	0.100	0.15
Guinea pig liver 1	68	84	70	3.4	—	1.01	0.15	0.089	—
Guinea pig liver 2	70	65	53	2.4	22	0.80	0.52	0.100	0.31
Horse liver 2	68	50	40.3	1.1	40	0.62	0.74	0.098	0.45
Horse liver 3	68	63	47	1.4	29	0.76	0.59	0.092	0.35

Hemochromogen spectra

Lemberg and Wyndham [9] found that their liver catalase preparations possessed a hemochromogen with an absorption band in red at 651 mμ. They assumed this was due to another hemochromogen besides protohemochromogen. The ratio of the absorption at 651 and 557 mμ was found to be 0.25.

Fig. 2 shows the hemochromogen spectrum of some of the catalases from Table 1. Horse blood has very little and horse liver catalase no. 1 has some absorption in the red, the ratio 650/557 mμ being about 0.1.

Horse liver nos. 2 and 3 have a much larger absorption in the red, the ratio being 0.36 and 0.25. A definite band can be seen at 651 mμ, which also was visible in a hand spectroscope.

Lemberg and Legge [3] have described the CO-hemochromogen spectra of liver catalase. They found an absorption band at 630 mμ, thus confirming Lemberg and Wyndham's assumption that another hemochromogen besides protohemochromogen was present in their liver catalase preparations. The ratio between the absorptions at 630 and 570 mμ was 0.50 in most cases. Assuming that the catalase CO-hemochromogen band at 630 mμ had the same molar extinction as CO-cholehemochromogen they could calculate the ratio bile pigment/total hematin from the formula $[\varepsilon(630/\varepsilon570)—0.047] / [(0.55\ \varepsilon630/\varepsilon570) + 0.96]$ ($\varepsilon = \log Io/I$) and found agreement with the results obtained from the direct determination of the bile pigment.

* Some cloudiness in the solution.

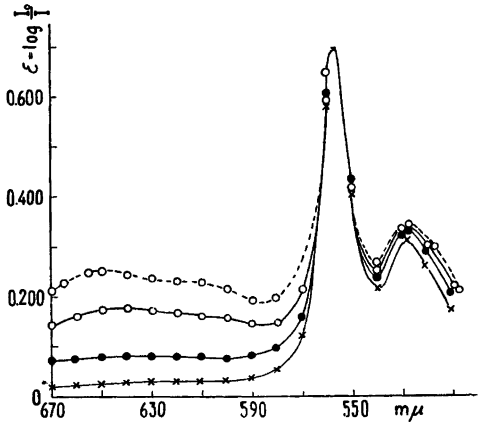

Fig. 2. Hemochromogen spectra of different catalases, extinction corrected to the same value at 557 mμ. ×—× *horse blood cat.,* ●—● *horse liver cat. no. 1,* ○—○ *horse liver cat. no. 3,* ○ — — ○ *horse liver cat. no.2.*

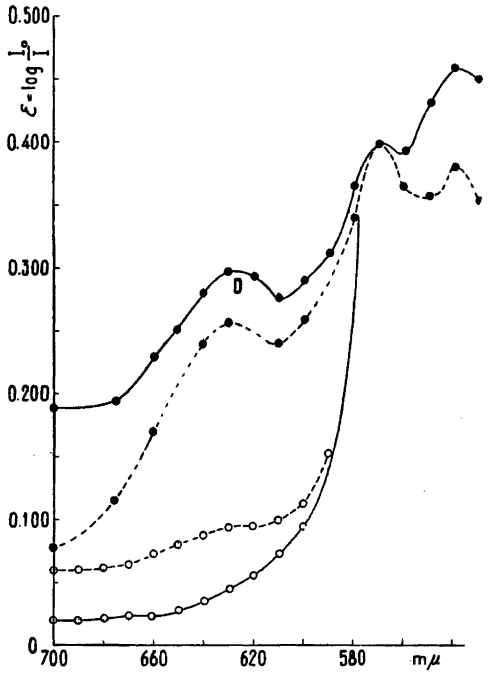

Fig. 3. CO-hemochromogen spectra of catalases with varying number of hematins. Extinction corrected to the same value at 570 mμ. ○—○ *horse blood cat.,* ○ — — ○ *horse liver cat. no. 1,* ● — — ● *horse liver cat. no. 3,* ●—● *horse liver cat. no. 2.*

Fig. 3 shows the CO-hemochromogen spectrum of some of the catalases from Table 1. In the table the bile pigment has also been calculated from the above mentioned formula, but the blood catalase absorption at 630 mμ has first been subtracted. With this modification we also found agreement between the determination of bile pigment from the above mentioned formula and the direct determination.

Analyses of liver catalase in the different steps of the preparation

A large number of experiments have been carried out on different liver catalase preparations in order to find the cause of the disagreement between our results and those of earlier authors concerning the bile pigments.

The reasons could be that:

1. Catalase where more than one hematin is converted to verdohematin might be more susceptible to denaturation by the reagents used by us in the preparation — thus the final preparation would consist of a more resistant catalase fraction with less bile pigment.

2. The bile pigment content could vary from horse to horse.

3. The methods of determining activity and bile pigment might give different results in the hands of different authors.

4. If hematin disintegrating enzymes are found in the liver, the way in which it is extracted and the time elapsed since the animal was killed could influence the bile pigment in the liver catalase.

The bile pigment content and the activity have been followed through the different steps in horse and guinea pig liver preparations. Some results are seen below. The activity had to be measured as a relative activity. This was done by determining the absorption at 405 mμ and using a β of $86 \cdot 10^7 \text{cm}^2 \cdot \text{mole}^{-1}$ in the calculation.

	Bile pigment % of total hematin	Ratio 630/570 mμ	k_1^4 mole^{-1} · seconds^{-1}
Before acetone fractioning	10.5 %	0.15	3.4
After acetone fractioning	12.8 %	0.12	3.3
After alcohol fractioning and crystallization	14.0 %	0.20	3.3

We have found that alcohol and chloroform used in a high concentration denatures the enzyme, but even the usual alcohol-chloroform amount slowly inactivates the catalase. Inactive hematins have a Soret band and gives the same pyridine hemochromogen as protohematin, but they have no activity and do not combine with CN^-. The figures below show a catalase before and after standing four days in chloroform-alcohol solution.

	Bile pigment % of total hematin	Ratio 630/570 mμ	k_1^4 mole^{-1} · seconds^{-1}
Immediately after adding alcohol-chloroform	8.5 %	0.12	—
After 4 days in the alcohol-chloroform	22 %	0.62	1.7

The same catalase after purification and crystallization is referred to as H. L. no. 3 in Table 1. It was not possible to obtain any fraction with more or less bile pigment.

In one single case in a horse liver containing numerous small and large parasitic granuloma, bile pigment in much greater proportion than usual was found in the catalase. The ratio bile pigment/total hematin kept nearly constant during the preparation though increasing a little, and even repeated fractionation with both alcohol and acetone did not denature any part of the enzyme, and no fraction with more or less bile pigment could be obtained. This catalase after crystallization is no. 2 in Table 1.

Catalase with about 2.3 hematins/molecule seems to be very resistant towards the reagent used for the preparation.

These two catalases with a low hematin content could not be crystallized from ammonium sulphate alone, but using dioxane as described by Sumner *et al.*[10] the enzyme could easily be crystallized. The crystals were green and not black as those of usual catalase.

The hematin content of intact catalase

Some control experiments have been carried out by Chance in this institute on the hematin content of the above mentioned catalases with a low hematin content. The discovery of the primary intermediate compounds of catalase with hydrogen peroxide and with alkyl hydro-peroxides bound to respectively one and all intact hematins in the catalase molecyle permits a determination of the number of intact hematins by a comparison of the spectral shifts on formation of these complexes [11].

Also the velocity of oxidation of alcohols by the primary alkyl hydro-peroxide complexes depends not only upon the catalase concentration but also upon the number of intact hematins in the catalase and thus enables us to determine the number of hematins in relation to blood catalase, which contains 4 hematins/molecule [11].

The density change at 405 mμ on the formation of catalase-hydrogen peroxide is 11.5 scale divisions and therefore four hematins bound to peroxide would give 46 scale divisions. But this liver catalase preparation gave only 26 scale divisions on reaction with methyl hydro-peroxide, thus there are $\frac{26}{46} \cdot 4 = 2.3$ hematins.

The velocity constant for the oxidation of alcohols is 590 mole^{-1} · seconds^{-1} for this preparation whereas blood catalase (of the same density at 405 mμ)

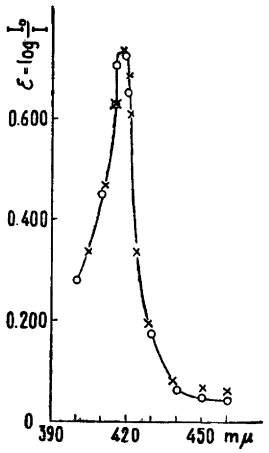

Fig. 4. CO-pyridine hemochromogen spectra of horse liver and blood catalase. 0.3 ml 1 N NaOH, 0.5 ml pyridine and 10.4 γ hematin, made up to 4 ml with water. × blood catalase, O liver catalase.

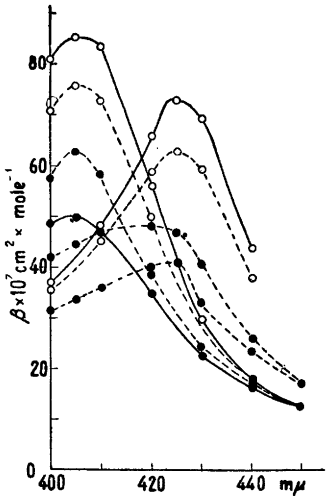

Fig. 5. The Soret band of different catalases from Table 1, and their CN^- compound.
O——O *horse blood catalase*
O--O *horse liver catalase no. 1*
●——● *horse liver catalase no. 2*
●--● *horse liver catalase no. 3*

gives a velocity constant of 1000 mole^{-1} · seconds^{-1}. The relative hematin content is $\frac{590}{1000} \cdot 4 = 2.4$ hematins.

Similar experiments with H. L. no. 2 gave 2.5 active hematins/molecule.

Post mortem changes of catalase in liver tissue and in liver extract.

No measureable increase in the bile pigment was found in the liver catalase prepared from liver tissue that was stored for two days in the ice-chest, but 14 days storing gave 20.7 % bile pigment.

In water extracts of liver tissues with 10 % ammonium sulphate kept at 20° C the catalatic activity is diminishing in a few days. The solution turns green due partly to conversion of hemoglobin into choleglobin. The catalase is destroyed if the solution is shaken with alcohol-chloroform, and fractionation with ammonium sulphate does not allow a complete separation of hemoglobin and choleglobin from the catalase.

The method of Bonnichsen, Chance and Theorell [4] used in this paper is more accurate than older methods and gives somewhat higher activity values

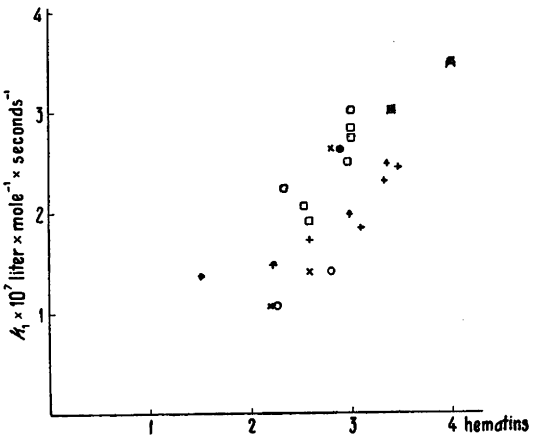

Fig. 6. k_1 plotted against the number of hematins determined from pyridine hemochromogen determinations and from the CN^- compound. △ horse blood cat., ● guinea pig liver cat., ○ horse liver cat., hematins determined from pyridine hemochromogen, × the CN^- compound of the same catalases. + values from Sumner et al.[2], □ *values of Lemberg and Legge*[3] *see text. 22° C.*

of the catalase preparations. The inaccuracy of older methods alone, however, cannot be the only cause of the great variation in the activity values of the different preparations prepared by other authors.

The bile pigments have been determined spectrophotometrically using the extinction coefficient used by Lemberg and Legge [3] and the values given in this paper are comparable to those of Lemberg and Legge.

Absorption spectra

Table 1 gives the extinction coefficient for the Soret band and the maximum at 280 mμ for several catalases in phosphate buffer pH 6.8. The purity of these preparations can be judged from the absorption at 280 mμ [1].

The human blood catalase since it was first described (1), has been further purified using acetate buffer as for the blood catalase from horse.

The horse blood and the human blood catalase have the same spectral properties. Horse kidney and one of the guinea pig liver catalases are almost identical to blood catalases. The extinction coefficient at 280 mμ is seen to be about the same for catalases from different animals.

The table shows further that in agreement with the results of Holden and Lemberg [12] on isolated verdohematin the bile pigment group in the liver cannot have any light absorption comparable to that of protohematin in the region of 405 mμ.

As the group of the liver catalases that yields bile pigment might interfere with the pyridine-hemochromogen determination, the hematin content was

also determined from the peak of the Soret band at 418 mμ of the CO-pyridine hemochromogen, as neither bile pigment nor verdohematin have any Soret band [12]. The result agreed as well as could be expected with the direct determination of hematins from the pyridine hemochromogen band at 557 mμ, thus the absorption of the verdohematin or the bile pigment is low at these two wavelengths compared to the strong band at 557 mμ and the still stronger band at 418 mμ.

Fig. 4 shows the Soret band of CO-pyridine hemochromogen at 418 mμ of blood and liver catalases from horse. There is no sign of any Soret band different from that of protohematin in this region.

The extinction coefficient for the peak of the cyanide compound at 425 mμ in the table seems only to be influenced by the number of active hematins in the molecule. Fig. 5 shows the Soret band of the different catalases in phosphate buffer and in cyanide. The band from horse liver no. 3 though seems to have its maximum shifted towards ultraviolet, but the absorption at 425 mμ was used to calculate the numbers of active hematins. This catalase as mentioned contained some inactive hematins.

In Fig. 6 the activity k_1 [4] is plotted against the number of hematins calculated from the pyridine hemochromogen determination and also from the cyanide compound $\beta \cdot 73 \cdot 10^7$ cm$^2 \cdot$ mole^{-1} from the blood catalase used as standard for 4 hematins per molecule.

DISCUSSION

Guinea pig liver catalase has been prepared without bile pigment so this cannot be a normal constituent of this catalase. Horse liver catalase has been prepared with about 8 % bile pigment in the first steps of the preparation increasing to about 14 % in the crystalline enzyme.

The conclusion is drawn that under normal conditions *in vivo* there is probably no bile pigment present in liver catalase. Several factors have been found to increase the bile pigment content of the liver catalase: post mortem changes, infections in the animals, the use of too much alcohol-chloroform or the standing for some time in the alcohol-chloroform solution.

No reliable method can be given to obtain horse liver catalase entirely free from bile pigment. Even if the greatest care is taken variable amounts of bile pigments or inactive hematins are found to be present.

In our usual preparations the number of active hematins are 3—3.5/ molecule. A rather drastic treatment of the catalase or the use of abnormal livers was necessary to prepare catalase with 2.3—2.5 hematins per molecule.

The former agrees with both Agners determination of protohematin in liver catalases [13, 8], and with Theorell and Agners [14] magnetic titration of liver catalase as well as with the data of Agner and Theorell [15] for titration with floride. These data show a variation of active hematins from 3 to about 3.5.

In blood catalases bile pigment has never been found in any preparation. On standing for some time in the alcohol-chloroform they are inactivated without any formation of bile pigment [16]. The only difference found in the behaviour of the protohematin of blood and liver catalase is the difficulty with which part of the liver hematins is split off from the protein, and this difficulty seems to vary from preparation to preparation.

Lemberg and Legge [3] increased the bile pigment content of their liver catalases by adding large amounts of hydrogen peroxide to an enzyme solution containing ascorbic acid. Some preliminary results have shown that if this experiment is repeated without ascorbic acid at pH 7 on a blood catalase it results in the hematins becoming more difficult to split off from the protein. At the same time the activity decreases somewhat. No bile pigment is formed. Hydrogen peroxide plus ascorbic acid oxidize hemoglobin [17] and probably the partly inactivated hematins of a catalase are also affected by ascorbic acid and hydrogen peroxide.

Nothing is yet known of the function of blood catalase but liver catalase may oxidize alcohols by coupled oxidations [18], or directly by the means of the catalase-peroxide compound [11]. Thus catalase may function as a peroxidase with hydrogen or alkyl hydroperoxides in the liver metabolism. If the function in the blood is similar the higher metabolic rate of the liver could explain the fragility of the liver enzyme.

More than one hematin/molecule seems to be necessary for the catalatic activity. This would speak in favor of the »internal» reaction type for the splitting of hydrogen-peroxide by catalase in agreement with the theory put forward by Theorell [19].

Sumner, Dounce and Frampton [2] have given some figures about the variation of activity with the bile pigment content in their catalase preparations. 4 hematins should have a Kat. f. of 60,000, 3 hematins 45,000 etc. The points of the curve published by them do not go through the origin, however, as already pointed out by Lemberg and Legge [3], and figures can also be taken in favor of one hematin/molecule being catalatically inactive. See Fig. 6 where the figures of Summer *et al.* have been plotted.

Lemberg and Legge [3] have also plotted bile pigment/total hematin against activity. They found the activity proportional to the number of active hematins. Assuming that the bile pigment value determined by them is too high,

this might change their curve in favor of our data. In figure 6 the data of Lemberg and Legge [3] are plotted having been recalculated on the base that the loss in bile pigment is constant and about 10 %; corrections have also been made for the Kat. F. They assumed that Kat. F. for a four hematin catalase was 52,000, whereas we found 61,000 [4]. None of their data would then speak against one hematin being catalatically inactive.

Since the catalase Soret band is due to the conjugated double bonds of the tetra pyrrol ring and this ring has been opened in the verdohematin [12], it could thus be expected, that the »verdohematin» in catalase does not have a noticable light absorption in region of the Soret band. The experimental results confirm this.

CN- is bound with covalent bonds to the hematin iron [14] and probably to the same place as is the hydrogen-peroxyde [11], to the hydroxyl group found by Agner and Theorell [15].

That peroxides do not combine with verdohematin has been shown by Chance [11]. The cyanide absorption band has also been found to be inversely proportional to the activity, and the peak of the cyanide band at 425 mμ seems to be a reliable value for the determination of the number of active hematins in the catalase molecule.

The content of hematins in catalase preparations can be determined if iron impurities can be excluded. The data in this paper indicate that pyridine-hemochromogen or CO-hemochromogen determinations give reliable values for the protohematins in liver catalases, the absorption of bile pigment in this region being negligible. Ferritin can be completely removed using acetate buffer in the preparation and by dialysis the rest of foreign iron can be removed. The difference between the total iron and the protohematin iron gives the bile pigment iron/molecule. The absorption coefficient for catalase from different animals at 280 mμ was found to be the same, and the hematins equal in catalatic activity in spite of the immunologic differences of the protein part. This indicates that only the heme-linked groups of the protein, as suggested by Theorell [20], take part in the catalatic activity, and that they are the same in the different animal catalases.

SUMMARY

A comparison has been made between different blood and liver catalases. Crystalline guinea pig liver catalase has been prepared without any demonstrable bile pigment. In horse liver catalase preparations bile pigment was found in an amount of 10 to 14 % of the protohematin content.

Chloroform-alcohol treatment especially increases the amount of bile pigments or of inactive hematins in the liver catalase molecule. A further increase in bile pigments was found in a preparation from an infected liver from horse.

The conclusion has been drawn that under normal conditions in vivo there is probably no bile pigment present in liver catalases. The small amounts invariably found in horse liver catalase can be explained as resulting from the preparation.

Part of the hematins in liver catalases, from 10 to 25 %, is much more difficult to split off from the protein than in blood catalases.

The catalatic activity has been found to be identical for catalases with the same hematin content but from different animals and with immunologically different proteins.

Catalase with little more than 2 active hematins/molecule was found to have so little catalatic activity that more than one hematin seems to be necessary for the catalatic activity.

CN^- was found to combine with active hematins only. The absorption band at 418 mμ of the CO-hemochromogen spectrum or at 557 mμ in the pyridine hemochromogen spectrum was found to be a reliable measure for the hematin content of liver catalases.

REFERENCES

1. Bonnichsen, R. K. *Arch. Bioch.* **12** (1947) 83.
2. Sumner, J. B., Dounce, A. L., and Frampton, V. L. *J. Biol. Chem.* **136** (1940) 343.
3. Lemberg, R., and Legge, J. W. *Biochem. J.* **37** (1943) 117.
4. Bonnichsen, R., Chance, B., and Theorell, H. *Acta Chem. Scand.* **1** (1947) 685.
5. Laskowski, M., and Sumner, J. B. *Science* **94** (1941) 615.
6. Agner, K. *Arkiv Kemi, Mineral. Geol.* **B 17** (1943) no. 9.
7. Lemberg, R., Lockwood, W. H., and Legge, J. W. *Biochem. J.* **35** (1941) 363.
8. Agner, K., *Arkiv Kemi, Mineral. Geol.* **A 16** (1942) no. 6.
9. Lemberg, R., and Wyndham, R. A. *Proc. Roy. Soc. N. S. W.* **70** (1937) 343.
10. Sumner, J. B., and Dounce, A. L. *Science* **85** (1937) 366.
11. Chance, B. *Acta Chem. Scand.* **1** (1947) 236.
12. Holden, H. F., and Lemberg, R. *Austral. J. Exptl. Biol. Med. Sci* **17** (1939) 133.
13. Agner, K. *Naturwissenschaften* **27** (1939) 418.
14. Theorell, H., and Agner, K. *Arkiv Kemi, Mineral. Geol.* **A 16** (1942) no. 7.
15. Agner, K., and Theorell, H. *Arch. Bioch.* **10** (1946) 321.
16. Bonnichsen, R. K. Unpublished experiments.
17. Lemberg, R., Legge, J. W., and Lockwood, W. H. *Biochem. J.* **35** (1941) 328.
18. Keilin, D., and Hartree, E. F. *Biochem. J.* **39** (1945) 293.
19. Theorell, H. *Experientia* **4** (1948) 100.
20. Theorell, H. *Advances in Enzymol.* **7** (1947) 265.

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