In vivo Metabolism of Hemoglobin

Report I. Pathway of Hemoglobin Catabolism in Rabbits

By

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It has been known for many years that hemoglobin is decomposed into green pigments when it was incubated aerobically in the presence of ascorbic acid and also that biliverdin can be obtained from the green products on addition of dilute hydrochloric acid. Thus the mentioned *in vitro* system has been considered to be the best model of hemoglobin catabolism *in vivo* and attracted many investigators¹⁻⁶⁾ Detailed studies of the *in vitro* system by Kaziro *et al.*²⁻³⁾ have revealed that the reaction in the model system proceeds in two steps: hemoglobin→choleglobin→verdohemoglobin. Also the immediate precursor of biliverdin was shown to be the verdoheme of verdohemoglobin.

It is not clear as yet, however, whether the *in vivo* catabolism of hemoglobin would actually proceed in a similar fashion as in the model system, although it is an established fact that the heme part of hemoglobin is metabolized into bile pigments in the living body. With the aim of elucidating this point, we intravenously administered hemoglobin or myoglobin to rabbits and followed the occurrence of their metabolic products in urine and blood stream. Results of these experiments will be reported in this paper. A preliminary report of some of this work has been published.⁷⁾

EXPERIMENTAL

Oxyhemoglobin was prepared from fresh rabbit erythrocytes. Well-washed packed erythrocytes were hemolyzed by the addition of equal volumes of distilled water and ether, then the hemolyzate was centrifuged at 8,000 x g for 10 minutes which yielded the destromatized oxyhemoglobin solution.

Methemoglobin was prepared by oxidizing oxyhemoglobin with potassium ferricyanide, followed by dialysis against 0.02 M phosphate buffer, pH 7.4. Metmyoglobin was obtained in crystalline form from horse heart by the method of Tsushima and Okazaki.⁸⁾ The sample was recrystallized before use. Male rabbits

were used throughout the experiments. Hemoglobin or myoglobin preparations, which had been adjusted to approximately 0.9% in terms of NaCl concentration and warmed at 37°, were infused to rabbit through auricular vein at a rate of about 3 ml. per minute, and thereafter urines were collected by means of continuous catheterization. Urine samples collected at intervals as designed in individual experiments were examined spectrophotometrically for the aimed compounds, using Hitachi Recording Spectrophotometer, Model EPS-2.

RESULTS

1) Experiments with oxyhemoglobin

When oxyhemoglobin was intravenously administered to rabbits, hemoglobin appeared in urine usually 20 to 22 minutes after the infusion.

Spectral properties of some urine samples are shown in Fig. 1, A and B. The spectra shown in Fig. 1, A are those of the urine sample collected in the initial 30 minutes after the intravenous administration of oxyhemoglobin. For the spectral examination, the urine was added with 3% NaOH and water at the ratio of 1:1:1. Insoluble substances were centrifuged off, and the resultant clear

Fig. 1. Absorption spectra of urine samples from rabbits which were intravenously administered with oxyhemoglobin.

A: Hemoglobin administered contained 19 μ moles heme. Initial 30 minutes urine sample was used. Solid line: treated with NaOH and $\text{Na}_2\text{S}_2\text{O}_4$. Broken line: CO-gas was passed through above sample.

B: Hemoglobin administered contained 47 μ moles heme. Initial 35 minutes urine sample was used. Solid line: urine sample was diluted with equal volume of water and reduced with $\text{Na}_2\text{S}_2\text{O}_4$. Broken line: CO-gas was passed through above sample.

solution was added with a pinch of pulverized sodium dithionite to convert heme compounds into hemochromes. Solid line in Fig. 1, A represents the absorption spectrum of the urine sample treated as such. There can be seen strong absorptions in the region of 500 to 600 $m\mu$, indicating the appearance of hemoglobin in the urine. In addition, a distinct absorption peak at 618 $m\mu$ can be recognized. The peak at 618 $m\mu$ shifted to 630 $m\mu$ when the sample was gassed with carbon monoxide (Fig. 1, A- broken line). In Fig. 1, B, spectra are given of another urine sample from a different rabbit. In this case, the urine sample was not treated with NaOH, but with $Na_2S_2O_4$. An additional absorption appeared also in this case, indicating its maximum at 630 $m\mu$ (solid line).

The absorption peak at 630 $m\mu$ became more distinct when the sample was passed with CO (broken line). Spectral characteristics of these absorptions at 618 $m\mu$ or 630 $m\mu$ coincided exactly with those of authentic choleglobin prepared by the reaction of hemoglobin-ascorbic acid-molecular oxygen system.

We were unable to definitely distinguish the occurrence of verdohemoglobin in urines by spectral examinations, but we could obtain small amounts of biliverdin from urines by applying procedures for extracting biliverdin.⁴⁾ The time courses of the appearance of protoheme and choleheme in urine as well as the yields of biliverdin are shown in TABLE I. Per cent contents of choleheme in total heme as listed in TABLE I were calculated according to the empirical equations presented by Kaziro *et al.*³⁾ Durations of hemoglobin and choleglobin excretion after intravenous administration of oxyhemoglobin differed between individual experiments, but usually were three to four hours. It appeared that amounts of choleglobin excreted were less when rates of hemoglobin excretion were lower.

TABLE I. Excretion of Hemes and Biliverdin in Urine after Intravenous Administration of Oxyhemoglobin

Time after HbO ₂ administration	Urine volume	Protoheme	Choleheme in total hemes	Biliverdin
min.	ml.	μ mole	%	$m\mu$ mole
35	20	1.40	27	49.2
70	4.0	0.278	27	16.4
100	7.6	0.429	38	71.7
130	18.0	0.056	56	28.3
160	5.0	0.001	75	—

HbO₂ administered: 30 μ moles as heme

The examination of circulating blood of a rabbit 35 and 70 minutes after the oxyhemoglobin administration revealed also the occurrence of choleglobin in the blood plasma although ratios of choleheme to protoheme were found to be far lower (approximately 3%) as compared with those in urine samples.

2) Experiments with methemoglobin

Intravenously administered methemoglobin was excreted into urine as fast as oxyhemoglobin. In urines collected soon after the methemoglobin administration, the main hemoglobin component was methemoglobin, but in urines collected at 60 minutes or later, methemoglobin was almost completely replaced with oxyhemoglobin, indicating the rapid reduction of methemoglobin in rabbits. The time courses of occurrence of protoheme and choleheme in urine are shown in TABLE II. Comparing the data to those in TABLE I, it appears that the occurrence of choleheme as well as the increase in per cent contents of choleheme in total hemes in urine were delayed when methemoglobin was infused in place of oxyhemoglobin. Also choleglobin was not detectable in the plasma of circulating blood in this case although fair amounts of oxyhemoglobin were found to exist together with methemoglobin.

TABLE II. Excretion of Hemes in Urine after Intravenous Administration of Methemoglobin

Time after MetHb administration	Urine volume	Protoheme	Choleheme in total hemes
min.	ml.	μ mole	%
20	2.3	none	none
40	1.4	1.270	17
60	1.3	1.092	12
80	1.2	0.865	14
100	2.6	0.229	50
120	1.8	0.115	64
150	3.6	0.024	67

MetHb administered: 19 μ moles as heme

3) Experiments with metmyoglobin

Recrystallized horse metmyoglobin was used in these experiments. The administered myoglobin was also rapidly excreted and its metabolites appeared in urine. Urine samples were collected at various time intervals, treated with NaOH, $\text{Na}_2\text{S}_2\text{O}_4$ and CO gas, and subjected to the spectral examination (Fig. 2). The shortage and difference in volumes of each urine samples made it necessary for spectrophotometric examination to dilute them to suitable volumes ranging from 5 to 10 folds, although the values given in Fig. 2 are expressed in terms of original volumes. It can be seen from Fig. 2 that the myoglobin molecule is fairly stable even when it was infused to rabbits. The appearance of decomposition products of myoglobin in urine was found to be far delayed as compared to those with oxyhemoglobin or methemoglobin. Of particular interest in Fig. 2 is that, in addition to cholemyoglobin, the occurrence of verdomyoglobin could clearly be evidenced by its specific absorption peak at $760\text{ m}\mu$ in the 80 and 100 minutes urine samples. The fact suggests that the pathway of catabolism of hemoglobin as well as myoglobin in the living body may proceed in a similar

Fig. 2. Absorption spectra of urine samples from a rabbit administered with 1.2 μ moles metmyoglobin.

Spectra were measured after the samples were treated with NaOH, $\text{Na}_2\text{S}_2\text{O}_4$ and CO gas. The values given in the figure are expressed in terms of original volumes of urine samples collected at intervals as designated in the figure.

way as in the *in vitro* model system.

4) Incubation of oxyhemoglobin with citrated whole blood

3.0 ml. of oxyhemoglobin ($6.3 \times 10^{-3} M$ as heme) were added to 48 ml. of citrated fresh whole blood of rabbits and incubated at 37° . Each 5 ml. were withdrawn

from the incubation mixture at 30 minutes intervals of incubation up to 3 hours. The sample was clarified by centrifugation, diluted 10 folds with 1.5% NaOH, then reduced by $\text{Na}_2\text{S}_2\text{O}_4$ and examined spectrophotometrically. Disturbing effect by hemolysis during the incubation was eliminated by running a control system which contained saline in place of oxyhemoglobin. The examination revealed that the choleglobin formation in the mentioned system was only meager, and most of the hemoglobin added remained unchanged during the course of 3 hours incubation (Fig. 3).

Fig. 3. Choleglobin formation in blood plasma during the incubation of oxyhemoglobin with citrated whole blood.

Spectra were taken after the samples were treated with NaOH, $\text{Na}_2\text{S}_2\text{O}_4$ and CO. For details, see text, Exp. 4).

DISCUSSION

The experimental results reported in this paper strongly suggest that the pathway of hemoglobin catabolism proceeds essentially in the same way as in the well-defined model system of hemoglobin breakdown as far as the reaction products and sequences are concerned, namely, protohemoglobin→choleglobin→verdo-

hemoglobin. Then verdohemoglobin will yield biliverdin in tissues by an so far unclarified manner. Also in this study, the occurrence of choleglobin in urine was found to be delayed when methemoglobin was administered in place of oxyhemoglobin and further delay was noticed when metmyoglobin was used. These results are consistent with the previous findings from the model reaction of hemoglobin breakdown using ascorbic acid: i) rates of choleglobin formation from methemoglobin were lower than those from oxyhemoglobin, in agreement with the generally accepted principle that the ferrous state of hemoglobin was essential to initiate the heme breakdown²⁾, ii) rates of heme breakdown in the model system with myoglobin were much lower than with hemoglobin.³⁾ On the basis of the observed similarity of the kinetic behaviors of heme breakdown in *in vivo* and *in vitro* systems, we are inclined further to presume that the mechanism of heme breakdown may be of a autocatalytic nature even in the *in vivo* system as was the case in the *in vitro* system.

Recently it was reported by Kosaka⁹⁾ that, when oxyhemoglobin or erythrocytes suspensions were subcutaneously injected to mammals or chickens to induce hematoma, the substances produced in hematoma as a consequence of hemoglobin breakdown were identical to those which could be found in the model system of hemoglobin-ascorbic acid-molecular oxygen. Also biliverdin and bilirubin were found in hematoma. However, the rate of hemoglobin breakdown in hematoma was low and usually it took a few days before considerable amounts of decomposition products accumulated. In our case, however, the rate of choleglobin formation was very high as evidenced by its rapid and massive excretion in urine. Also the high rate of hemoglobin breakdown *in vivo* thus observed could account for Yamada's observation¹⁰⁾ that, when hemoglobin was intravenously administered to rabbits, the biliverdin excretion into fistula bile increased rapidly with a small initial lag, reaching the maximum rate about two hours after the hemoglobin administration. Thus we may safely conclude that the pathway as assumed in this paper would reflect the physiological pattern of hemoglobin catabolism.

Lastly, it should be pointed out that, in contrast to the rapid formation of choleglobin *in vivo*, only a trace amount of choleglobin was formed during *in vitro* incubation of hemoglobin with citrated whole blood of rabbits under conditions as desinged to be comparable to those in *in vivo* experiments. This would suggest that the reaction of hemoglobin catabolism *in vivo* may be participated by activities of living cells, although their roles in the reaction are not clearly understood.

SUMMARY

Rabbit oxyhemoglobin, rabbit methemoglobin and equine metmyoglobin were intravenously administered to male rabbits, respectively, and the occurrence of their metabolic products in urine and blood stream was followed.

1) Rapid and massive excretion of choleglobin, together with hemoglobin, was observed to occur after the oxyhemoglobin administration. Also biliverdin could be obtained from urines, though attempts to demonstrate the occurrence of verdohemoglobin in urines failed. A small amount of choleglobin was also found in the circulating blood.

2) When methemoglobin was infused in place of oxyhemoglobin, the occurrence of choleglobin in urine was delayed.

3) Further delay was noticed when metmyoglobin was used. In this case, however, the appearance of verdoheme in urine was clearly demonstrated.

4) Extents of heme breakdown were very small when oxyhemoglobin was incubated with citrated whole blood.

On the basis of the above experimental results, the path of hemoglobin catabolism was assumed to proceed in a similar way as in the well-defined model system of hemoglobin breakdown using ascorbic acid. Also the reaction of hemoglobin catabolism *in vivo* was presumed to be participated by activities of living cells.

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