Effect of hemoglobin concentration on the oxidation of linoleic acid

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ABSTRACT The inhibitory effect of high concentrations of hemoglobin on the oxidation of linoleic acid was related to the ability of hemoglobin to associate with the fatty acid. Ultracentrifugation of the mixture of hemoglobin and potassium linoleate revealed that approximately 880 moles of linoleic acid could associate with 1 mole of hemoglobin. High concentrations of hemoglobin apparently reduced the amount of free linoleic acid accessible to the heme group, thus preventing the oxidation of the fatty acid. With low hemoglobin concentrations, at which the oxidation began immediately after the addition of the catalyst, the amount of free linoleic acid was considerably greater than that of bound linoleic acid.

SUPPLEMENTARY KEY WORDS heme · induction period

HEMATIN COMPOUNDS have long been known to accelerate oxidation of lipids (1–5). Since hematin compounds are widely distributed in biological systems, their involvement in the in vivo oxidation of lipids under pathological conditions has been suspected (6, 7). We have previously studied the nature of oxidative destruction of low density lipoproteins catalyzed by hemin and hemoglobin (8, 9). Molecular and surface properties of both low density lipoproteins and hemin or hemoglobin molecules were found to be directly involved in the catalytic activities of these hematin compounds.

The rate of oxidation of fatty acids catalyzed by hemoprotein seems to be greatly influenced by the ratio of the hemoprotein concentration to the fatty acid concentration. Cytochrome c in high concentrations inhibited oxidation of unsaturated fatty acids, while in low concentrations it accelerated the oxidation (10). This observation was confirmed with hemoglobin (11). The present communication describes the nature of potassium linoleate oxidation as governed by hemoglobin concentrations.

METHODS

Human hemoglobin was prepared according to the method previously described (9). Hemoglobin concentration was determined by a standard cyanomethemoglobin method (12). Methyl linoleate was prepared from safflower oil by the bromination and debromination method described by Frankel and Brown (13). The purity of the methyl linoleate preparation was estimated to be greater than 99.5% by gas–liquid chromatography. Linoleic acid was obtained by hydrolysis of methyl linoleate and was purified by distillation under high vacuum. The fatty acid obtained was neutralized with potassium hydroxide. The concentration of potassium linoleate in aqueous solution was determined by the method of Dole (14).

The rate of potassium linoleate oxidation was estimated by measuring the oxygen uptake of incubation mixtures at 30°C in a Warburg manometer. The final volume of the mixtures was 2.5 ml; 0.05 M phosphate buffer, pH 7.5, was used.

To study the sedimentation behavior of hemoglobin–potassium linoleate mixtures, various concentrations of hemoglobin were placed in the bottom of centrifuge tubes; buffer and potassium linoleate solution were then layered on the hemoglobin. The air in the tubes was replaced by nitrogen through the Spinco cap hole, the contents were mixed, and the mixtures were kept at room temperature for 30 min. Layering and replacing the air by nitrogen were necessary to prevent the oxidation of potassium linoleate in the presence of hemoglobin. After the mixtures had been centrifuged for 24 hr at 17°C in a

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Spinco rotor 40.3 at 109,000 *g*, the amount of linoleic acid associated with the bottom hemoglobin fraction was determined according to the method described by Dole (14).

Spectrophotometric analyses of hemoglobin and incubation mixtures were performed with a Cary recording spectrophotometer.

RESULTS

Ultracentrifugation of mixtures of 10 mM potassium linoleate and various concentrations of hemoglobin revealed an extensive association of linoleic acid with hemoglobin. The samples containing only potassium linoleate showed almost complete flotation of the fatty acid, and those containing only hemoglobin showed complete sedimentation of hemoglobin. Centrifugation of samples containing both potassium linoleate and hemoglobin, however, showed sedimentation of potassium linoleate with the hemoglobin. The amount of linoleic acid associated with the hemoglobin fraction increased with increases in hemoglobin concentration, as shown in Fig. 1 and Table 1. At hemoglobin concentrations above 11 μM, most of the linoleic acid added was associated with hemoglobin. Table 1 also lists the number of moles of linoleic acid associated with 1 mole of hemoglobin in the mixtures of 10 mM potassium linoleate and various concentrations of hemoglobin. At hemoglobin concentrations below 10 μM, the average number of moles of linoleic acid associated per mole of hemoglobin was approximately 880.

The association of fatty acid with hemoglobin seems to be responsible for the dependence of potassium linoleate oxidation on hemoglobin concentration. The change in oxygen uptake upon incubation of 10 mM potassium linoleate with hemoglobin at concentrations of

TABLE 1 ASSOCIATION OF LINOLEIC ACID WITH HEMOGLOBIN

Hemoglobin		Linoleic Acid Sedimented with Hemoglobin	Linoleic Acid/ Hemoglobin Molar Ratio	
M × 10^6	*moles* × 10^8	*moles* × 10^5		
2	1.0	0.87	867	↑
4	2.0	1.70	850	|
6	3.0	2.70	900	Average
8	4.0	3.56	890	877
10	5.0	4.38	876	↓
11	5.5	4.66	847	
12	6.0	4.50	750	
13	6.5	4.73	728	
16	8.0	4.52	565	

All experimental conditions are given in the legend to Fig. 1. The average linoleic acid/hemoglobin molar ratio, 877, represents the value obtained with hemoglobin concentrations at which hemoglobin appeared to be associated stoichiometrically with the added potassium linoleate.

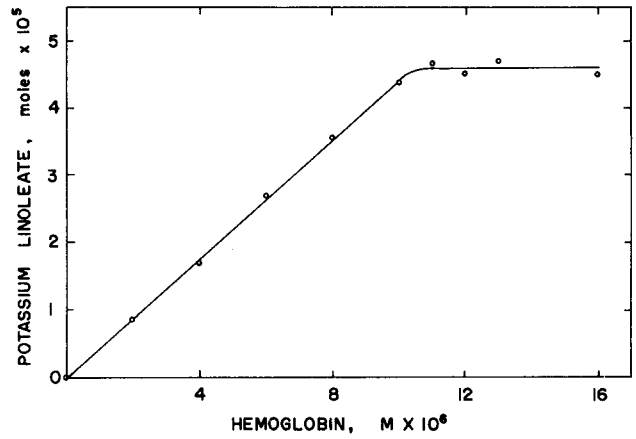

FIG. 1. Recovery of potassium linoleate from the hemoglobin fraction obtained by centrifugation of mixtures of 10 mM potassium linoleate and hemoglobin at different concentrations. The amount of potassium linoleate associated with the hemoglobin fraction is given on the ordinate and the hemoglobin concentration on the abscissa. The samples for centrifugation contained 50 μmoles of potassium linoleate and hemoglobin at various concentrations in a final volume of 5.0 ml. The medium contained 0.05 M phosphate buffer, pH 7.6.

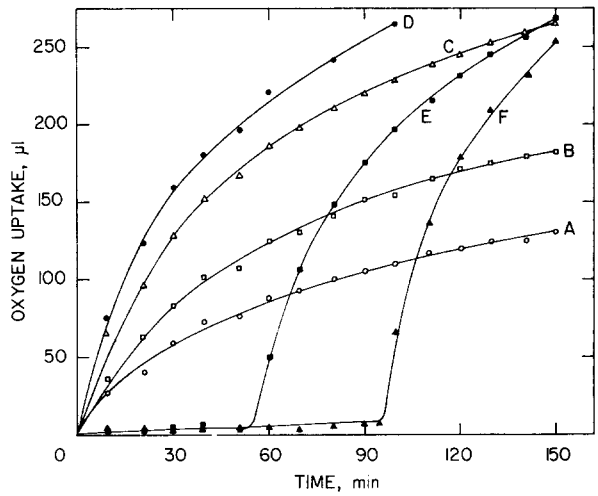

FIG. 2. The effect of hemoglobin concentration on the oxidation of 10 mM potassium linoleate. The oxygen uptake of 10 mM potassium linoleate at pH 7.5 in 2.5 ml of reaction mixture was measured at 30°C for a period of 150 min in the presence of hemoglobin. The hemoglobin concentrations used were: curve *A*, 0.5 μM; *B*, 1.0 μM; *C*, 2.0 μM; *D*, 2.5 μM; *E*, 3.0 μM; and *F*, 4.0 μM. No significant oxygen uptake was observed in the absence of hemoglobin.

0.5–4.0 μM is shown in Fig. 2. At a hemoglobin concentration of 0.5 μM (curve *A*), oxidation began immediately but at a slow rate. When the hemoglobin concentration was increased to 1.0 μM (curve *B*), 2.0 μM (curve *C*), and 2.5 μM (curve *D*), the rate of oxidation was accelerated. Further increases in hemoglobin concentration (curve *E*, 3.0 μM; and *F*, 4.0 μM), however, caused an induction period which was prolonged with increasing concentration.

When the concentration of potassium linoleate was increased from 10 to 20 mM, an induction period was no longer observed at hemoglobin concentrations of 3.0 μM (Fig. 3, curve *C*) and 4.0 μM (curve *D*). The critical hemoglobin concentration, above which a period of induction became detectable, approximately doubled in the presence of 20 mM potassium linoleate; an induction period was noted at hemoglobin concentrations above 5.2 μM. The induction period as a function of hemoglobin concentration in the presence of 20 mM potassium linoleate is shown in Fig. 4; an increase in the concentration prolonged the period of induction. We noted that with 24 μM hemoglobin, oxidation of 20 mM potassium linoleate was delayed approximately 10 hr. Fig. 5 shows a linear relationship between potassium linoleate concentrations of 6–30 mM and the critical hemoglobin concentration above which induction occurred. At critical hemoglobin concentrations, the molar ratio of hemoglobin to potassium linoleate in the medium was approximately 1 to 3800. We noted that the change in potassium linoleate concentration does not significantly influence the number of moles of potassium linoleate, approximately 880, which could associate per mole of hemoglobin. Thus, free potassium linoleate concentration corresponding to each experimental point in Fig. 5 was calculated by subtracting the concentration of potassium linoleate associated with hemoglobin from the total potassium linoleate concentration and was plotted against total potassium linoleate concentration (Fig. 6). The slope of the line, 0.77, implied that induction occurred when free potassium linoleate concentration was reduced below 77% of total potassium linoleate. The

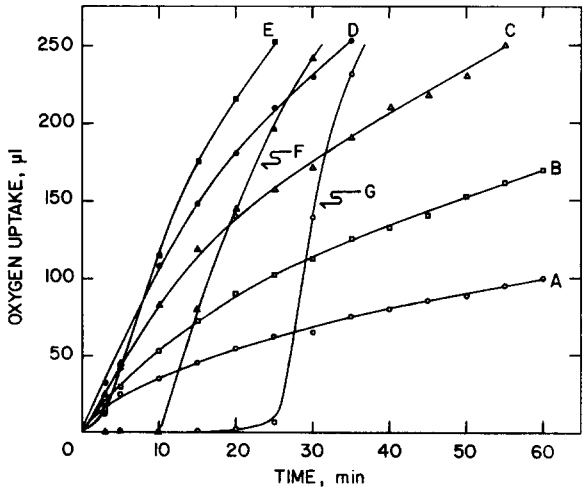

FIG. 3. The effect of hemoglobin concentration on the oxidation of 20 mM potassium linoleate. The oxygen uptake of 20 mM potassium linoleate was measured for 60 min under the same conditions described for Fig. 2. The hemoglobin concentrations used were: curve *A*, 0.5 μM; *B*, 1.5 μM; *C*, 3.0 μM; *D*, 4.0 μM; *E*, 5.2 μM; *F*, 6.0 μM; and *G*, 7.6 μM.

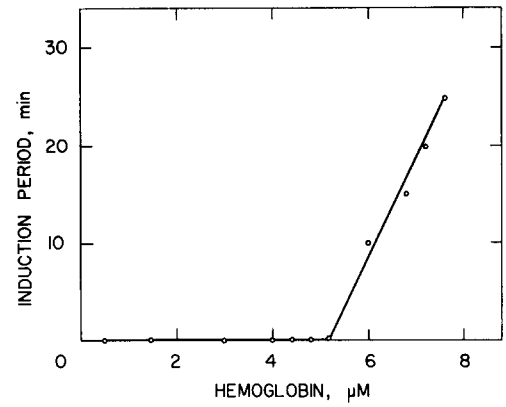

FIG. 4. Induction period as a function of hemoglobin concentration in the presence of 20 mM potassium linoleate. The experimental conditions are the same as those given for Fig. 3.

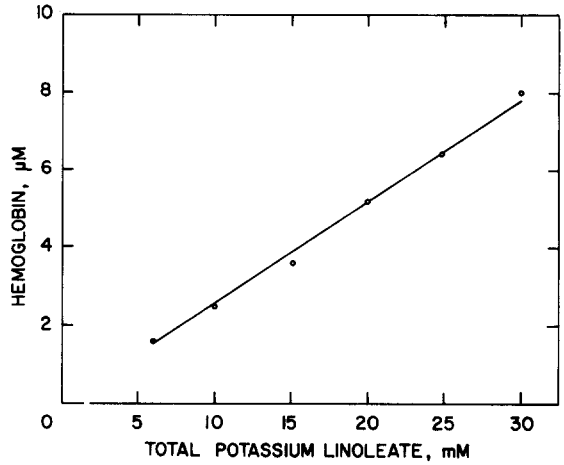

FIG. 5. Effect of potassium linoleate concentration on the critical hemoglobin concentration above which a period of induction was observed. The critical hemoglobin concentrations given on the ordinate were determined in the presence of various concentrations of potassium linoleate by measuring oxygen uptake as a function of hemoglobin concentration as described for Fig. 2.

rapid oxidation of potassium linoleate apparently required the presence of potassium linoleate predominately in the free form. The increase in the induction period with increases in hemoglobin concentration above the critical level seems to have been caused by a progressive decrease in the relative amount of free potassium linoleate.

The oxidative destruction of hemoglobin in the presence of potassium linoleate was also greatly influenced by the concentration of hemoglobin, reflecting the amount of free fatty acid available for catalytic oxidation. The spectrum of 1.1 μM oxyhemoglobin, measured in a cell with a 10.0-cm light path, is shown in Fig. 7, curve *A*. Within 1 min after the addition of 10 mM potassium linoleate to the oxyhemoglobin solution, the oxyhemoglobin visible bands disappeared (curve *B*), indicating the destruction of the heme groups. When samples con-

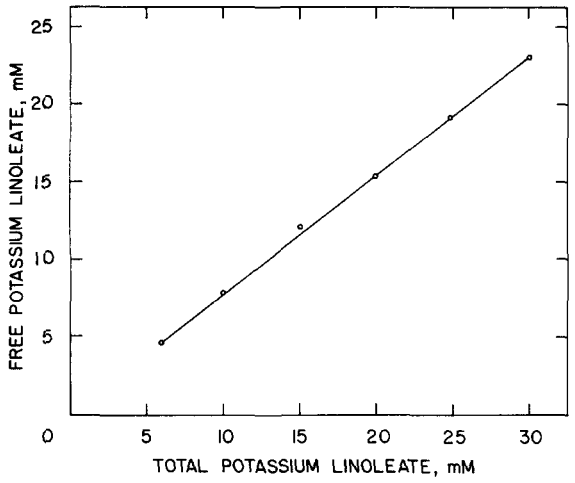

FIG. 6. Relationship between free and total potassium linoleate concentrations at critical hemoglobin concentrations. The concentration of free potassium linoleate was obtained by subtracting the concentration of potassium linoleate associated with hemoglobin at the critical concentration from the total potassium linoleate concentration. The number of moles of potassium linoleate which could associate per mole of hemoglobin was considered to be 880.

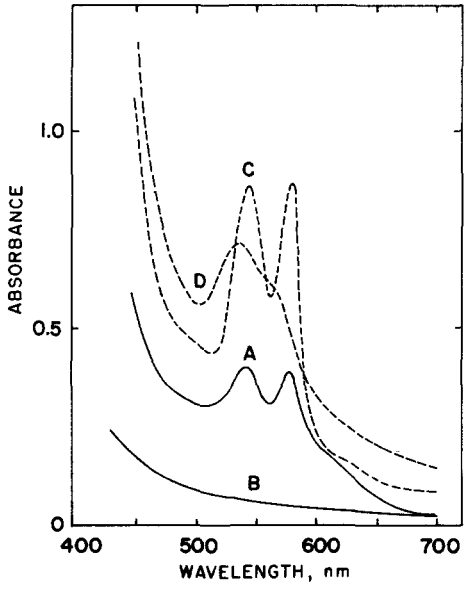

FIG. 7. Change in the absorption spectrum of hemoglobin upon addition of potassium linoleate. Curve *A* represents the absorption spectrum of 1.1 μM oxyhemoglobin; and curve *B*, the spectrum of 1.1 μM oxyhemoglobin measured 1 min after the addition of 10.0 mM potassium linoleate. Both were measured in a cell with a 10.0-cm light path. Curve *C* shows the spectrum of 15.0 μM oxyhemoglobin; and curve *D*, the spectrum measured 20 hr after the addition of 10.0 mM potassium linoleate; both were determined in a cell with a 1.0-cm light path.

taining higher concentrations of oxyhemoglobin were used, the change in the absorption spectrum was less pronounced. The absorption spectrum of 15 μM oxyhemoglobin, measured in a cell with 1.0-cm light path, is shown by curve *C*. 20 hr after 10 mM potassium linoleate was added to 15 μM oxyhemoglobin, the absorption spectrum shown by curve *D* was observed; the induction period lasted over 24 hr. This spectrum, showing absorption bands at 535 and 560 nm, was similar to that of a low-spin ferric porphyrin compound (15) such as imidazol–hemoglobin (16), formamide-denatured hemoglobin (17), or histidine–hemin complex (18). The visible bands of the absorption spectrum, however, rapidly disappeared once the oxidation of potassium linoleate was initiated after the induction period.

DISCUSSION

The present study revealed that approximately 880 moles of linoleic acid were associated with 1 mole of hemoglobin in the presence of excess potassium linoleate. The relative amounts of free and bound potassium linoleate as determined by the association appear to govern primarily the length of the induction period in the hemoglobin-catalyzed oxidation of potassium linoleate. At lower hemoglobin concentrations, the potassium linoleate is present mostly in the free form and is rapidly oxidized. On the other hand, at higher concentrations the decrease in free potassium linoleate prolongs the induction period.

The immediate oxidation of potassium linoleate at lower hemoglobin concentrations appears to indicate that the catalytic site of hemoglobin is not masked by the bound fatty acid but is available to effect oxidation of the free fatty acid. It was observed that at all potassium linoleate concentrations used the initial oxidation rate progressively increased as the concentration of hemoglobin was increased from zero to critical concentrations, above which induction occurred, despite the corresponding decrease in the concentration of free fatty acid. Thus, in the hemoglobin concentration range from zero to the critical level, the rate of oxidation at each substrate level is governed primarily by the hemoglobin concentration rather than by the free fatty acid concentration. However, this dependence on hemoglobin concentration no longer applied at higher hemoglobin concentrations; increase in the concentration above critical levels progressively increased the induction period. It was computed that at critical hemoglobin concentrations, where the molar ratio of hemoglobin to potassium linoleate was approximately 1:3800, 23% of the potassium linoleate added was associated with the hemoglobin and the rest, 77%, was in the free form. In this computation, the number of moles of potassium linoleate that could associate with 1 mole of hemoglobin was considered to be 880. It must be noted that this value, 880, was obtained after centrifugal separation of bound potassium linoleate from free linoleate. Some of the free potassium linoleate might have been in loose association with the hemoglobin–potassium linoleate complex and not readily

available for oxidation. It is possible that the loosely associated linoleate was cleaved during centrifugation and contributed to the amount of free linoleate. Therefore, the amount of free potassium linoleate readily available to the catalytic site of hemoglobin at critical hemoglobin concentrations could have been lower than 77%.

Although the induction period was prolonged with increases in hemoglobin concentration above the critical level, the rate of oxidation was greater at higher hemoglobin concentrations once the oxidation had been initiated (Figs. 2 and 3). Furthermore, even with hemoglobin concentrations at which all or most of the potassium linoleate is associated with hemoglobin, oxidation of the potassium linoleate took place after long induction periods. These observations suggest that the bound fatty acid molecules, though not readily available for oxidation, eventually have access to the catalytic site of hemoglobin for oxidation. It appears that at high hemoglobin concentrations the amount of free linoleate available at the hemoglobin catalytic site is not sufficient to induce an effective chain reaction for catalytic oxidation. However, some of the bound as well as free linoleate may gradually be subject to catalytic or auto-oxidation, thus leading to the formation of linoleic acid hydroperoxide and other oxidation products. Since the polar groups of these oxidation products have a high affinity for aqueous solvents, accumulation of the oxidation products may lead to the disruption of the hemoglobin–linoleate complex or to rearrangement of the bound fatty acids in such a way that fatty acid molecules have access to the hemoglobin catalytic site.

The oxidation of methyl linoleate catalyzed by hemoproteins seems to be considerably different from the oxidation of potassium linoleate (19). We noted that increases in the concentration of the hemoglobin progressively increased the oxidation rate of methyl linoleate, but no induction was observed even at high hemoglobin concentrations. Furthermore, no association between these two components was noted upon ultracentrifugation of the mixtures. It is apparent that the lack of methyl linoleate–hemoprotein interaction is responsible for the absence of an induction period as well as for the progressive increase in the oxidation rate of methyl linoleate as the hemoglobin concentration is increased.

Although the nature of the association of linoleic acid with hemoglobin was not investigated in the present study, the absence of a methyl linoleate–hemoglobin interaction suggests that the association requires the carboxylate ion of the fatty acid. The association of a large number of linoleic acid molecules with hemoglobin may imply that only a part of the bound fatty acids are directly associated with hemoglobin. The rest may be arranged as stable micelles on the surface of hemoglobin molecules in such a way as to prevent the fatty acids from orienting to the heme group of the hemoglobin. Our preliminary study indicated that the association of fatty acid with hemoglobin requires, in addition to a free carboxyl group, the presence of at least one *cis* double bond. Studies are currently in progress to clarify various factors involved in the fatty acid–hemoglobin interaction.

The authors wish to thank Miss J. Warfel and Mrs. S. Hoffman for skillful assistance during the course of this work. Appreciation is also given to Dr. F. A. Kummerow for his interest in this work.

This investigation was supported by Grant HE 03063 from the National Institutes of Health, U.S. Public Health Service and by a grant from the Chicago and Illinois Heart Association.

Manuscript received 25 August 1969 and in revised form 19 October 1970; accepted 11 November 1970.

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