Human whole blood and washed intact erythrocytes from human and sheep exhibit aniline hydroxylase activity indicative of oxygen activation by the erythrocytes. Formation of p-aminophenol by human and sheep erythrocytes was similar under similar conditions, 5.8 ± 0.26 and 5.3 ± 0.14 nmol of p-aminophenol/60 min/ml, respectively, at 1 mM hemoglobin and 60 mM aniline. The amount of p-aminophenol produced was directly proportional to the concentration of oxy-hemoglobin. Erythrocytes in which the hemoglobin had been oxidized completely to methemoglobin were essentially inert. Hence, oxyhemoglobin plays a key role in the catalysis. Furthermore, studies of the stimulatory effects of lactate, glucose, and methylene blue revealed that NADPH apparently is the important cofactor for the aniline hydroxylase activity of the erythrocytes. Thus, lactate which serves to produce NADH via the lactate dehydrogenase reaction had little, if any, effect on the hydroxylase activity, even under conditions where its ability to support reduction of methemoglobin was demonstrated. In contrast, the addition of glucose with methylene blue led to a 17-fold stimulation of the activity with both human and sheep erythrocytes. Glucose serves to supply the initial substrate for the pentose phosphate pathway which produces NADPH, and methylene blue predominantly affects NADPH-dependent electron transport. The combination of lactate with methylene blue produced no such stimulation.

Hemoglobin is an oxygen-carrier protein whose physiological function depends upon its ability to bind oxygen reversibly. In contrast, the related hemoprotein cytochrome P-450 is characterized as an oxygen-activating enzyme, capable of incorporating oxygen into drug substrates. The contrast, however, is not an absolute one. For example, we and others (1-3) have shown that isolated hemoglobin can catalyze the hydroxylation of aniline, a reaction that is typical of the monooxygenases, 5.8 ± 0.26 and 5.3 ± 0.14 nmol of p-aminophenol/60 min/ml, respectively, at 1 mM hemoglobin and 60 mM aniline. This unusual reactivity of hemoglobin was recognized by Juchau and Symms during an investigation of the subcellular localization of aniline hydroxylase in human placenta (1). Subsequently, we demonstrated that hemoglobin could substitute for P-450 in a reconstituted aniline hydroxylase system composed of human hemoglobin, NADPH, P-450-reductase, and oxygen (2). Our studies of the mechanism of the reaction implicated oxyhemoglobin as the form which interacts with substrate (2, 4). This finding emphasized the importance of examining hemoglobin reactivity in intact erythrocytes and whole blood where it is maintained in the oxyhemoglobin form. Whether the erythrocytes would display hydroxylase activity could not be predicted a priori from our studies of isolated hemoglobin because of the many differences between the reconstituted system and the erythrocytes. For example, the endogenous reductase activity of the red blood cell would have to function in place of liver P-450 reductase. In addition, the erythrocytes contain several enzymes which scavenge activated forms of molecular oxygen including catalase, superoxide dismutase and glutathione peroxidase. We had demonstrated that catalase could inhibit completely the activity of the isolated hemoglobin system (2). In the report by Juchau and Symms, they had indicated that whole blood (diluted 5-fold) did hydroxylate aniline, but no data were reported (1). Likewise, during our studies of the reconstituted system we confirmed the reactivity of whole blood; our characterization of that activity comprises the present report. In a recent note Tomoda et al. also reported p-aminophenol formation by resuspended erythrocytes (5).

Besides the similarity between the terminal electron acceptors hemoglobin and P-450, other components of their respective electron transport systems are also similar. Each contains an NADH-dependent reductase and an NADPH-dependent reductase. In erythrocytes, the NADH pathway is the principal system for maintaining hemoglobin in the reduced, oxygenated form (6). With microsomes, the NADPH system is quantitatively more important in supporting both P-450 reduction and P-450-catalyzed hydroxylation reactions, but the NADH system may also participate (7-9).

The results of this study suggest a predominant role for the NADPH pathway in the aniline hydroxylation reaction in erythrocytes as well. This hydroxylase activity reflects their ability to activate molecular oxygen, and the reactivity appears to require oxyhemoglobin.

**EXPERIMENTAL PROCEDURES**

Methylene blue, NADPH, NADH, and lithium lactate were obtained from Sigma. Aniline obtained from Fisher was distilled and divided into aliquots which were purged with nitrogen and then stored frozen until used. All other chemicals were reagent grade.

Blood from a single female sheep was obtained in heparin from the Case Western Reserve University Animal Resource Center at weekly intervals. The most extensive data on the erythrocytic aniline hydroxylase activity under various conditions were obtained from this source because it was readily and abundantly available. After each characteristic of the aniline hydroxylase activity was examined with sheep blood, fewer experiments could be performed with human blood.

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Karen S. Rilisardj and John J. Mieyal
From the Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

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The abbreviations used are: P-450, liver microsomal cytochrome P-450; HbO2, oxyhemoglobin; Hb+, ferrihemoglobin.

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2. J. L. Blumer and J. J. Mieyal (1976) unpublished observations.
to define the same property. In most cases, the values reported for human blood represent a composite from experiments for which several different individuals served as donors. The blood samples were prevented from clotting with heparin.

Erythrocytes were isolated by low speed centrifugation in a clinical centrifuge and washed with an equal volume of isotonic saline four times. They were then either resuspended in an appropriate volume of isotonic saline or hemolyzed with an appropriate volume of distilled water. In all cases, erythrocyte concentration or hemolysate concentration was quantitated by measurement of the hemoglobin concentration, which was assayed as the cyanomethemoglobin complex ($e_{540}$ = 44 mM$^{-1}$ cm$^{-1}$) for the hemoglobin tetramer. Methemoglobin content was determined by a modification of the method of Hegesh et al. (10), or by direct spectrophotometric measurements ($e_{540}$ = 1.48 mM$^{-1}$ cm$^{-1}$) at pH 7.5.

The aniline hydroxylation reaction was initiated by addition of substrate. Incubation was continued at 38°C for the designated time period. The reaction was terminated by immersing the test tubes containing the reaction mixtures in an ice bath. After cooling the samples, intact cells were removed by centrifugation at 4°C in a clinical centrifuge. The product p-aminophenol was quantitated by a modification of the method of Brodie and Axelrod (12). Thus, the supernatant from the intact cell suspension or the uncentrifuged hemolysate (1.0 ml total volume in each case) was extracted twice with 5-ml volumes of diethyl ether$^9$ which had been prewashed with 0.1 M sodium bicarbonate. The combined ether fractions then were extracted with 1.3 ml of 0.2 N HCl. Phenol in alkaline solution was added to the acid extracts, and the absorption of the resultant indophenol derivative of p-aminophenol was measured at 630 nm (2). Controls for each condition of incubation (i.e. ±glucose, lactate, etc. (see "Results")) were prepared routinely by adding aniline after the sample was cooled. It was confirmed that no significant amount of p-aminophenol was formed even when samples containing aniline were incubated at 4°C for the designated period.

Separate complete standard curves for quantitating p-aminophenol for each experimental system were constructed as follows. Various known amounts of p-aminophenol were incubated at 38°C with samples of intact cells or hemolysates from each species for 60 min and then extracted and assayed as described above. A representative standard curve is shown in Fig. 1. By frequently including samples containing known quantities of p-aminophenol among the test samples, it was confirmed that this procedure yielded standard curves which were very reproducible for every experimental system tested.

Addition of other components such as glucose, lactate, methylene blue, etc., as described under "Results" was found to have little effect on the standard curves. The actual recovery of p-aminophenol from the incubation mixtures was determined in each case by comparing these standard curves to one for which the same quantities of p-aminophenol were added directly to the 1.3 ml of 0.2 N HCl before assay. The recoveries were comparable for all systems (±50%), except for dog whole blood (±22%). The recovery of p-aminophenol from whole blood could be increased by the following modifications of the standard procedure. Uncentrifuged samples of whole blood were extracted with six 10-ml portions of ether. The combined fractions were extracted into 8 ml of 0.2 N HCl which were concentrated to 1.3 ml, 0.1 ml of 3 N NaOH was added, and then p-aminophenol content was assayed as usual. This technique resulted in a 50% recovery. Intermediate volumes of ether and acid gave intermediate recoveries. Nevertheless, after correcting for the actual recovery in each case, the calculated amounts of p-aminophenol formed by whole blood were found to be the same. Therefore, the assay procedure was demonstrated to give accurate and reproducible results irrespective of the absolute value of the recovery.

$^9$ The cooling method of terminating the reaction and ether extraction procedure were developed to replace the common method of precipitating the protein with trichloroacetic acid because the latter was found to produce an artificial burst of p-aminophenol formation, possibly due to the production of activated oxygen species generated by the abrupt denaturation of oxyhemoglobin.

[FIG. 1. Standard curve for p-aminophenol (PAP) assay with intact sheep erythrocytes. Known amounts of p-aminophenol were incubated with intact sheep erythrocytes at a hemoglobin concentration of 1 mM for 60 min at 38°C, extracted, and assayed as described under "Experimental Procedures." The absorbance at 630 nm of the indophenol derivative of p-aminophenol was plotted against the known concentration of p-aminophenol. Each point represents the average ± the standard error of at least four determinations. Interpolation of this graph then was used to determine the concentration of p-aminophenol in experimental samples.]

[FIG. 2. Dependence of p-aminophenol (PAP) formation on aniline concentration: sheep intact cells and hemolysates. Suspensions of sheep intact erythrocytes (A) or hemolysates (C) at 1 mM hemoglobin were incubated as described under Table I with aniline at the appropriate concentration as shown. Each point represents the average ± the standard error of at least four determinations.]

grammed flow rate from 0.5 to 1.5 ml/min over a 15-min period. p-Aminophenol was found to elute at 8.2 min. Quantitation by this method was essentially identical to that obtained by the colorimetric assay described above. In addition, p-aminophenol was documented to be the predominant product of the reaction by gas chromatographic-mass spectroscopic analysis. A sample reaction mixture and a sample of intact cells containing standard p-aminophenol were preextracted with benzene to remove the aniline and then with ether and hydrochloric acid as described above. The acid fractions were evaporated to dryness and methylated by the procedure of Kapetanovic et al. (14). The mass
spectrum of the methylated derivative of p-aminophenol produced by erythrocytes was equivalent to that of authentic p-aminophenol. It was confirmed separately that o- and m-aminophenol were easily distinguished from p-aminophenol via this technique. We are grateful to Dr. Edward H. Fairchild, Department of Pharmacology, Case Western Reserve University School of Medicine, for assistance with this analysis.

The dependence of p-aminophenol formation on aniline concentration was characterized in order to select an appropriate concentration to use in all assays of aniline hydroxylase activity. Fig. 2 displays the relationships for sheep erythrocytes and hemolysates. The concentration dependence was essentially the same in both cases. Moreover, the data for human erythrocytes was superimposable on these data. At intermediate aniline concentrations (10 to 70 mM) typical hyperbolic kinetics were observed (Fig. 2); i.e. these data gave linear double reciprocal replots from which $K_m$ values for aniline of $\sim$30 mM were obtained in all cases. As the standard concentration, 60 mM aniline ($\sim$2 $K_m$) was chosen because it was near the upper limit of the region of typical substrate concentration dependence. It is not surprising that the rate values at low aniline concentrations deviate from the hyperbolic relationship because they are not sufficiently greater than the concentration of the catalyst hemoglobin (1 mM) in order that the initial concentrations of aniline can be assumed to be equivalent to unbound aniline. At high aniline concentrations (>70 mM), the rate dependence deviated in an upward fashion. The reason for this unusual behavior is unknown, but several trivial explanations were eliminated. For example, it was confirmed separately that the phenomenon was neither a consequence of a deviation from linearity of product formation with respect to time nor due to a change in recovery of p-aminophenol.

**RESULTS**

Aniline Hydroxylase Activity of Unstimulated Blood Components—Human whole blood, as well as washed erythrocytes from human and sheep sources, were able to catalyze the production of p-aminophenol from aniline (Table I). The net amounts of p-aminophenol formed by each were essentially identical. Aniline hydroxylation by plasma alone was negligible (Table I). These results indicate that the hydroxylase activity of blood is a property of the erythrocytes.

At constant aniline concentration, p-aminophenol formation was linearly dependent upon the concentration of intact human or sheep erythrocytes, up to the concentration corresponding to 1 mM hemoglobin (Fig. 3). At 2 mM, which corresponds to the concentration of hemoglobin in whole blood, the amount of p-aminophenol deviated downward from the line by $\sim$15%. The activity for sheep hemolysates displayed essentially the same relationship within this region of hemoglobin concentrations (Fig. 3).

**Correlation of Aniline Hydroxylase Activity with Oxyhemoglobin Concentration**—Hemoglobin of intact sheep erythrocytes was equivalent to that of authentic p-aminophenol. It corresponded to the concentration of hemoglobin in whole blood. Before testing, the plasma was mixed with an equal volume of isotonic saline in order that the quantity of plasma/unit volume would approximate that of whole blood.

**TABLE I**

| Sample       | p-Aminophenol formed (nmol/60 min/ml) |
|--------------|--------------------------------------|
| A. Human     |                                       |
| Whole blood  | 10.0 ± 0.7 n = 15                     |
| Plasma \(^a\)| 0.0 ± 0.2 n = 6                        |
| Erythrocytes | 9.6 ± 0.5 n = 22                      |
| Erythrocytes | 5.8 ± 0.3 n = 32                      |
| Erythrocytes | 9.7 ± 0.4 n = 4                        |
| Erythrocytes | 5.3 ± 0.1 n = 27                      |
| B. Sheep     |                                       |
| Erythrocytes |                                       |
| Erythrocytes |                                       |

\(^a\) Plasma was obtained as the supernatant after centrifugation of whole blood. Before testing, the plasma was mixed with an equal volume of isotonic saline in order that the quantity of plasma/unit volume would approximate that of whole blood.
Erythrocytes were oxidized completely to methemoglobin by sodium nitrite. After washing, the cells were incubated with aniline in the presence of methylene blue and glucose in order to promote the reduction of the methemoglobin (Fig. 4). The conversion of methemoglobin to oxyhemoglobin and the production of p-aminophenol were followed with respect to time. Very little p-aminophenol was produced when the levels of methemoglobin were high (i.e. oxyhemoglobin concentration low); p-aminophenol production increased as the percentage of methemoglobin decreased.

The correlation of aniline hydroxylase activity with oxyhemoglobin content was demonstrated more directly by using erythrocytes only partially oxidized by nitrite (Fig. 5). The relative contents of methemoglobin and oxyhemoglobin for each test point remained essentially constant during the period of incubation with aniline, because glucose and methylene blue were omitted. The individual samples contained the same quantity of erythrocytes (i.e. total hemoglobin concentration = 1 mM), but they were oxidized to different extents. The dependence of p-aminophenol formation on per cent oxyhemoglobin yielded a straight line which is essentially superimposable on that of Fig. 3.

Aniline Hydroxylase Activity of Metabolically Stimulated Erythrocytes and Hemolysates—Within the erythrocyte, metabolism of glucose produces NADPH via the pentose phosphate pathway and NADH via glycolysis; lactate effects production of only NADH via lactate dehydrogenase (6). With intact sheep erythrocytes addition of glucose elicited a marked increase in p-aminophenol formation (Figs. 6 and 7). The production of p-aminophenol by intact sheep cells in the absence of glucose was approximately linear with time for at least 2 h (Fig. 6). In the presence of glucose, the p-aminophenol production increased and the time course appeared exponential, suggesting an autocatalytic process. Unlike glucose, addition of lactate had no apparent effect on the aniline hydroxylase activity of sheep erythrocytes (Fig. 7). This result contrasts with the documented ability of lactate to support methemoglobin reduction in intact erythrocytes (6). Therefore, an experiment was designed to retest the ability of added lactate to affect the aniline hydroxylase activity under conditions comparable to those of Fig. 3.

4 These results suggest that p-aminophenol itself may act as an intermediate electron carrier and thus stimulate the reaction analogously to methylene blue (see Figs. 7 and 8). It is known that p-aminophenol may be reversibly oxidized to semiquinoid and iminoquinone forms (16). Moreover, experiments in which a known quantity of p-aminophenol was added to a mixture of aniline and erythrocytes resulted in increased product formation so that the total amount of p-aminophenol recovered was greater than the sum of the exogenously added amount and the amount normally formed.
tions where its ability to support methemoglobin reduction could be demonstrated. The use of partially oxidized erythrocytes allows one to examine the effects of added lactate or glucose on methemoglobin reduction and p-aminophenol formation simultaneously. Consistent with previous reports (17, 18), Table II (second column) shows that both lactate and glucose were effective in supporting methemoglobin reduction. On the other hand, only glucose elicited an increase in the aniline hydroxylase activity of the erythrocytes (Table II, columns 3 and 4).

Addition of NADPH and NADH directly had little effect on p-aminophenol formation by intact sheep erythrocytes (Fig. 7). This result might be ascribed to a permeability barrier since each did enhance the activity of hemolysates (Fig. 8). The stimulation of the hemolysate activity by NADPH was much greater than that by NADH.

Glucose and lactate were also tested with the sheep hemolysates (Fig. 8); little change in the aniline hydroxylase activity occurred in either case. The lack of a lactate effect is consistent with the data for the intact erythrocytes (Fig. 7, Table II). The lack of glucose effect with sheep hemolysates, however, could be overcome by also adding ATP or by replacing glucose with glucose 6-phosphate (data not shown). These findings are analogous to the earlier studies of Warburg and Christian (19) who showed that glucose 6-phosphate, but not glucose, could stimulate oxygen consumption in hemolysates, while glucose was effective with intact cells. Thus the activity of hexokinase appears to be the limiting factor in hemolysates.

Glucose and lactate were also tested with human erythrocytes and hemolysates were examined also in a parallel fashion for their responses to added effectors. As expected, the aniline hydroxylase activity of neither responded significantly to lactate addition (Figs. 7 and 8). NADPH and NADH again did not affect the activity of the intact erythrocytes (Fig. 7), but they were equipotent in stimulating p-aminophenol formation by the hemolysates (Fig. 8). As with the sheep hemolysates, glucose had no apparent effect on the activity of human hemolysates (Fig. 8). The results for glucose with human erythrocytes contrasted to those for sheep erythrocytes (Fig. 7). Although erythrocytes from one individual showed a stimulation of activity of as much as 60%, the pooled results for multiple tests of erythrocytes from four individuals showed only a slight stimulation, whereas a 3-fold stimulation was noted for sheep. Glucose was found to be effective with human erythrocytes, however, in the presence of methylene blue (see below).

**Stimulation of Aniline Hydroxylase Activity by Methylene Blue**—The addition of the artificial electron carrier methylene blue stimulated p-aminophenol formation in intact erythrocytes, especially in human erythrocytes where an increase of about 7-fold was observed (Fig. 7). Such stimulation was also reported by Tomoda et al. (5). When both glucose and methylene blue were added simultaneously, a stimulation resulted

**Table II**

| Time | Change in Hb$^{3+}$ concentration | p-Aminophenol predicted | p-Aminophenol observed |
|------|----------------------------------|------------------------|------------------------|
| n    | %                               | mmoles/ml              | mmoles/ml              |
| A. Lactate effects |                                  |                        |                        |
| Experiment 1 | 1 | 11 | 3.0 | 2.6 |
|         | 2 | 20 | 6.3 | 5.0 |
| Experiment 2 | 1 | 12 | 3.5 | 3.4 |
|         | 2 | 24 | 7.3 | 7.0 |
| B. Glucose effects |                                  |                        |                        |
| Experiment 1 | 1 | 16 | 3.1 | 6.0 |
|         | 2 | 18 | 6.2 | 15.9 |
| Experiment 2 | 1 | 18 | 3.6 | 6.4 |
|         | 2 | 15 | 7.0 | 19.6 |

$^a$ Per cent change in Hb$^{3+}$ concentration was calculated according to the relationship:

\[
\text{initial Hb}^{3+} \text{ concentration} - \text{Hb}^{3+} \text{ concentration at designated time} \times 100
\]

\[
\% \text{ change} = \text{initial Hb}^{3+} \text{ concentration}
\]

$^b$ P-Aminophenol predicted was determined in the following manner. 1) Oxyhemoglobin concentration at the designated time (1 or 2 h) was calculated as the difference between total hemoglobin concentration (1 mM) and the methemoglobin concentration; 2) the values for oxyhemoglobin concentration at 1 h were used directly with Fig. 4 to interpolate the corresponding amounts of p-aminophenol which would be produced. For the values of oxyhemoglobin concentration at 2 h, Fig. 4 was again interpolated, but the corresponding amounts for p-aminophenol were multiplied by 2, with the assumption that p-aminophenol formation was linear for the 2-h period (the validity of this assumption is shown by the lower curve of Fig. 5). Since oxyhemoglobin contents increased to their final values over the course of the incubation, the predicted amounts represent slight overestimates.

$^c$ P-Aminophenol observed was measured directly as described under "Experimental Procedures."
that was much in excess of the sum of the separate effects of the two substances. In contrast, the effect of adding lactate plus methylene blue was not different from the effect of methylene blue alone.

**DISCUSSION**

An increasing awareness (1-3, 21-26) has developed regarding certain structural and functional similarities of the hemoglobin and hemoglobin reductase systems of erythrocytes and the cytochrome P-450 and cytochrome P-450 reductase systems of the endoplasmic reticulum of hepatocytes, both of which are involved in the utilization of molecular oxygen. P-450 and hemoglobin are both b-type cytochromes with relatively hydrophobic heme sites which act as terminal electron acceptors (27, 28). Other components of the electron transport systems in each cell type are also similar. Each contains an NADH-dependent reductase and an NADPH-dependent reductase. The former apparently mediates reduction of the hemoprotein via cytochrome b, in both erythrocytes (22) and hepatocytes (7). Moreover, the NADH-dependent reductases from both cell types have been shown to be immunologically identical (23, 24), and the cytochromes b, to be immunologically similar (24, 25). It has been shown also that the isolated NADPH-dependent liver enzyme can catalyze the NADPH-dependent reduction of methemoglobin (2, 29). Several studies (1-3, 5) have shown that isolated hemoglobin can act as a mixed function oxidase; this and other unusual catalytic activities of human hemoglobin have been reviewed recently (26). The demonstration in this report that isolated erythrocytes possess aniline hydroxylase activity like that of hepatic microsomes further the analogy between the two systems.

**Erythrocyte Electron Transport Systems and Aniline Hydroxylase Activity**—As related above, one of the electron transport systems in the erythrocyte is dependent upon NADH, and the other on NADPH. Glucose metabolism serves to produce both pyridine cofactors, while lactate generates only NADH. Reports of genetic deficiencies and concomitant methemoglobinemia have shown that the NADH system apparently is more important for maintaining hemoglobin in the reduced oxygenated form (6). In contrast, the results of the present study suggest that the NADPH-dependent electron transport system in the erythrocyte is the predominant source of reducing equivalents for the hydroxylase activity of the erythrocytes. Such behavior parallels that of the liver microsomal P-450 system where NADPH is the chief cofactor for monoxygenase function (7-9).

The predominant role of NADPH in erythrocytic hydroxylase activity was most clearly demonstrated by the data for sheep. Thus, addition of glucose elicited a 3-fold increase in p-aminophenol formation by intact erythrocytes, whereas lactate had essentially no effect (Fig. 7). Lactate was found ineffective as a stimulant of p-aminophenol formation even under conditions where its ability to support reduction of methemoglobin was demonstrated (Table II). With sheep hemolysates, NADPH was a better effector of the hydroxylase activity than NADH (Fig. 8). The pattern of predominance of the NADPH system was further evident from the combined effects of methylene blue with glucose or lactate; glucose, but not lactate, caused a marked supra-additive effect (Fig. 7).

With human erythrocytes from several individuals, the average results suggested that neither glucose nor lactate alone were effective stimulants (Fig. 7). In the presence of methylene blue, however, the effectiveness of glucose was unmasked and a marked synergism was observed. Lactate plus methylene blue did not show this effect (Fig. 7). With human hemolysates, NADH addition resulted in a stimulation of activity similar to its effect on sheep hemolysates, but NADPH was not more effective than NADH (Fig. 8). Overall, these data suggest that the NADPH electron transport system is favored for the hydroxylase activity of human erythrocytes as well, but it appears to be less efficiently coupled to hemoglobin than it is in sheep.

**Role of Oxyhemoglobin in Aniline Hydroxylation**—In our previous studies with a reconstituted aniline hydroxylase system, hemoglobin was specifically identified as the catalyst (2). Furthermore, characterization of the kinetics of the direct interaction of aniline with various forms of hemoglobin implicated oxyhemoglobin as the important form (2, 4). The results of the present study (Figs. 4 and 5) suggest that oxyhemoglobin is also required for the hydroxylase activity in the intact erythrocyte. This conclusion is based on the interpretation that oxyhemoglobin is the only one of the component necessary for the hydroxylase activity of erythrocytes that is affected by nitrite. The following evidence supports this interpretation. It has been shown that treatment of erythrocytes with sodium nitrite affects the oxidation state of hemoglobin without altering the level of reduced glutathione (15). Since production of NADPH from glucose is sensitive to changes in the level of reduced glutathione (30), maintenance of the reduced glutathione level may reflect maintenance of the NADPH level, as long as glucose is not depleted. In our experimental design, the erythrocytes which were partially oxidized by nitrite were extensively washed in the presence of glucose in order to remove excess nitrite and avoid glucose depletion before testing their hydroxylase activity. This procedure is expected to maintain NADH levels as well as NADPH levels. The former may not be important, however, since the NADH system apparently does not play a key role in p-aminophenol formation. Thus, the change in aniline hydroxylase activity expressed by Fig. 5 is probably a direct consequence of the change in oxyhemoglobin content only. Hence, the virtual superimposition of Figs. 3 and 5 implicates oxyhemoglobin as a necessary component of the erythrocyte aniline hydroxylase system.

**Mechanism of Aniline Hydroxylation by Erythrocytes**—From our model study of an artificial reconstituted hemoglobin-dependent hydroxylase system (2, 4), we concluded that interaction of aniline with oxyhemoglobin in the presence of a reducing system initiated the sequence of events leading to p-aminophenol formation. The current data for the actual intact erythrocyte appear to be consistent with such a scheme but inconsistent with the mechanism usually proposed for P-450 action (31), wherein formation of a substrate-ferrihemoprotein complex is the first step. The P-450 scheme has been based on the information that substrates do interact with the ferric form of that hemoprotein (32, 33) and may alter its oxidation-reduction behavior (34). Furthermore, estimates of the oxidation-reduction state of P-450 in isolated hepatocytes (35) have indicated that in the absence of substrates most of the P-450 exists in the ferric form and addition of substrates shifts the ratio toward the reduced form. In contrast, hemoglobin exists predominantly in the reduced oxygenated form in erythrocytes and this is the form that apparently is necessary for the hydroxylation of aniline (Figs. 4 and 6). A possible sequence of events is depicted below:

\[
\text{Aniline} + \text{HbO}_2 \rightarrow \text{aniline} - \text{HbO}_2
\]  
\[
\text{Aniline} - \text{HbO}_2 + e^- \xrightleftharpoons{2H^+} \text{p-aminophenol} + \text{Hb}^{3+} + \text{H}_2\text{O}
\]  
\[
\text{Hb}^{3+} + e^- + \text{O}_2 \rightarrow \text{HbO}_2
\]  
\[
\text{Sum:Aniline} + 2e^- + \text{O}_2 \xrightleftharpoons{2H^+} \text{p-aminophenol} + \text{H}_2\text{O}
\]

It is proposed that the NADPH-dependent electron transport system supplies the electron to the aniline-HbO₂ complex.
capacity is already compromised. The normal oxygen-carrying function of oxyhemoglobin, especially for certain diseased individuals whose blood oxygen to be toxic via that mechanism, they still might interfere with produce drug metabolites or generate enough methemoglobin system. Although all such interactions may not mon drugs related to it (e.g. phenacetin, lidocaine, acetami-

Toxicant in supporting the reduction of methemoglobin (Step 3). The operation of such a system was suggested by the data of Table II. Lactate was shown to support methemoglobin reduction without enhancing the aniline hydroxylase activity. On the other hand, glucose, whose metabolism by erythrocytes generates NADPH as well as NADH, stimulated p-

We might speculate as a basis for further study that the relative hydroxylase activities of the erythrocyte and hepatocyte systems besides being related to the intrinsic properties of the hemoproteins themselves, such as autoxidizability (23), also might be a function of the ratio of efficiencies of the NAUDP/H and NADH electron transport systems in the two tissues.

Potential Consequences of Drug-Erythrocyte Interacti-

Aniline is a prototype substrate for P-450-catalyzed hydroxylations. Its reactivity with erythrocytes suggests that a hemoglobin generator (namely p-aminophenol) can be formed directly in the blood. Accordingly, it was observed that during long term incubations of aniline with erythrocytes or hemolysates, the solutions darkened in a fashion characteristic of methemoglobin accumulation. A quantitative estimation of methemoglobin formation showed that little accumulated during the first 60 min of incubation of aniline with erythrocytes, but as much as 20% methemoglobin was present after 3 h.

Aniline is a prototype substrate for P-450-catalyzed hydroxylations. Its reactivity with erythrocytes suggests that common drugs related to it (e.g. phenacetin, lidocaine, acetaminophen, amphetamine, etc.) might also interact with the hemoglobin system. Although all such interactions may not produce drug metabolites or generate enough methemoglobin to be toxic via that mechanism, they still might interfere with the normal oxygen-carrying function of oxyhemoglobin, especially for certain diseased individuals whose blood oxygen capacity is already compromised.

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