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INVOLVEMENT IN AFFECTING OXYGEN DISSOCIATION

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Pentobarbital binding to hemoglobin, evidence of an interaction between pentobarbital and 2,3-DPG in binding to hemoglobin, and the effect of altered oxygen dissociation on the outcome of barbiturate poisoning were studied.

Ultrafiltration and equilibrium dialysis techniques were used to determine the binding of pentobarbital to solutions of purified hemoglobin, diluted whole blood, diluted suspended red blood cells, diluted plasma and to hemoglobin obtained from suspended red blood cells. Ultrafiltration was used to study whether the binding of pentobarbital to hemoglobin affected the binding of 2,3-DPG and if there is evidence of competition between these two molecules for a common binding site. Mice were dosed with 10 ml/kg of 0.1 M inosine, 0.1 M pyruvate, and 0.25 M inorganic phosphate which produced an elevation of 2,3-DPG levels and subsequently received either 75 mg/kg or 100 mg/kg of sodium pentobarbital. The recovery times and mortality rates were compared to a control population which received pentobarbital alone.

The percent of pentobarbital bound to purified hemoglobin and to hemoglobin obtained from fresh red blood cells was 8-12% and 9-13%, respectively. The percent of pentobarbital bound to the diluted whole blood was 22-27%, to the diluted blood cell suspension was 7-8%, and to the diluted plasma was 10-13%. There was no change in the percent of pentobarbital bound to hemoglobin in the presence of varying concentrations of 2,3-DPG ranging from zero to twice physiological. The effect of pentobarbital on the binding of 2,3-DPG to hemoglobin could not be determined due to an unreliable assay method. There was no significant effect of altered oxygen dissociation produced by an elevated 2,3-DPG concentration on the outcome of pentobarbital poisoning in terms of survival rate or revival time.

The study suggests that pentobarbital binds to the hemoglobin molecule in whole blood. However, whether this binding interferes with the binding of 2,3-DPG to the central cavity of hemoglobin could not be concluded. It appears that an elevated 2,3-DPG concentration does not affect the outcome of barbiturate poisoning. Since suicidal doses of barbiturates have been shown to significantly affect the oxygen dissociation curve, this study suggests the need for determining the plasma concentration at which a significant effect is observed.

THE BINDING OF PENTOBARBITAL TO HEMOGLOBIN AND
ITS POSSIBLE INVOLVEMENT IN AFFECTING
OXYGEN DISSOCIATION

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

THE BINDING OF PENTOBARBITAL TO HEMOGLOBIN AND ITS POSSIBLE
INVOLVEMENT IN AFFECTING OXYGEN DISSOCIATION

INTRODUCTION

Tissue Hypoxia in Barbiturate Poisoning

For such a long-standing and recurrent problem as barbiturate
poisoning, surprisingly little attention has been given to the patterns
of disturbance that occur in the varying depths of coma they produce
(Sutherland, 1977). Precise studies on the respiratory effects of the
hypnotics are not plentiful, and the literature contains little
information on the relationship of the barbiturates to oxygen uptake
and transport (Shideman, 1961; Carlon, 1978).

The barbiturates are progressive depressants of respiration, the
degree depending on the dose. Respiration is normally maintained by
three physiological influences: (1) a "neurogenic drive", originating
in the reticular activating system; (2) a chemical drive which is
dependent on the CO_2 and pH of arterial blood; and (3) a hypoxic
drive which is mediated by chemoreceptors of the carotid and aortic
bodies (Sharpless, 1970). The neurogenic drive is diminished by
hypnotic doses of barbiturates and respiration is depressed as in
natural sleep. As the dose is increased to amounts sufficient to
produce anesthesia, the hypoxic and chemical drives are proportionately
depressed. The hypoxic drive persists at levels of intoxication that
eliminate the response to CO_2, but if the dose is increased further,
the powerful hypoxic drive will also eventually fail (Harvey, 1980;
Shideman, 1961).

An overdose of a barbiturate drug induces the classical picture of progressive central nervous system depression. In its most severe form this syndrome leads to respiratory arrest as a result of general reflex paralysis; in its milder forms, it may mimic any stage of clinical anesthesia (Eckenhoff and Dam, 1956). Respirations may be rapid and shallow or slow and labored, but as a result of a decrease in depth, the minute volume (pulmonary ventilation) is always reduced. Respirations become irregular, sometimes Cheynes-Stokes in character, and eventually cease (Reed et al., 1952).

Even when barbiturate intoxicated patients appear to be ventilating adequately, hypoxia and hypercarbia may be so marked as to be immediately life-threatening. The electrocardiographic changes which can occur in barbiturate poisoning are chiefly the result of anoxia, and in rare cases, extensive ischemic muscular necrosis of large areas and whole extremities may occur (Moeschlin, 1971). The arterial oxygen tension may be markedly reduced, but a corresponding decrease in the mixed venous oxygen content is not necessarily found. Decreased cellular metabolism and reduced oxygen consumption are implicated (Shubin and Weil, 1971). But, an increase in the affinity of hemoglobin for oxygen could also account for this observation. Meyer and co-workers (1974) revealed that the effect of suicidal doses of barbiturates on oxygen dissociation was an increase in the affinity of hemoglobin for oxygen resulting in a significant shift to the left of the oxygen dissociation curve. The P_{50}, the partial pressure of oxygen required to result in 50% saturation of hemoglobin with oxygen,

was found to average 23.1 ± 1.6 mmHg, compared to 27 mmHg normally.
This represents a decrease in oxygen dissociation at the tissue level
of about 27% (Meyer et al; 1974). This increase in affinity of
hemoglobin for oxygen might contribute to the hypoxia produced by
medullary depression of respiration as a result of barbiturate
intoxication.

Among the mechanisms which could produce such an increase in
oxygen affinity are these possibilities: (1) a binding of barbiturate
to the hemoglobin molecule resulting in either blockage of salt bridge
formation or inhibition of 2,3-diphosphoglycerate (2,3-DPG) binding to
the central cavity, or (2) a modification of intra-erythrocytic metab-
olism resulting in a reduction in the concentration of 2,3-DPG.

Binding of Drugs to Blood Proteins

The binding of most drugs to plasma proteins has been well
documented, and has provided information pertinent to the
pharmacodynamics of various drugs. It is thought that the
physico-chemical factors involved in the binding of a drug to a plasma
protein may be of some relevance to the behavior of that drug at its
site of action, particularly when that site consists of a protein
surface (Goldstein, 1949).

Potentially all proteins of the plasma may bind drugs. Albumin,
the principal protein of the plasma, appears to be most widely involved
in the binding of most drugs, and is by far the most extensively
studied. Albumin has a molecular weight of 69,000 and at plasma pH
carries a net negative charge. The relative affinity of albumin for

anions is explained in terms of the hydrogen bonds formed preferentially between the hydroxyl and carboxy groups of amino acids, serving to leave unbonded cationic nitrogen groups available for binding with anionic substances (Thorp, 1964).

The barbiturates in their enol form behave as weak anions. The R-groups on carbon #5 of the barbiturate ring determine the lipophilic properties of the molecule. It appears that protein-binding depends on the same structural features that determine affinity for nonpolar solvents. That is, those with the highest methylene chloride:water partition coefficients tend to be bound to various proteins to a greater extent. Also, the capacity of various tissues (other than fat) to concentrate barbiturates depends largely on protein binding, and the various barbiturates show the same relative affinities to tissue proteins as to plasma protein (Goldbaum and Smith, 1954).

Pentobarbital, with a methylene chloride:water partition coefficient of 39 has been reported to be bound to plasma proteins at a mean percentage of 61.0 $\pm$ 1.2% (Ehrnebo and Odar-Cederlof, 1975). In addition, Ehrnebo and co-workers (1975) have shown that in healthy subjects approximately 33% of the total pentobarbital in whole blood is present in or on the red blood cells. This suggests that at least a portion of the drug might be carried bound to hemoglobin.

Hemoglobin Structure and Oxygen Transport

Hemoglobin with a molecular weight of 67,000 constitutes about 90% of the total protein in erythrocytes. It is comprised of four polypeptide chains, two identical alpha chains (141 amino acid

residues) and two identical beta chains (146 residues) to each of which is bound a heme residue. A single polypeptide chain combined with a single heme is called a subunit of hemoglobin. Each of the four heme residues consists of a porphyrin ring with an atom of iron (in the ferrous state) at its center. Four of the six possible coordination positions of the iron atom are filled by the nitrogen atoms of the porphyrin ring and a fifth is filled by the imidazole group of the proximal histidine. The sixth position is available for coordination with an oxygen molecule in oxyhemoglobin and is vacant in deoxyhemoglobin (Ten Eyck, 1972).

Hemoglobin must be capable of taking up oxygen in the lungs, transporting it to the periphery and releasing it to the tissues. In the muscle cell, for example, the released oxygen is taken up by myoglobin which subsequently stores it until needed by the cell. A plot of the oxygen dissociation curve of myoglobin takes the shape of a rectangular hyperbola, whereas that of hemoglobin is sigmoidal (Figure 1). The transfer of oxygen from hemoglobin to myoglobin is due to the differences in affinities of the two carriers for oxygen. In the lungs, where the partial pressure of oxygen is high (about 100 mmHg) and the pH also relatively high, hemoglobin tends to become almost maximally saturated with oxygen. In the interior of the peripheral tissues where the oxygen tension is low (about 40 mmHg) and the pH also relatively low, the hemoglobin binds oxygen less strongly and will thus unload a portion of its oxygen to the cells.
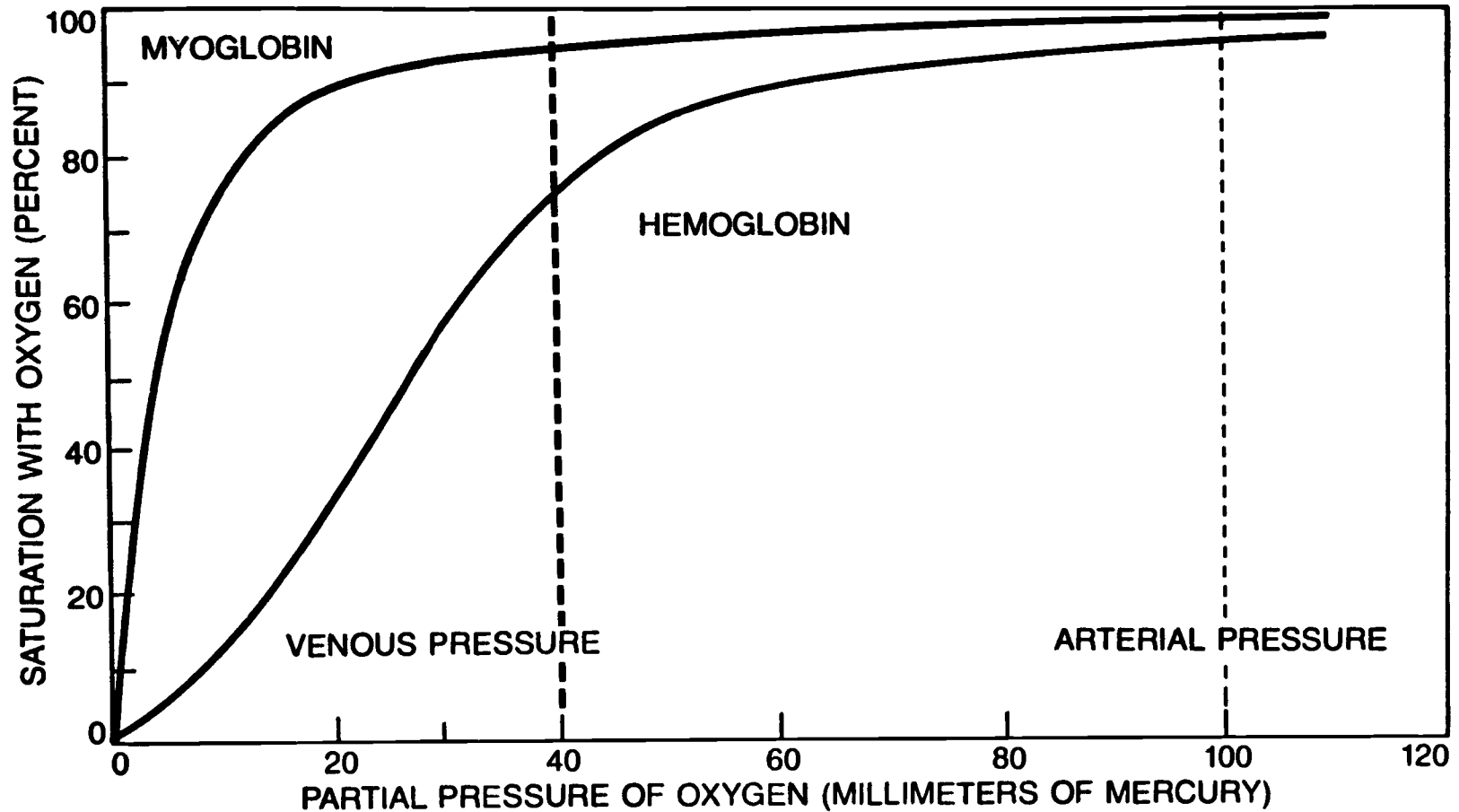
FIGURE 1. Oxygen dissociation curves of myoglobin and hemoglobin.
Myoglobin, a protein of muscle, has just one heme group and one
polypeptide chain and resembles a single subunit of hemoglobin. At
any partial pressure myoglobin has a higher affinity than hemoglobin,
which allows oxygen to be transferred from blood to muscle.

Each heme residue has the capability of combining with one molecule of oxygen. However, the consecutive binding of oxygen to the four hemes of a hemoglobin molecule is not independent, but does occur without contact between them, since each heme lies in isolated pockets on the surface of the subunits. The first oxygen attaches very slowly, the second and third more readily, and the fourth oxygen molecule binds several hundred times more rapidly than the first. Perutz (1970a) proposed an allosteric theory to explain this apparent heme-heme interaction or cooperative effect. Deoxyhemoglobin contains salt bridges which are linkages between adjacent subunits formed by the attraction of oppositely charged amino acid residues in close proximity to each other only in the deoxy conformation. This form of hemoglobin has been labelled T, for "tense". The binding of oxygen to the heme group of a subunit is accompanied by a movement of the iron atom and the proximal histidine into the plane of the porphyrin ring. This results in a change in the tertiary structure of the subunit and breaks the salt bridges binding the subunit to its neighboring chains. By removing the constraints which stabilized the deoxy conformation, the oxygen affinity will increase. By a similar process, oxygenation of the second and third hemes breaks additional salt bridges and shifts the equilibrium toward the high affinity oxyhemoglobin conformation. The oxy form of hemoglobin has been labelled R, for "relaxed" (Arnone, 1974; Ten Eyck, 1972).

The physiological importance of heme-heme interaction lies not so much in ensuring an increased oxygen affinity as successive molecules

of oxygen combine, as it is in making the affinity fall as successive oxygen molecules dissociate. Without cooperativity only a small fraction of the oxygen carried by hemoglobin would be extracted, resulting in asphyxiation even if man inspired pure oxygen. The Bohr effect and 2,3-DPG both increase the efficiency of oxygen transport further by lowering the oxygen affinity of hemoglobin in the tissues (Perutz, 1970b).

The Bohr Effect

As the hydrogen ion concentration increases the oxygen dissociation curve shifts to the right, decreasing hemoglobin's affinity for oxygen. Since metabolism of nutrients by tissues liberates lactic acid and carbonic acid, which in turn liberates protons, the hemoglobin will release more oxygen at the periphery, where it is needed. This is known as the Bohr effect, and can be expressed as:

$$HbO_2 + H^+ \rightleftharpoons HHb^+ + O_2$$

in which HHb^+ is a protonated subunit of a deoxyhemoglobin molecule.

Both the lowering of the oxygen affinity by protons and the uptake of protons on release of oxygen can be explained in terms of salt bridges (Perutz, 1979). As the concentration of protons increases there is a greater chance of a histidine becoming positively charged and forming a salt bridge with a carboxyl group carrying a negative charge. Also, during the transition from the R structure to the T structure, negatively charged oxygen atoms of carboxyl groups are brought into proximity of uncharged nitrogen atoms. This results in a

greater uptake of protons from the solution by hemoglobin in the T structure.

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The Role of 2,3-Diphosphoglycerate (2,3-DPG) in Oxygen Affinity

</div>

The opening between the two beta chains in the T structure is tailor-made to fit the molecule of 2,3-DPG and to complement its negative charges which result in another set of salt bridges. In the R structure the gap narrows and 2,3-DPG has to drop out (Perutz, 1970b). Benesch and Benesch (1969) showed that one mole of 2,3-DPG binds with one mole of deoxyhemoglobin resulting in a shift of the oxygen dissociation curve to the right, toward lower affinity.

Production of 2,3-DPG occurs through an offshoot of the glycolytic pathway termed the Rapoport-Luebering shuttle (Figure 2). The substrate 1,3-DPG can either be converted to 2,3-DPG by phosphoglycerate mutase, or to 3-phosphoglycerate by phosphoglycerate kinase with the production of ATP. Hence, the two pathways compete for the common substrate 1,3-DPG producing either ATP or 2,3-DPG. The enzyme phosphoglycerate mutase is inhibited by 2,3-DPG and phosphoglycerate kinase activity is increased by a rise in the ADP/ATP ratio. Thus, the Rapoport-Luebering shuttle is a self-regulating system designed to maintain levels of ATP and 2,3-DPG, one at the expense of the other (Rapoport, 1968).

The physiological effects of 2,3-DPG on oxygen transport are best demonstrated by the use of oxygen dissociation curves (Figures 3 and 4). Normally, at the partial pressure of oxygen in arterial blood
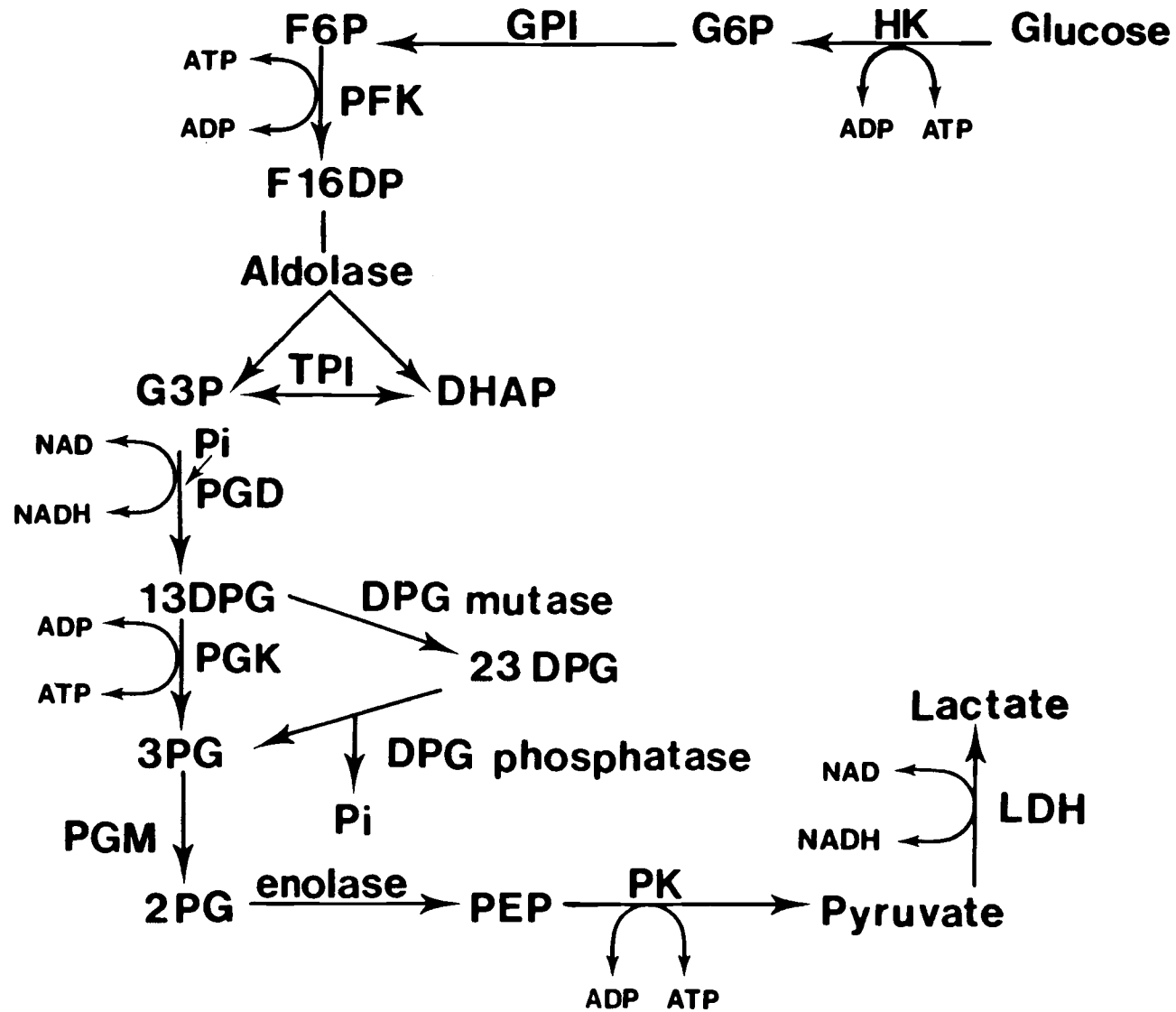
FIGURE 2. Glycolytic pathway showing the Rapoport-Luebering cycle.

(100 mmHg) hemoglobin is 98% saturated with oxygen. As the partial
pressure of oxygen falls in the capillaries to the venous value of 40
mmHg, oxygen is released until the hemoglobin is only 75% saturated.
Thus, 23% of the oxygen originally bound to hemoglobin is released to
the tissues.

In the absence of 2,3-DPG the oxygen affinity of the blood
increases so that at a given PO_2 more oxygen is bound (Figure 3).
This is described as a shift to the left of the oxygen dissociation
curve. In normal blood the P_{50} has a value of 27 mmHg at pH 7.4
and 37°C. The P_{50} of blood depleted of 2,3-DPG is 12 mmHg (Bunn et
al., 1969). Elevation of red cell 2,3-DPG above normal baseline levels
has the opposite effect. This is shown in Figure 4 by a shift to the
right of the oxygen dissociation curve and a P_{50} of 36 mmHg
indicating a decreased affinity of hemoglobin for oxygen.

These changes in oxygen affinity are important because they affect
the amount of oxygen delivered to the tissues. When the oxygen
affinity is increased in the absence of 2,3-DPG (the left-hand curve in
Figure 3), the hemoglobin in arterial blood becomes 99% saturated with
oxygen but the saturation in venous blood would still be 96%. This
results in the release of only 3% of the oxygen to the tissues. When
the affinity of hemoglobin for oxygen is decreased by an elevated
2,3-DPG (right-hand curve in Figure 4), the arterial saturation falls
slightly to 93% but the saturation in venous blood drops to 58%. This
results in the release of 35% of the oxygen to the tissues (Metcalf and
Dhindsa, 1972). Various agents have been shown to affect the
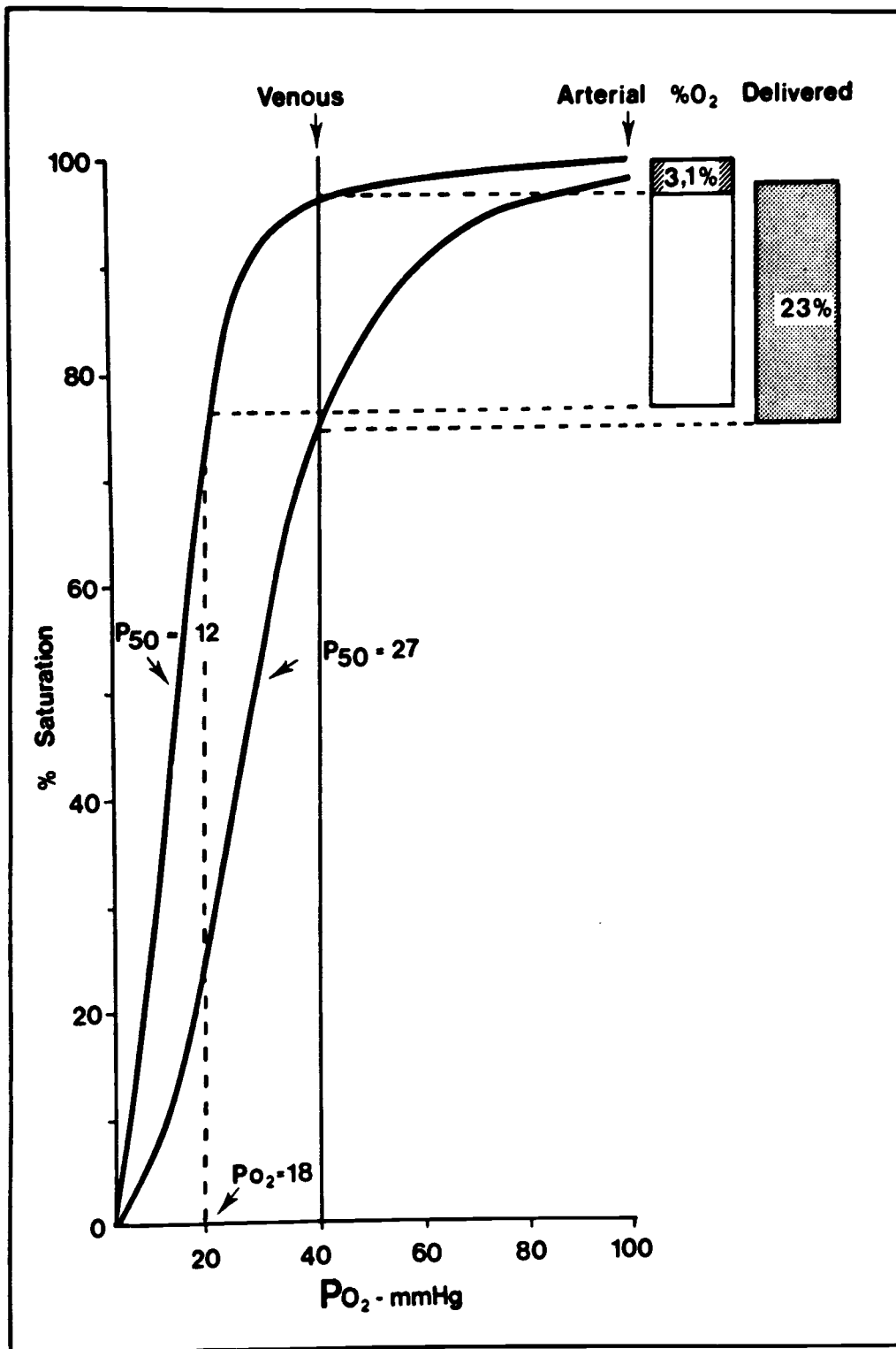
FIGURE 3. A normal oxygen dissociation curve (P50 = 27mm Hg) showing the delivery of 23% of the oxygen as the PO_2 drops from 100mm Hg to 40mm Hg. The left-hand curve represents the oxygen dissociation curve of blood depleted of 2,3-DPG. The P50 is 12mm Hg and this blood would release only 3,1% of its oxygen as the PO_2 drops to 40mm Hg.
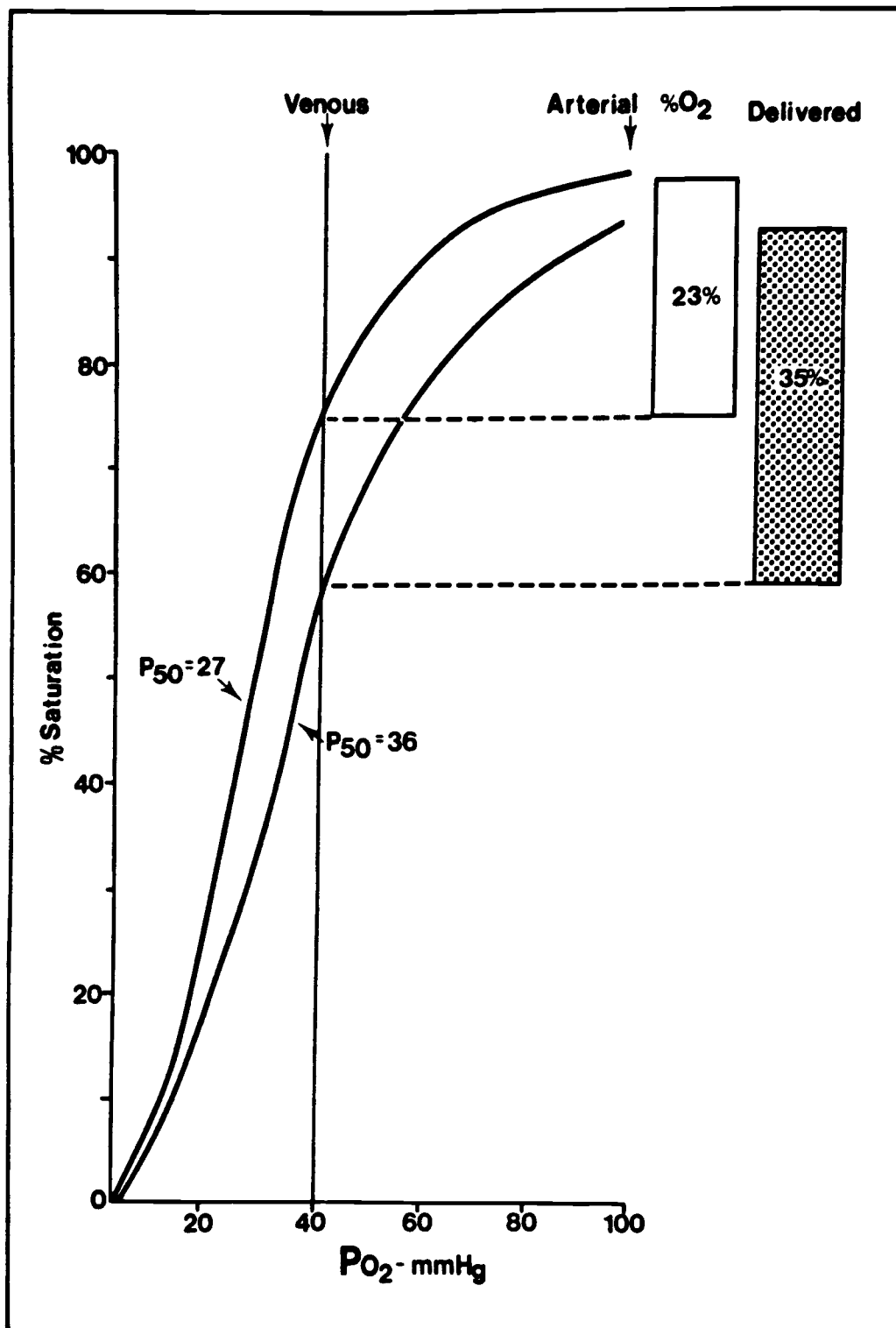
FIGURE 4. A normal oxygen dissociation curve (P50 = 27mm Hg) showing the delivery of 23% of the oxygen as the PO_2 drops from 100mm Hg to 40mm Hg. The right-hand curve is the oxygen dissociation curve of blood with an elevated 2,3-DPG level. The P50 is 36mm Hg and this blood would release 35% of its oxygen as the PO_2 drops to 40mm Hg.

concentration of red cell 2,3-DPG and subsequently to alter the
affinity of hemoglobin for oxygen.

Agents Affecting Oxygen Affinity

Agents That May Decrease the Oxygen
Affinity of Hemoglobin

By utilizing erythrocyte glycolysis, a mixture of inosine,
phosphate, and pyruvate (IPP) can produce an increase in the 2,3-DPG
concentration. Through several enzymatic steps inosine and phosphate
undergo phosphorolysis in the presence of nucleoside phosphorylase to
yield hypoxanthine and ribose-1-phosphate and eventually provide
substrate for 2,3-DPG synthesis in the form of glyceraldehyde-
3-phosphate (G-3-P). The reduction of the added pyruvate provides NAD
for the conversion of G-3-P to 1,3-diphosphoglycerate (Oski et al.,
1971). Sugerman and co-workers (1972) showed that infusion of 20 ml/kg
of a 0.1 M solution of inosine, pyruvate, and disodium phosphate in
rhesus monkeys produced an increase in mean red cell 2,3-DPG from 5.1
to 7.6 mM/L RBC at six hours after infusion and demonstrated a mean
rise in P_{50} from 33.5 to 36.1 mmHg. Proctor and co-workers (1974)
observed an increase in rabbit 2,3-DPG levels from 8.1 to 12.05 mM/L
RBC four hours after infusion of 10 ml/kg of 0.1 M inosine, 0.1 M
pyruvate, and 0.25 M inorganic phosphate.

In ten patients with acute lymphoblastic leukemia, prednisone (1.5
to 2.2 mg/kg per day by mouth for three weeks) significantly increased
(21 percent) the red cell concentration of 2,3-DPG (Silken, 1975). The
increase in 2,3-DPG was accompanied by a decrease in plasma inorganic

phosphate and an increase in the hematocrit and total hemoglobin. Six patients in chronic renal failure increased their mean red cell 2,3-DPG levels from 5.67 to 9.1 mM/L RBC after 12 weeks of weekly intramuscular injections of 400-600 mg of testosterone enanthate (Parker et al., 1972). Androgens also display an erythropoietic response. Therefore, corticosteroids and androgens have a two-fold effect in improving oxygen delivery by increasing the hemoglobin and by facilitating its ability to release oxygen to the tissues.

Methylene blue produces an increase in the P_{50} of human blood by increasing the intracellular levels of 2,3-DPG in a concentration-dependent manner (Bernasconi et al., 1974).

Lichtman and co-workers (1974) showed that propranolol in concentrations of 1-5 X 10^{-4} M produces an increase in the mean P_{50} by 6-8 mmHg. Their conclusion was that propranolol causes a massive leakage of potassium, chloride, and water from the erythrocyte resulting in an increase in red cell hydrogen ion concentration. Therefore, the fall in pH explains the change in affinity on the basis of the Bohr effect. Oski and co-workers (1972) showed that propranolol produces a decrease in the affinity of hemoglobin for oxygen by causing a redistribution of 2,3-DPG in the red cell. Their conclusion was that propranolol produces a release of 2,3-DPG that is bound to the intracellular membrane of the red cell, thus increasing the free concentration of 2,3-DPG without changing the total red cell concentration.

Agents That May Increase the Oxygen
Affinity of Hemoglobin

Hemoglobin treated with hydrazine, which prevents the carboxyl

terminus of each alpha chain from forming a salt bridge with a lysine

on the neighboring alpha chain, diminishes the stability of the T

structure, increases the affinity for oxygen, and shifts the oxygen

dissociation curve to the left (Kilmartin et al., 1977).

Sodium salicylate in large, but not lethal doses produces a

dose-dependent reduction in red cell 2,3-DPG in rats. Salicylate

inhibits several steps in the glycolytic sequence and presumably one or

more of these accounts for its effects on 2,3-DPG levels. The effect

of salicylate on 2,3-DPG may account for the 10-20 percent increase in

venous oxygen saturation produced by high doses of salicylates in man

and rats (Kravath et al., 1972).

Cyanate produces an increase in oxygen affinity by combining

irreversibly with the N-terminal residues of the beta chains of

hemoglobin which comprise part of the 2,3-DPG binding site (Caldwell et

al., 1971). The cyanate binding interferes with the ability of the

tetramer to assume the deoxy configuration by reducing the

possibilities for salt bridge formation or 2,3-DPG binding (Manning et

al., 1972). Harkness and co-workers (1974) found no effect of cyanate

in concentrations up to 10 mM on the activities of 2,3-DPG phosphatase

or mutase. Nevertheless, a dose-dependent reduction in intracellular

2,3-DPG was noted together with the increase in oxygen affinity. The

authors speculate that 2,3-DPG mutase is very sensitive to end-product

inhibition, and that the increase in unbound 2,3-DPG in the red cell
after carbamylation of hemoglobin may result in a lower level of
2,3-DPG secondary to decreased activity of the mutase enzyme.

STATEMENT OF PROBLEM

The objectives of this study were to determine if pentobarbital would bind to the hemoglobin molecule in whole blood, to determine if an interaction exists between pentobarbital and 2,3-DPG in binding to the hemoglobin molecule, and to investigate the potential role of altered oxygen dissociation on the outcome of barbiturate poisoning.

Studies were performed to investigate whether pentobarbital would bind to the purified hemoglobin molecule using both equilibrium dialysis and ultrafiltration techniques to assess binding (Rosenburg and Klotz, 1960). In order to bind to hemoglobin in vivo, pentobarbital must have the capacity to partition through the red blood cell. Even though the binding to purified hemoglobin might be extensive, the degree to which it would bind to hemoglobin in whole blood could be reduced by the limitation of partitioning through the red blood cell and by the number of other protein surfaces available for binding. Therefore, the extent of binding of pentobarbital to whole blood, suspended red blood cells, and albumin were compared to the studies of binding to purified hemoglobin and to hemoglobin obtained from suspended red blood cells.

Binding of pentobarbital to hemoglobin could inhibit or reduce the occurrence of necessary salt bridge formation resulting in the increase in affinity of hemoglobin for oxygen as observed by Meyer (1974). Also, blockage of the binding of the modulator 2,3-DPG to the central cavity of hemoglobin could produce similar results. Both 2,3-DPG (MW

260) and pentobarbital (MW 226) are small molecules and both behave as anions. In addition, pentobarbital possesses lipophilic properties necessary to facilitate entry into the central cavity of hemoglobin. Studies were performed to investigate whether the binding of pentobarbital to hemoglobin affected the binding of 2,3-DPG and if there is evidence of competition between these two molecules for a common binding site.

Mice were used to determine whether the administration of pentobarbital had any influence on the in vivo concentration of 2,3-DPG in erythrocytes. Mice were also used to determine the effect of pharmacologically increased 2,3-DPG pretreatment on the outcome of administration of lethal and sub-lethal doses of pentobarbital. If the increase in affinity of hemoglobin for oxygen produced by barbiturates could be overcome by an increase in the concentration of 2,3-DPG, another therapeutic tool could be added to the treatment of barbiturate poisoning.

MATERIALS AND METHODS

Binding of Pentobarbital to Hemoglobin

Binding of Pentobarbital to Purified Hemoglobin

Purified hemoglobin was obtained from Sigma Chemical Co., and a 2% w/v (2.7×10^{-4} M) hemoglobin solution prepared in 0.07 M isotonic phosphate buffer, pH 7.36 (14 mM KH_2PO_4, 57 mM Na_2HPO_4, and 70 mM NaCl). To insure that the hemoglobin was present in the deoxygenated and ferrous form, 500 mg of sodium dithionite was added per liter of hemoglobin solution. ^{14}C-pentobarbital sodium (40 mCi/mmole) was obtained from New England Nuclear.

Equilibrium Dialysis Experiments. The following components were added to a series of 50 ml Erlenmeyer flasks: 0.1 ml of varying concentrations of unlabelled sodium pentobarbital to result in final concentrations ranging from 1.25 to 20 mcg/ml ($5 \times 10^{-6} - 8 \times 10^{-5}$ M); 0.1 ml ^{14}C-pentobarbital to result in final concentrations of 60,000 CPM/ml; and 15 ml of 2% w/v hemoglobin. The Erlenmeyer flasks were incubated in a Dubnoff shaker bath at 37°C for 30 minutes under a constant nitrogen atmosphere.

Ten ml of this solution was pipetted into a 1/4" cellulose dialysis bag. This was positioned into a 40 ml conical centrifuge tube containing 15 ml of 0.07 M phosphate buffer, pH 7.36. Nitrogen gas was bubbled through each centrifuge tube for 30 minutes. Each tube was stoppered and placed in an incubation bath at 37°C for 24 hours under a

constant nitrogen atmosphere. A 0.1 ml aliquant of the hemoglobin solution and 0.1 ml aliquant of dialysate were assayed for pentobarbital concentration.

Ultrafiltration Experiments. Identical methods of sample preparation were employed as in the equilibrium dialysis experiments, except the sodium pentobarbital final concentrations were in the range of 2.5 to 67 mcg/ml (10^{-5} - 2.7 X 10^{-4} M). Upon completion of incubation in the Dubnoff shaker bath, 10.0 ml of the hemoglobin solution was pipetted into 1/4" cellulose dialysis tubing and suspended in a 40 ml conical centrifuge tube and stoppered. The tubes were then centrifuged at 200-300 RPM for eight hours, or until approximately 0.3 to 0.4 ml of dialysate had been collected. Pentobarbital concentrations were assayed as before.

Assay of Pentobarbital

For radioassay 0.1 ml samples were pipetted into liquid scintillation vials containing 10 ml of Dimilume-30 (Packard Inst. Co.). Each vial was counted for ten minutes using a Packard Model 3385 liquid scintillation spectrometer. The counting efficiency was approximately 77% for hemoglobin samples and 89% for dialysate. Results were converted to DPM before calculation of binding.

Binding of Pentobarbital to Diluted Whole Blood, Diluted Blood Cell Suspension, and Diluted Plasma

A sample of fresh human whole blood was obtained by a licensed Medical Technologist and the hemoglobin concentration and hematocrit

determined. The sample was diluted with 0.07 M isotonic phosphate buffer, pH 7.36, to yield diluted whole blood with a concentration of hemoglobin equal to 2% w/v. One-half of the diluted sample was centrifuged and the diluted plasma decanted and saved for subsequent experimentation. A blood cell suspension (BCS) was prepared by washing the centrifuged blood cells three times with 0.07 M isotonic phosphate buffer, pH 7.36. The volume of BCS was finally adjusted to that of the initial whole blood sample with the phosphate buffer. This provided a suspension of washed erythrocytes in an isotonic buffer system with the same hematocrit as the diluted whole blood sample.

Ten ml aliquants of either diluted whole blood, diluted blood cell suspension, or diluted plasma were incubated as before with ^{14}C-pentobarbital and with various concentrations of unlabelled sodium pentobarbital ranging from 2.5 to 20 mcg/ml. Ultrafiltration of samples and subsequent assay of pentobarbital concentrations were performed. Undiluted whole blood and plasma were analyzed for binding to insure that the results were in agreement with previously reported observations.

Binding of Pentobarbital to the Hemoglobin Obtained from the Diluted Blood Cell Suspension

Experiment A. After ultrafiltration of the diluted blood cell suspension in the previous experiment, the remaining blood cell suspension present in the dialysis tubing was collected and pooled according to pentobarbital concentration. The red blood cells were

lysed by alternate freezing and thawing in an acetone-dry ice bath and 37°C water bath, followed by sonication. The individual samples were then centrifuged at 20,000 X g for 20 minutes. The supernatant or hemoglobin solution was transferred to dialysis tubing and ultrafiltration was again performed.

Experiment B. Ten ml aliquants of diluted BCS were incubated with ^{14}C-pentobarbital and unlabelled sodium pentobarbital in concentrations ranging from 2.5 to 20 mcg/ml. The samples were then lysed, centrifuged and the hemoglobin solution dialyzed by ultrafiltration as in Experiment A.

Binding Interaction Between 2,3-DPG and Pentobarbital

Binding of 2,3-DPG to Purified Hemoglobin

To a series of 50 ml Erlenmeyer flasks containing 15 ml of 2% w/v hemoglobin was added 0.1 ml of varying concentrations of 2,3-DPG resulting in final concentrations of 40, 80, and 160 mcg/ml. This corresponded to molar ratios of 2,3-DPG to hemoglobin of 0.5:1, 1:1, and 2:1, respectively. Samples were incubated in a Dubnoff shaker bath at 37°C for 30 minutes under a constant nitrogen atmosphere. Ten ml of sample solution was transferred to dialysis tubing and subjected to ultrafiltration. The 2,3-DPG concentration of the hemoglobin solution and of the dialysate were analyzed using the fluorometric assay.

Interaction of Pentobarbital and 2,3-DPG in Binding to Hemoglobin

Fifteen ml aliquants of 2% w/v hemoglobin containing ^{14}C-pentobarbital, unlabelled sodium pentobarbital (67 mcg/ml), and

2,3-DPG (40 to 160 mcg/ml) were incubated in a Dubnoff shaker bath at 37°C for 30 minutes under a constant nitrogen atmosphere. Ten ml of this solution was pipetted into 1/4" cellulose dialysis tubing, positioned into a 40 ml conical centrifuge tube, stoppered, and dialyzed by ultrafiltration. Two 0.1 ml aliquants of the hemoglobin solution were obtained and analyzed for either pentobarbital concentration or 2,3-DPG concentration (fluorometric assay). Similarly, two 0.1 ml aliquants of dialysate were obtained and assayed for pentobarbital concentration and for 2,3-DPG concentration.

Assay of 2,3-DPG

Fluorometric Assay. The fluorometric enzymatic endpoint assay of Keitt was used to determine 2,3-DPG content of hemoglobin samples in vitro (Keitt, 1971). Samples were treated with equal volumes of 10% TCA, mixed thoroughly, and left on ice for at least 15 minutes. The denatured protein was then centrifuged at 27,000 X g for 20 minutes at 2°C. The clear supernatant was neutralized with 2 M $KHCO_3$. The assay solution consisted of the following components: Tris buffer, 50 mM; hydrazine SO_4, 5 mM; $MgCl_2$, 5 mM; glutathione (reduced), 2.5 mM; albumin (BSA), 0.01% w/v; ATP, 0.3 mM; 2-phosphoglycolate, 1.32 mM; NADH, 0.006 mM; glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), 20 mcg/ml; phosphoglycerate kinase (EC 2.7.2.3), 3 mcg/ml; and phosphoglycerate mutase (EC 2.7.5.3), 10 mcg/ml. A 25 ul sample of the neutralized supernatant was added to 4.0 ml of the assay solution. NADH fluorescence was measured with a Turner Model III fluorometer with filters Nos. 0-53 + 7-54 (primary) and 2A + 48 (secondary) at time zero

and 30 minutes later. The change in fluorescence was dependent upon the 2,3-DPG concentration.

Spectrophotometric Assay. Analysis of 2,3-DPG in mouse erythrocytes (subsequent experiments) was accomplished by the spectrophotometric method of Keitt with minor modifications (Keitt, 1971). The TCA treated blood samples were centrifuged at 27,000 X g for 20 minutes at 2°C. The clear supernatant was removed and assayed for 2,3-DPG content. The assay solution consisted of the following components: Tris buffer, 50 mM; hydrazine SO_4, 5 mM; $MgCl_2$, 5 mM; glutathione (reduced), 2.5 mM; ATP, 1 mM; NADH, 0.15 mM; glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), 20 mcg/ml; phosphoglycerate kinase (EC 2.7.2.3), 3 mcg/ml; and phosphoglycerate mutase (EC 2.7.5.3), 25 mcg/ml. A 0.1 ml sample of supernatant was added to 3.0 ml of the assay solution. After allowing ten minutes for all 2-phosphoglyceric acid and 3-phosphoglyceric acid to react, 0.1 ml of 66 mM 2-phosphoglycolate was added. Immediately a measurement was taken using a Beckman Model spectrophotometer at 340 nm. One hour later a second reading was taken and the 2,3-DPG quantified by the change in absorbance.

Stimulation of 2,3-DPG Synthesis and Pentobarbital Toxicity

Increase in 2,3-DPG Concentration
by Administration of IPP

Male mice averaging 30 g in weight and 40 days in age were administered 10 ml/kg of 0.1 M inosine, 0.1 M pyruvate, and 0.25 M inorganic phosphate intraperitoneally. A control group received 10

ml/kg of saline. Five mice were sacrificed each hour and blood samples obtained at zero, one, two, three, and four hours post-injection via intra-aortic puncture under ether anesthesia. Samples were mixed with equal volumes of 10% TCA and kept on ice until analysis of 2,3-DPG was performed. The 2,3-DPG concentration was assayed spectrophotometrically.

Effect of Pentobarbital Administration on 2,3-DPG Concentration In Vivo

Male mice were administered 75 mg/kg of sodium pentobarbital intraperitoneally. Five mice were sacrificed each hour and blood samples were obtained at zero, one, two, three, and four hours post-injection via intra-aortic puncture under ether anesthesia. Samples were mixed with equal volumes of 10% TCA and kept on ice until analysis of 2,3-DPG was performed. The spectrophotometric assay was used to determine 2,3-DPG concentration.

Effect of Pretreatment with IPP on Toxic Doses of Pentobarbital

Male mice were administered 10 ml/kg of 0.1 M inosine, 0.1 M pyruvate, and 0.25 M inorganic phosphate intraperitoneally. A control group received 10 ml/kg of saline. One hour post-injection both groups received 75 mg/kg of sodium pentobarbital intraperitoneally. In a separate experiment, one hour post-IPP or post-saline injection both groups received 100 mg/kg of sodium pentobarbital intraperitoneally. The mice were observed until death or until all had revived. Revival time was recorded as well as objective and subjective observations related to behavior.

RESULTS

Binding of Pentobarbital to Hemoglobin

Binding of Pentobarbital to Purified Hemoglobin

The percent of sodium pentobarbital bound to purified
hemoglobin over the range of concentrations tested for both the
equilibrium dialysis and ultrafiltration methods is shown in Table I.
The percent of pentobarbital bound was between 11-12% at all
concentrations employed as assayed by equilibrium dialysis. At each
concentration of pentobarbital the ultrafiltration method tended to
give consistently lower values as compared to the equilibrium dialysis
method. There was a significant decrease in binding obtained via
ultrafiltration at the 20 mcg/ml concentration of pentobarbital
($p < 0.05$), but otherwise there was no significant difference in binding
between the two methods ($p > 0.05$). In addition, the results obtained
via the ultrafiltration method revealed a consistent but insignificant
($p > 0.05$) decrease in the percent of binding as the concentration of
pentobarbital increased. This is consistent with saturation kinetics
of protein binding.

Binding of Pentobarbital to Diluted
Whole Blood, Diluted Blood Cell
Suspension, and Diluted Plasma

The percent of pentobarbital (10 mcg/ml) bound to whole blood
and to plasma was 45.4% and 58.5%, respectively. One sample for each
was analyzed simply to determine if the binding was in agreement with
other published data. The results agree well with those obtained by

Table 1. Binding of pentobarbital to purified hemoglobin.

Per cent Bound

Pentobarbital Conc (mcg/ml)	Dialysis		Ultrafiltration	
1.25	12.18 + 2.44	(4)*		
2.5	12.13 + 5.18	(8)	10.49 + 5.65	(5)
5.0	10.94 + 2.75	(8)	10.18 + 2.8	(5)
10.0	10.58 + 2.32	(8)	9.37 + 1.6	(6)
20.0	12.35 + 3.85	(8)	8.7 + 2.92	(7)
67.0			8.08 + 1.53	(6)

*number in parentheses is the number of assays at that pentobarbital concentration. Data are expressed as $\overline{X} \pm$ S.D.

Ehrnebo and Odar-Cederlof (1975) of 50.4% and 61.0%, respectively.

The results for the binding of pentobarbital to diluted whole blood, diluted blood cell suspension, and diluted plasma are presented in Table 2. The percent of binding to diluted whole blood (22-27%) was significantly greater than the binding to the diluted blood cell suspension (7-9%) at all pentobarbital concentrations tested ($p < 0.025$). In fact, at a pentobarbital concentration of 2.5 mcg/ml, the binding to diluted whole blood (27.1 $\pm$ 5.36%) was significantly greater than the additive binding to the diluted blood cell suspension and diluted plasma (17.9 $\pm$ 1.93%) ($p < 0.025$).

Binding of Pentobarbital to the Hemoglobin Obtained from the Diluted Blood Cell Suspension

Table 3 shows the percent of pentobarbital bound to the hemoglobin obtained from the lysed red blood cells in Experiment A and Experiment B. There was no significant difference in binding to hemoglobin between Experiment A or Experiment B at all concentrations of pentobarbital tested ($p > 0.05$). Also, there was no significant difference between the binding of pentobarbital to purified hemoglobin (ultrafiltration method) and the binding to hemoglobin obtained from lysed blood cells in Experiment A ($p > 0.05$). Although the values obtained for binding to the diluted BCS (7-9%) were lower than the values obtained for binding to the hemoglobin derived from those cells in Experiment A (9-13%), there was no significant difference in binding between them ($p > 0.1$). The binding of pentobarbital to diluted whole blood (22-27%) was significantly greater than the binding to hemoglobin

Table 2. Binding of pentobarbital to diluted whole blood, diluted
blood cell suspension, and diluted plasma.

Percent Bound

Pentobarbital Conc (mcg/ml)	Diluted Whole Blood		Diluted BCS		Diluted Plasma	
2.5	27.11 ± 5.36	(3)*	7.13 ± 1.67	(3)	10.72 ± 0.29	(3)
5.0	25.06 ± 6.04	(3)	8.49 ± 0.95	(3)	11.31 ± 0.2	(2)
10.0	23.19 ± 4.99	(2)	8.79 ± 2.86	(3)	10.72 ± 1.17	(2)
20.0	22.15 ± 3.31	(3)	7.67 ± 1.83	(3)	13.32 ± 1.83	(2)

*number in parentheses is the number of assays at that pentobarbital
concentration. Data are expressed as $\overline{X}$ ± S.D.

Table 3. Binding of pentobarbital to the hemoglobin obtained from the diluted blood cell suspension.

Percent Bound

Pentobarbital conc (mcg/ml)	Experiment A	Experiment B
2.5	13.29 ± 0.83 (3)*	15.13 ± 2.88 (2)
5.0	10.0 ± 2.38 (3)	13.93 ± 5.25 (2)
10.0	11.52 ± 4.09 (3)	9.83 ± 4.1 (3)
20.0	9.18 ± 2.05 (3)	10.54 ± 3.29 (3)

*number in parentheses is the number of assays at that pentobarbital concentration. Data are expressed as $\overline{X}$ ± S.D.

in Experiment A (9-13%) at all pentobarbital concentrations (p<0.025).

Binding Interaction Between 2,3-DPG and Pentobarbital

The effect of the presence of 2,3-DPG on the binding of pentobarbital to hemoglobin is shown in Figure 5. At a pentobarbital concentration of 67 mcg/ml there was no significant difference in binding to hemoglobin at all concentrations of 2,3-DPG tested (p>0.1).

Unfortunately, the binding of 2,3-DPG to hemoglobin and the effect of pentobarbital on the binding of 2,3-DPG to hemoglobin were not elucidated, due to the inability of interpreting the data generated from the fluorometric technique for the assay of 2,3-DPG. It was discovered that sodium dithionite activates the enzyme 2,3-diphosphoglycerate 2-phosphohydrolase (EC 3.1.3.13) (Sauer and Kramer, 1970). Although this enzyme was not present in the assay solution, the dithionite ion may affect other glycolytic enzymes and account for the inability to enzymatically quantify 2,3-DPG.

Stimulation of 2,3-DPG Synthesis and Pentobarbital Toxicity

Increase in 2,3-DPG Concentration
by Administration of IPP

The results of the injection of 10 ml/kg of 0.1 M inosine, 0.1 M pyruvate, and 0.25 M inorganic phosphate and the effect on the 2,3-DPG concentration of mouse erythrocytes are presented in Figure 6. The concentration of 2,3-DPG rose to a peak of 5.4 mM 2,3-DPG/L of blood at one hour post-injection which was a significant increase from a baseline value of 4.06 mM 2,3-DPG/L of blood (p<0.005). The 2,3-DPG
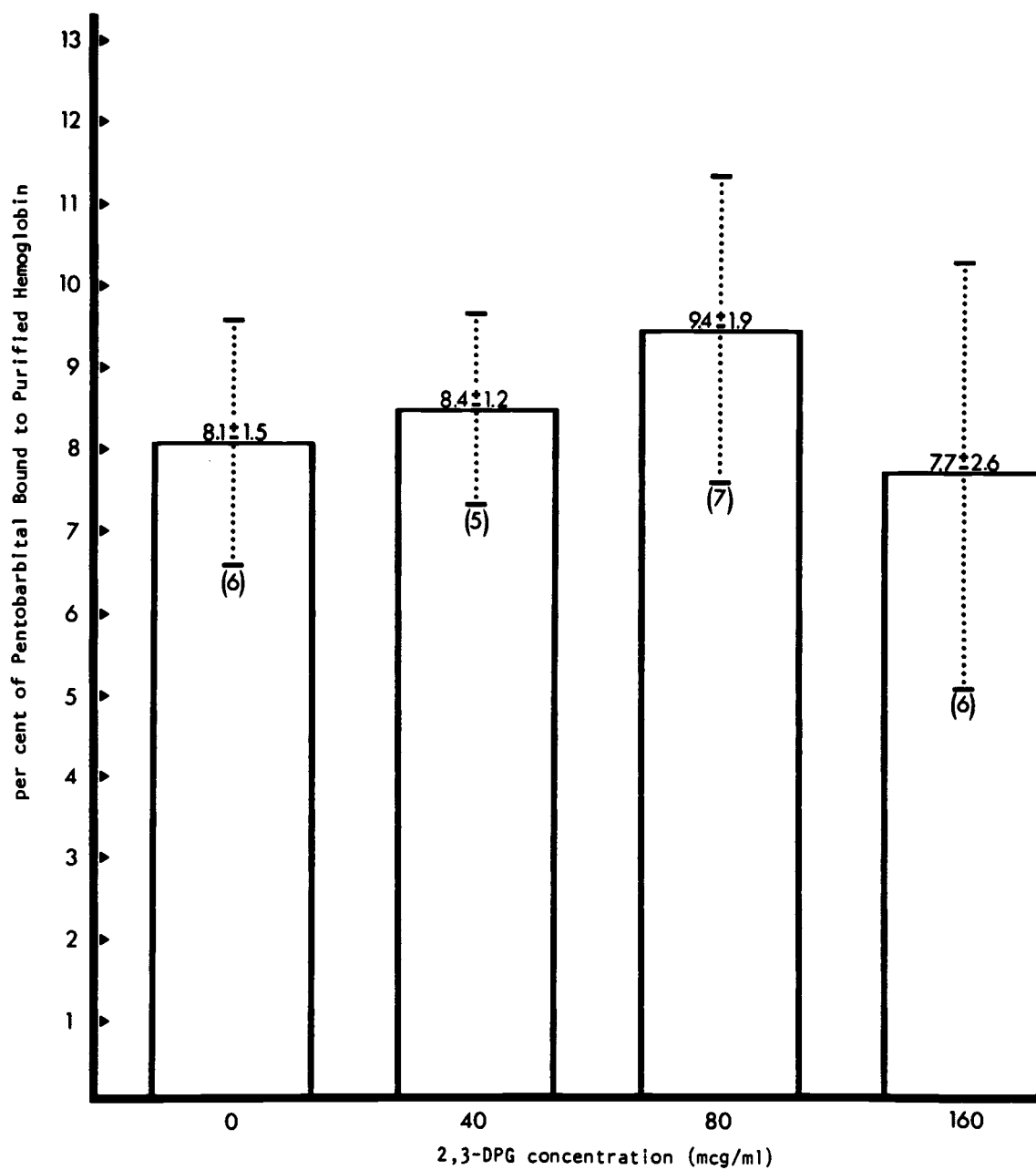
FIGURE 5. The effect of 2,3-DPG on the binding of pentobarbital (67 mcg/ml) to purified hemoglobin. Data are expressed as $\bar{X} \pm$ S.D. The number in parentheses is the number of assays at that concentration.
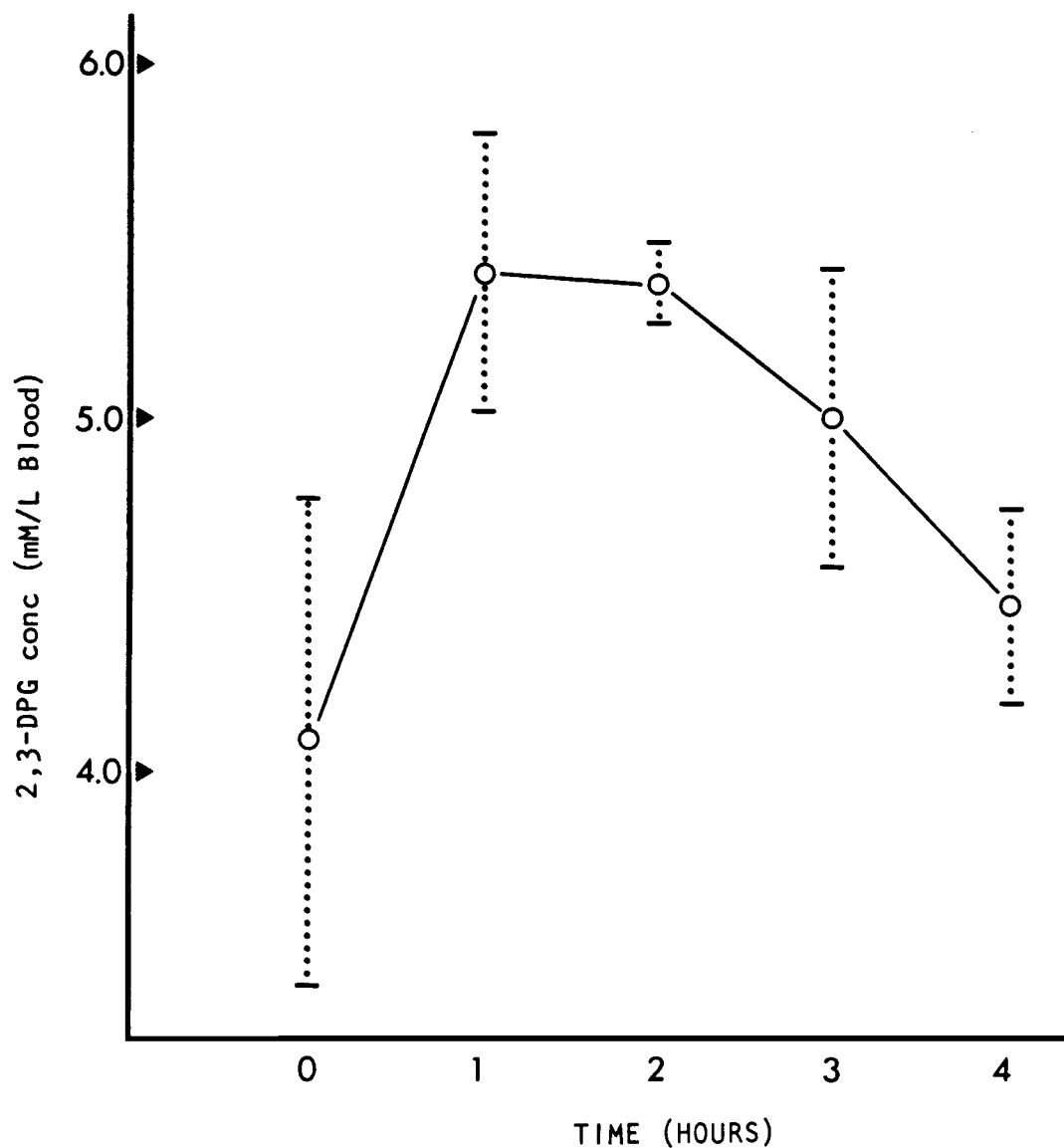
FIGURE 6.   Increase in 2,3-DPG concentration by i.p. administration of 10 ml/kg of 0.1 M inosine, 0.1 M pyruvate, and 0.25 M inorganic phosphate.   Each point represents the mean ± S.D. of five mice. The increase in 2,3-DPG concentration at one hour post-injection is statistically significant as compared to control (p<0.005).

levels began to decline at three hours post-injection returning to near the baseline values by four hours post-injection.

Effect of Pentobarbital Administration
on 2,3-DPG Concentration In Vivo

The effect of injection of 75 mg/kg of pentobarbital on the 2,3-DPG concentration in mouse erythrocytes is shown in Figure 7. The concentration of 2,3-DPG did not change from normal baseline levels throughout the entire four hour period of 2,3-DPG determination ($p > 0.25$).

Effect of Pretreatment with IPP
on Toxic Doses of Pentobarbital

The results of pretreatment with the inosine, pyruvate, and phosphate (IPP) mixture on recovery time and lethality after toxic doses of pentobarbital are presented in Table 4. Since the 2,3-DPG levels were near baseline values by four hours after injection of IPP, only those mice reviving within four hours of pentobarbital dosing were used in determining revival time.

The mice which received 75 mg/kg of pentobarbital after IPP pretreatment had an average revival time of 111.5 ± 7.1 minutes as compared to 102.6 ± 17.5 minutes for the control. There was no significant difference between these two groups for both revival time and survival rate ($p > 0.05$). The mean revival time for the IPP pretreatment group was 132.7 ± 15 minutes as compared to 134.2 ± 42 minutes for the control group at a dose of 100 mg/kg of pentobarbital. There was no significant difference at this dose for both revival time
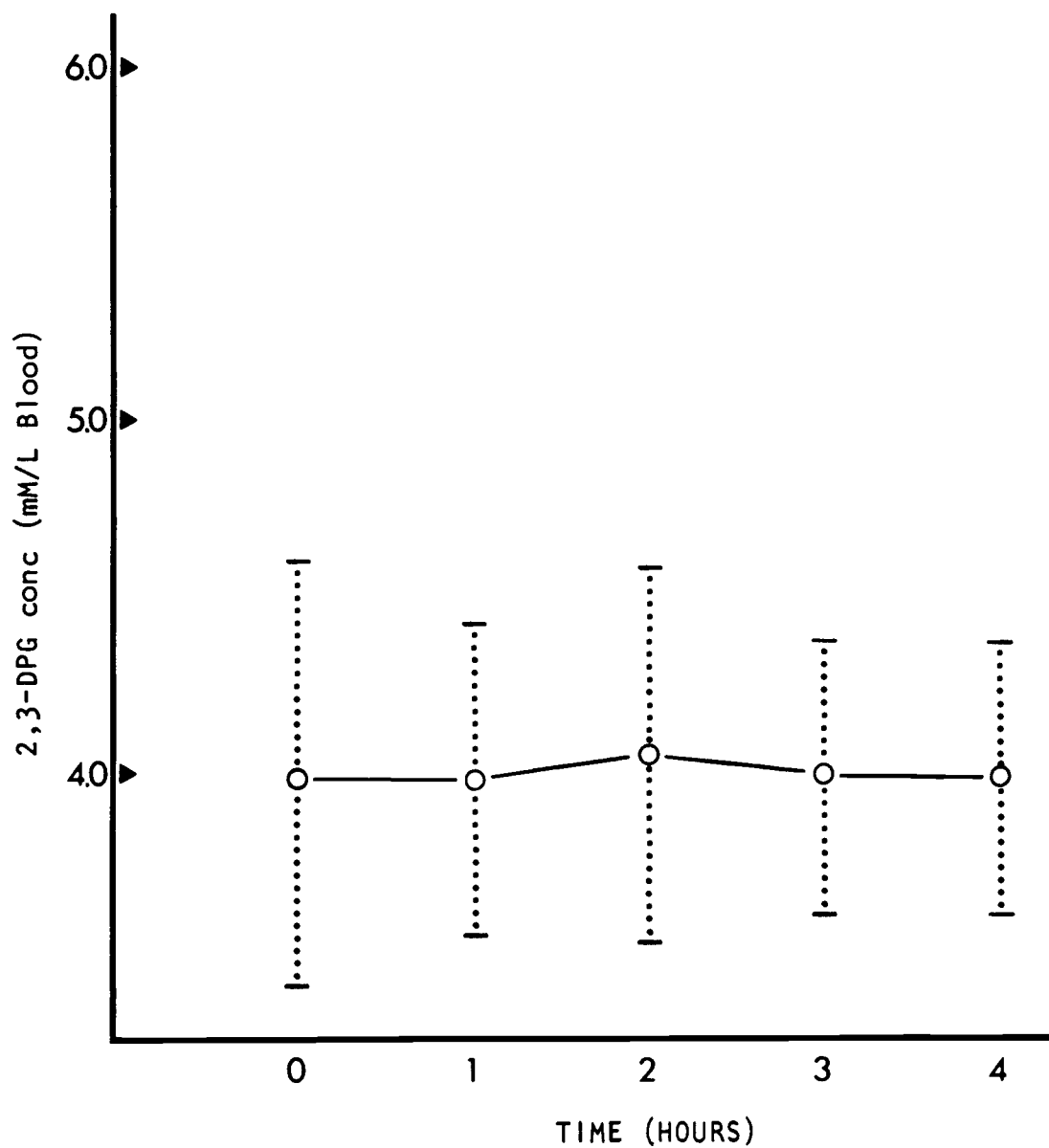
FIGURE 7. Effect of i.p. injection of 75 mg/kg of sodium
pentobarbital on 2,3-DPG concentration. Each point represents
the mean ± S.D. of five mice. There was no significant change
in 2,3-DPG concentration (p>0.25).

Table 4. Number of survivors and average time to recover from toxic doses of pentobarbital given one hour after inosine, pyruvate, and phosphate pretreatment.

Pentobarbital Dose		IPP		Control	
75 mg/kg	n/n_1 $\overline{X} \pm SD$ Range	18/20 111.5 $\pm$ 7.1" (85 - 138)	(15)*	19/20 102.6 $\pm$ 17.5" (80 - 151)	(14)
100 mg/kg	n/n_1 $\overline{X} \pm SD$ Range	8/20 132.7 $\pm$ 15" (117 - 157)	(7)	9/19 134.2 $\pm$ 42" (85 - 190)	(6)

n = number of survivors
n_1 = number of mice dosed
* = number of survivors reviving in < 4 hours.

or survival rate (p>0.05). Although subjective, the mice which received the IPP pretreatment appeared to be more alert and active upon awakening as opposed to the control group which appeared disoriented and sluggish and took longer to resume full activity.

DISCUSSION

Binding of Pentobarbital to Hemoglobin

The data support the conclusion that pentobarbital binds to the hemoglobin molecule in whole blood. The percent of binding of pentobarbital to hemoglobin was not different in preparations of purified hemoglobin or in preparations of hemoglobin obtained from human red blood cells. The binding of pentobarbital to whole blood was roughly three times as great as the binding to the hemoglobin preparations. Since Ehrnebo and Odar-Cederlof (1975) have shown that 33% of the total pentobarbital in whole blood is present in or on the red blood cells, our data suggest that hemoglobin has the capacity to bind this fraction of total drug present in whole blood. This data was obtained from diluted samples and therefore cannot be extrapolated exactly to the normal in vivo situation without further experimentation.

The percent of pentobarbital binding to hemoglobin was very similar to the percent of binding to albumin in the preparations tested. Both proteins are of similar molecular weight and it can be assumed that the affinity of both proteins for pentobarbital could be explained by the availability of unbonded cationic nitrogen groups. This raises the question of whether pentobarbital would bind differently to hemoglobin depending on which configuration it was in. The salt bridge formation which occurs in the deoxy conformation could reduce the available binding sites for anions. Conversely, the binding of barbiturates might be greater to oxyhemoglobin which would be devoid

of salt bridges and thus, provide more cationic sites for barbiturate
binding. In addition, the occupation of a cationic site by a
barbiturate to oxyhemoglobin would preclude the occurrence of salt
bridge formation at that site. The subsequent deoxy configuration
would result in the formation of fewer salt bridges and the oxygen
affinity would be increased. Since the present study investigated the
binding of pentobarbital to deoxyhemoglobin, it is necessary to further
study the binding to oxyhemoglobin and compare the extent of binding
between the two conformations.

Analysis of binding data using Scatchard or Lineweaver-Burk
double-reciprocal plots was not done. In order to determine affinity
constants for the binding of a drug to a protein, a wide enough range
of concentrations is needed to eventually saturate the protein to which
it is binding. Since in the present study the percent of bound drug
did not change significantly over the range of concentrations tested,
use of analyses of drug-binding data was not possible. Future studies
need to be done in which the range of concentrations of pentobarbital
is wide enough to determine the affinity constant and the number of
binding sites present on the hemoglobin molecule.

Binding Interaction Between 2,3-DPG and Pentobarbital

The data from Figure 5 support the conclusion that the binding
of pentobarbital to hemoglobin is independent of 2,3-DPG concentration.
This suggests that the binding either does not occur at the 2,3-DPG
binding site or that the binding might be noncompetitive between the
two molecules. Since we were unable to compare the percent of binding

of 2,3-DPG to purified hemoglobin with the percent of binding in the
presence of varying concentrations of pentobarbital, we were unable to
conclude whether there is evidence of competition between these two
molecules for a common binding site.

It appears that the dithionite ion has an effect on the enzymatic
assay for 2,3-DPG. The fluorometric assay was very reliable in
producing a standard curve which was reproducible with samples of
2,3-DPG in saline at concentrations ranging from 3.75×10^{-5} M to 6
$\times 10^{-4}$ M. But, samples of the purified hemoglobin preparations
which were analyzed produced inconsistent results. The values obtained
for free 2,3-DPG (dialysate) were greater than the quantity of 2,3-DPG
which had been added to the hemoglobin preparations. Conversely, the
values obtained for total 2,3-DPG were considerably lower than the
actual quantities of 2,3-DPG added. The spectrophotometric assay was
extremely reliable in producing a standard curve and in quantifying
2,3-DPG from mouse red blood cells. However, when spectrophotometric
analyses of the 2,3-DPG concentration in the purified hemoglobin
preparations was attempted, the results were again unexplainably
inconsistent. The spectrophotometric method of de Leeuw (1971) in
which 2,3-DPG is isolated as the lead salt, converted to the free acid
with H_2S and measured colorimetrically at 693 nm after reaction with
chromotropic acid proved also to be unusable. It was found that
glycerin interfered in the determination of 2,3-DPG producing greatly
elevated values. Since glycerin is present in the dialysis tubing, the
results using this method were not reported.

The elucidation of any binding interaction between 2,3-DPG and pentobarbital is vital to the determination of the mechanism of increased oxygen affinity produced by barbiturates. Further studies need to be attempted in hemoglobin preparations in which the dithionite ion is not present. Although glycerin would not be expected to interfere with the enzymatic assay, perhaps soaking the dialysis tubing in buffer solution for 24 hours prior to experimentation might reduce any interference that glycerin may have on the assay.

Stimulation of 2,3-DPG Synthesis and Pentobarbital Toxicity

Figure 6 shows that the intraperitoneal injection of IPP produces an increase in 2,3-DPG in mouse blood in vivo. Therefore, if pentobarbital produces an increase in oxygen affinity, pretreatment with IPP should elevate the 2,3-DPG levels and overcome this change. It is important to note that it was not shown whether the IPP-induced increase in 2,3-DPG actually results in a decrease in oxygen affinity. Nor was it shown whether pentobarbital would affect the production of 2,3-DPG resulting from IPP administration.

The data in Figure 7 reveal that pentobarbital does not affect the intra-erythrocytic concentration of 2,3-DPG in mouse red blood cells. This rules out the possibility of pentobarbital producing an increase in oxygen affinity via a modification of intra-erythrocytic glycolytic metabolism resulting in a decrease in the concentration of 2,3-DPG. However, the binding of pentobarbital to hemoglobin could inhibit the binding of 2,3-DPG to the central cavity resulting in an increased unbound concentration of 2,3-DPG without affecting the total

concentration. This seems unlikely since you would expect an increased
concentration of unbound 2,3-DPG to result in a decrease in activity of
the phosphoglycerate mutase enzyme eventually resulting in a lower
level of 2,3-DPG similar to that seen with cyanate as observed by
Harkness (1974).

If pentobarbital does cause an increase in oxygen affinity,
perhaps this is in part responsible for some of the anoxic insults
resulting from barbiturate poisoning. The fact that in barbiturate
poisoning the mixed venous oxygen content is not decreased as
profoundly as would be expected with a decreased arterial oxygen
tension is consistent with an increased oxygen affinity and reduced
oxygen delivery. This is in concordance with the observation of a
10-20 percent increase in venous oxygen saturation produced by high
doses of salicylates, presumably as a result of a reduction in 2,3-DPG
levels (Kravath et al., 1972).

The results of pretreatment with IPP on recovery time and
lethality after toxic doses of pentobarbital revealed no significant
differences between these variables. In other words, altered oxygen
dissociation produced by an elevated 2,3-DPG concentration did not
affect the outcome of barbiturate poisoning in terms of survival rate
or revival time. This is consistent with pentobarbital binding to the
2,3-DPG binding site and blocking the effect of 2,3-DPG. It is also
consistent with pentobarbital binding to the hemoglobin molecule
resulting in a decrease in salt bridge formation. This mechanism would
be unaffected or affected only slightly by a change in the 2,3-DPG

levels. Another consideration is that perhaps the elevation of 2,3-DPG

was not of sufficient magnitude to result in a significant decrease in

oxygen affinity. Also, perhaps the dose of pentobarbital was

insufficient to result in a significant increase in oxygen affinity.

The data suggest the need for determining the plasma concentration of

pentobarbital at which the oxygen dissociation curve is significantly

affected.

In normal blood the average P_{50} is 27 mmHg at pH 7.4 and 37°C.

The values found by Meyer (1974) after suicidal doses of barbiturates

averaged 23.1 mmHg. Although this is only a 4 mmHg reduction in the

P_{50}, it represents a profound reduction in oxygen dissociation at

the level of the tissues. However, at present there is disagreement in

the literature as to what effect the barbiturates actually have on the

oxygen dissociation curve.

Gillies and co-workers (1970) induced anesthesia with thiopental

in eight mongrel dogs. In two dogs anesthesia was maintained with

pentobarbital and in six with halothane (1.5%) for two hours, following

which pentobarbital was used. Arterial blood samples were obtained at

intervals during each experiment and the P_{50} determined. Their

conclusion was that the barbiturates had no effect on the oxygen

dissociation curve, but that halothane produced a shift to the right of

between 3.8 and 6 mmHg. There was no change in intra-erythrocytic

2,3-DPG concentration in any of the samples. There are several

problems inherent in this study. There was no mention of doses used

for either thiopental or pentobarbital, nor was there mention of the

blood levels obtained. Even though the change in P_{50} produced by halothane is described, there are no values given for baseline P_{50} levels or standard deviations and no values reported at any times during the experiment. Consequently, no statistical tests were performed and the reader has no basis for judgement. Unfortunately, this study is cited frequently in reference to the effect of barbiturates on oxygen transport.

Petty and Bageant (1974) postulated that since thiopental is highly lipid soluble, has a dissociation constant of 7.6 at 37°C, and binds extensively with serum proteins, it could diffuse through the lipid membrane of the erythrocyte and possibly affect the oxygen dissociation curve. Their purpose was to determine the in vitro effect of thiopental on the oxygen dissociation curve in human blood. They obtained venous samples and added sodium thiopental for a final concentration of 110 mcg/ml. This value corresponds to the expected peak level which would be achieved immediately after an intravenous bolus of 300-600 mg which is similar to the dose used for induction of anesthesia. Tonometry was utilized for determining oxygen dissociation and the linear portion of the oxygen dissociation curve was between 60 and 70 percent saturation. Therefore, P_{62} and P_{68} values were reported rather than P_{50}. They found no significant change in 2,3-DPG concentration between groups and found no alteration of the oxygen dissociation curve. The control value at P_{62} was 31.9 ± 0.4 (S.E.) mmHg, compared with the thiopental P_{62} of 30.5 ± 1.1 mmHg ($p<0.1$). At P_{68}, the control value was 35.2 ± 0.4 mmHg vs.

thiopental of 33.9 $\pm$ 0.9 mmHg (p<0.2). It is important to note that the values obtained for thiopental were consistently lower than control although not significant. In addition, these were levels of thiopental sufficient to induce anesthesia and not doses which would coincide with barbiturate poisoning.

In conclusion, it is necessary to determine the plasma concentration of pentobarbital at which the oxygen dissociation curve is significantly shifted to the left resulting in an increase in the affinity of hemoglobin for oxygen. Actually, it would be ideal to perform studies to evaluate any pentobarbital induced dose-dependent change in the oxygen dissociation curve concomitant with any change in the binding of 2,3-DPG to hemoglobin. This would delineate whether the increase in oxygen affinity is the result of pentobarbital binding to hemoglobin or of competition with 2,3-DPG binding to the central cavity. Also, knowledge of the pentobarbital concentration at which a significant change in the oxygen dissociation curve occurs is necessary to determine if the effect would be clinically significant.

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