STUDIES ON COMPENSATIVE MECHANISMS IN CHRONIC ANOXIA

I. ANOXIA DUE TO CONGENITAL HEART DISEASE IN CHILDREN.

II. ANOXIA IN EXPERIMENTAL ANIMALS.

by

RICHARD J. HAVEL

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

June 1949

APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The various pathologic states usually grouped under the heading of congenital heart disease include a great number of distinct entities. They are conveniently divided into those cases which show normal arterial oxygen saturation (the acyanotic type) and those in which it is reduced (the cyanotic type). In the latter polycythemia is present. This is presumed to constitute a physiologic response to decreased oxygen tension in the tissues. As a result of this polycythemia, hemoglobin loses a smaller proportion of its oxygen as it passes through the capillaries. Since blood oxygen tension is a function of the amount of oxyhemoglobin present, the decreased tissue oxygen tension is mitigated, but not eliminated. It is to be expected, therefore, that additional compensative mechanisms exist as Haldane pointed out many years ago in his treatise on respiration. The following quotation is taken from this work:

> "In considering the effects of anoxaemia a factor comes in which must always be borne in mind--namely that of adaptation or acclimatization. This may act in two different ways. In the first place adaptation may bring it about that the anoxaemia which would, without adaptation, exist is greatly diminished....In the second place the tissues may adapt themselves to a lower partial pressure of oxygen. About this second form of adaptation our knowledge is at present very imperfect; but it seems to me that clinical evidence points strongly to its existence. Perhaps the clearest evidence is afforded by cases of congenital heart defect, in which part of the venous blood passes direct to the left side of

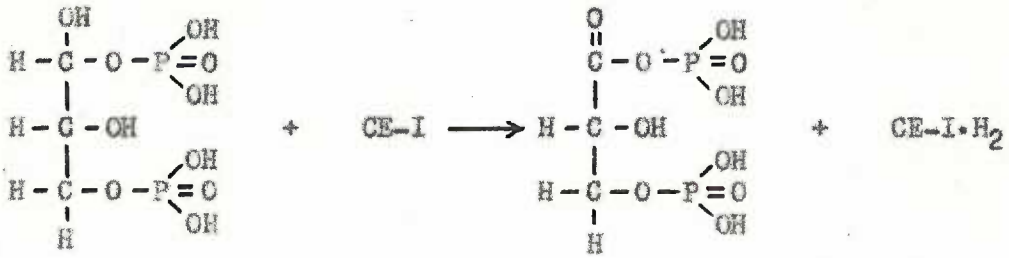
the heart without first passing through the lungs. In these cases of "Morbus coeruleus" the arterial blood is always more or less blue, and becomes extremely blue on muscular exertion, so that one can always recognize this condition in persons walking in the street. The remarkable point, however, is that in spite of the anoxaemic condition of the arterial blood these persons may get on quite well, and be able to walk at a good pace. On account of the large increase in their hemoglobin percentage, they have plenty of oxygen in their blood, but at a low partial pressure. It seems hardly possible to doubt, therefore, that their tissues have become adapted to the low partial pressure of oxygen...."(1).

At present, as a result of the recent spectacular development of surgical methods of treatment, intensive work is being carried out on the altered circulatory dynamics existing in the various types of congenital heart disease in an effort to improve methods of diagnosis (2). Relatively little, however, has been done to elucidate further the mechanisms by which compensation for the altered internal environment is achieved in such cases. Metabolic studies may give a clue as to the compensative mechanisms permitting survival under conditions of severe chronic oxygen lack.

This study was undertaken in an attempt to answer two questions. 1. To what degree do individuals with cyanotic congenital heart disease compensate for their lowered tissue oxygenation? 2. What are the mechanisms by which this compensation is realized? The answers to these questions are important not only to an understanding of the altered physiology of individuals with congenital heart disease, but to the fundamental problem of the physiology of hypoxia as well.

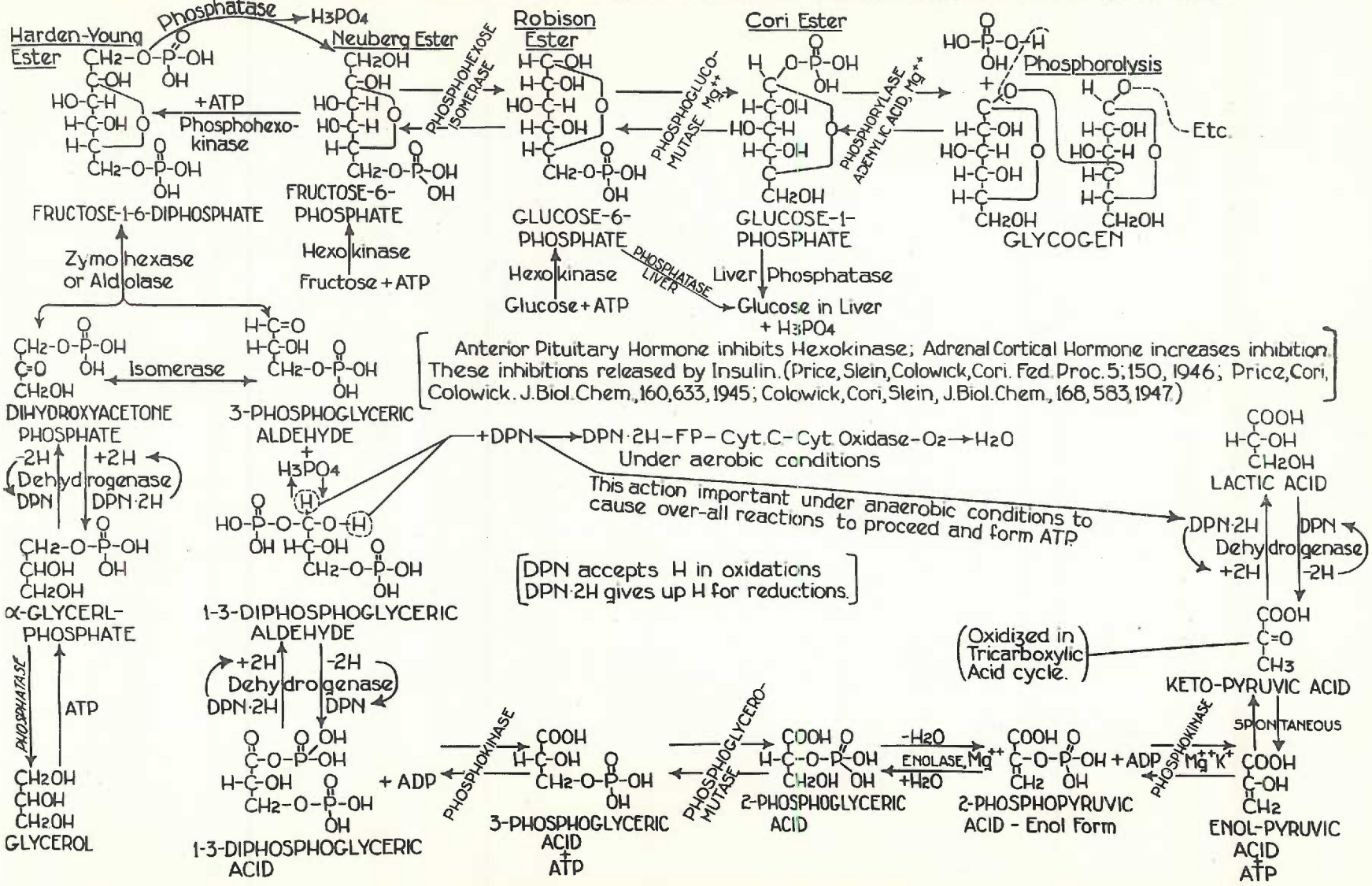
One of the most notable accompaniments of acute anoxia is the accumulation of lactic acid in the blood. A clear explanation of the mechanism of this phenomenon may be drawn from the work of Green, Needham and Dewan (3) in the light of our knowledge of carbohydrate catabolism.

As seen in Figure 1 and Figure 2 carbohydrate breakdown can be divided into two stages, the anaerobic or 'glycolytic' and the aerobic. In the absence of adequate oxygen the tissues must rely on the anaerobic phase to obtain the energy needed for vital processes. The portion of this phase in which energy is built up (synthesis of high-energy phosphate bonds) lies between 1,3-diphosphoglyceric acid and pyruvic acid. In order to produce 1,3-diphosphoglyceric acid from 1,3-diphosphoglyceraldehyde the latter must be oxidized by giving hydrogen to diphosphopyridine nucleotide (coenzyme I), which in turn is reduced. If the reduced coenzyme were not in some way reoxidized so that it could be utilized repeatedly, it would soon all be in the reduced form and the generation of energy-rich phosphate bonds would terminate. Green and coworkers found that the following reactions readily occur in muscle extracts:

(1) 

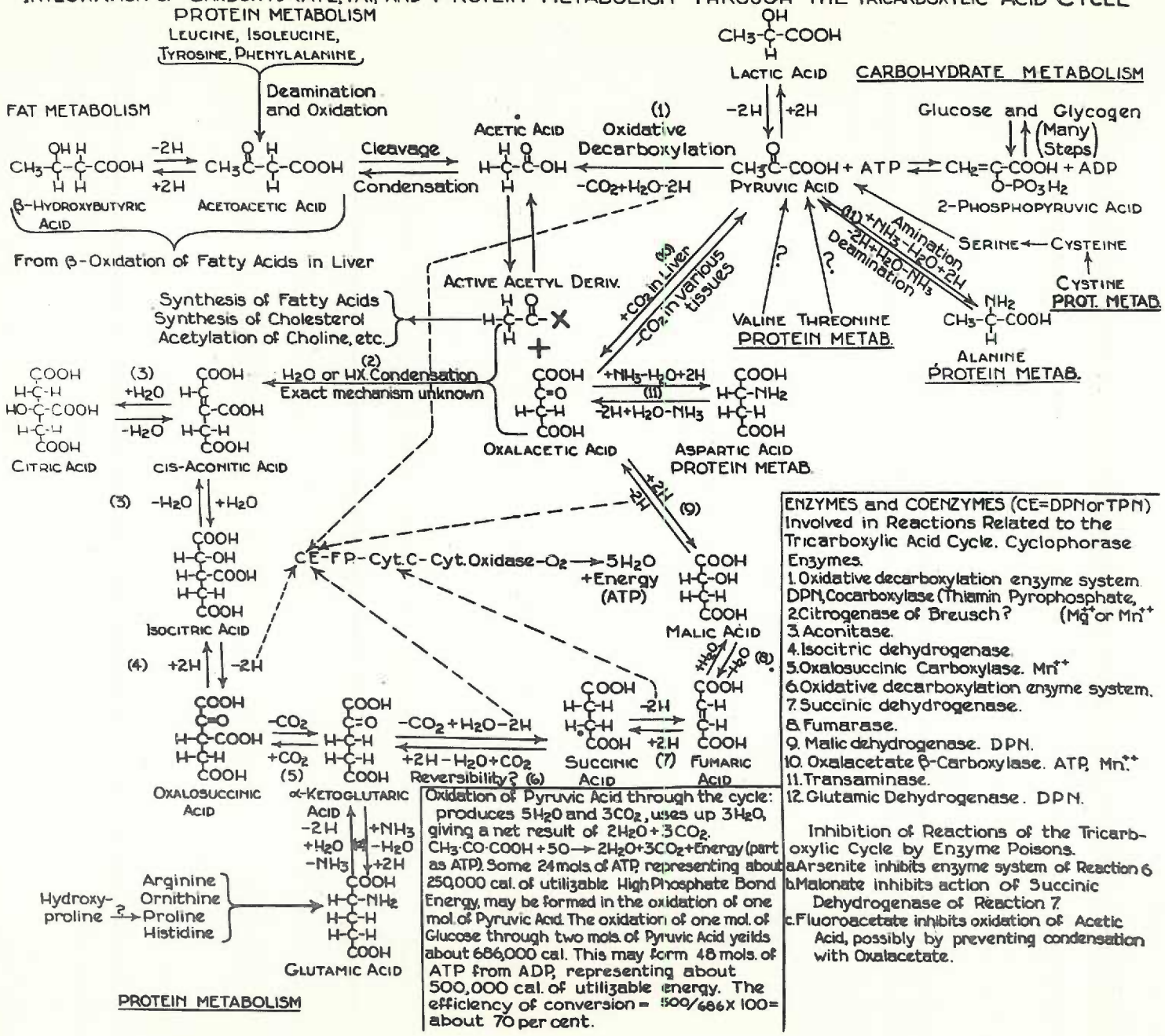
1,3-diphosphoglyceraldehyde | coenzyme I (oxidized form) | 1,3-diphosphoglyceric acid | coenzyme I (reduced form)

ANAEROBIC CARBOHYDRATE METABOLISM—ANAEROBIC GLYCOLYSIS



Breakdown of one mol. of Glucose forms 4 ATP (forms 2 mols of 3-Phosphoglyceric Acid and subsequent products) and requires 2 mols of ATP. Thus, anaerobically, one mol of Glucose gives a net yield of 2 mols ATP. However, the breakdown of one 6 C unit of Glycogen requires only one mol of ATP while yielding 4 ATP. Thus, Glycogen gives a net yield of 3 ATP per 6C unit as contrasted with 2 ATP per mol of Glucose.

Integration of Carbohydrate, Fat, and Protein Metabolism Through the Tricarboxylic Acid Cycle



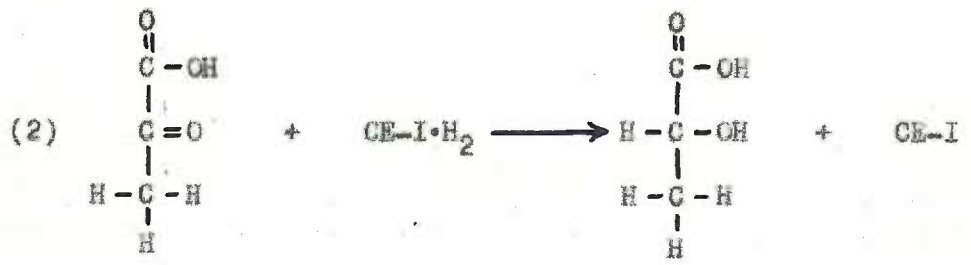
Oxidation of Pyruvic Acid through the cycle: produces $5H_2O$ and $3CO_2$, uses up $3H_2O$, giving a net result of $2H_2O + 3CO_2$.
$CH_3{\cdot}CO{\cdot}COOH + 5O \rightarrow 2H_2O + 3CO_2$ + Energy (part as ATP). Some 24 mols. of ATP, representing about 250,000 cal. of utilizable High Phosphate Bond Energy, may be formed in the oxidation of one mol. of Pyruvic Acid. The oxidation of one mol. of Glucose through two mols. of Pyruvic Acid yeilds about 686,000 cal. This may form 48 mols. of ATP from ADP, representing about 500,000 cal. of utilizable energy. The efficiency of conversion = $500/686 \times 100$ = about 70 per cent.

ENZYMES and COENZYMES (CE=DPN or TPN) Involved in Reactions Related to the Tricarboxylic Acid Cycle. Cyclophorase Enzymes.

1. Oxidative decarboxylation enzyme system. DPN, Cocarboxylase (Thiamin Pyrophosphate, Mg^{++} or Mn^{++})
2. Citrogenase of Breusch?
3. Aconitase.
4. Isocitric dehydrogenase.
5. Oxalosuccinic Carboxylase. Mn^{++}
6. Oxidative decarboxylation enzyme system.
7. Succinic dehydrogenase.
8. Fumarase.
9. Malic dehydrogenase. DPN.
10. Oxalacetate β-Carboxylase. ATP, Mn^{++}
11. Transaminase.
12. Glutamic Dehydrogenase. DPN.

Inhibition of Reactions of the Tricarboxylic Cycle by Enzyme Poisons.

a. Arsenite inhibits enzyme system of Reaction 6
b. Malonate inhibits action of Succinic Dehydrogenase of Reaction 7
c. Fluoroacetate inhibits oxidation of Acetic Acid, possibly by preventing condensation with Oxalacetate.



(2) pyruvic acid + coenzyme I (reduced form) → lactic acid + coenzyme I (oxidized form)

These reactions provide for the continued production of 1,3-diphosphoglyceric acid and the consequent resynthesis of energy-rich phosphate bonds. The lactate produced diffuses into the blood stream.

Under conditions of tissue hypoxia it is to be expected, therefore, that the ratio of the concentration of lactate to the concentration of pyruvate (L-P ratio) in the blood will increase. Such has been found to be the case during this and other investigations.

Stotz and Bessey (4) studied the L-P ratio in the blood of humans and found it to be constant under various physiologic conditions; much more so than the absolute concentrations of the two constituents. They also demonstrated a marked decrease in the L-P ratio in experimental thiamin deficiency in pigeons, due chiefly to an increase in the pyruvate concentration. This was to be expected, as it had been shown previously be Peters and Thompson (5) that thiamin is essential to the oxidation of pyruvate in animal tissue.

Friedemann and Barborka (6) studied the L-P ratio in the blood of humans after exercise. They found that the pyruvate concentration increases and then decreases, both logarithmically, with time, while the lactate concentration rises rapidly to a maximum and then slowly

returns to normal. Consequently, the ratio rises rapidly to a maximum, drops rapidly at first and then slowly returns to normal. These facts are seen in the following graph, taken from their work.

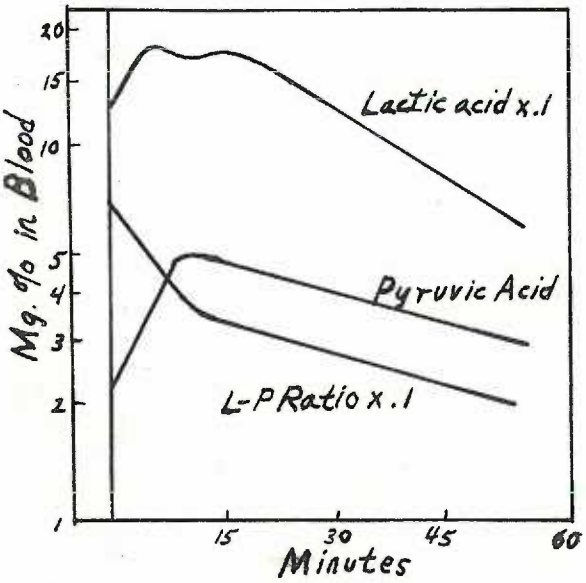


Figure 3 - Lactate, Pyruvate and L-P Ratio in the Blood of Man After Exercise.

At the point of inflection of the time curve of the ratio, pulse and respirations were rapidly returning to normal. They concluded from their work that the ratio is an indicator of the adequacy of tissue oxygenation.

Tepperman and Tepperman (7) studied the changes in the L-P ratio during exercise in trained human subjects breathing air under reduced barometric pressure. The ratio was increased when the pressure was reduced to a simulated altitude of 5,000 feet. This increase could be suppressed by breathing 100% oxygen at this altitude. The investigation further demonstrated the relationship between the L-P ratio and tissue oxygenation.

The L-P ratio has not been studied under conditions of chronic

anoxia, such as exist in individuals with cyanotic congenital heart disease. The first part of this study is devoted to an investigation of the L-P ratio in children with congenital heart disease and its relation to the blood gases. In addition, the lactic dehydrogenase content of red blood cells was estimated in several of these children in order to determine if alterations in the concentration of this enzyme could explain some of the results obtained early in the study.

The second part of the investigation is concerned with some of the intracellular constituents involved in energy transformation and oxygen transfer under conditions of chronic anoxia in an attempt to find further compensative mechanisms at the cellular level.

The first step considered to take place in the liberation of energy within the cell is the breakdown of adenosine triphosphate (ATP) to yield adenosine diphosphate (ADP), inorganic phosphate and about 11,000 calories per mol. The ADP is immediately resynthesized by reaction with creatine phosphate (phosphagen) in what is known as the Lohmann reaction:

$$\text{ADP} + \text{creatine phosphate} \rightleftharpoons \text{ATP} + \text{creatine}$$

After all the available creatine phosphate has been used up, ADP can be further phosphorylated by transfer of high-energy phosphate groups produced as a result of glycolysis and through subsequent oxidations in the aerobic tricarboxylic acid cycle. When a tissue is functioning anaerobically the supply of high-energy phosphate derived from the tricarboxylic cycle, which normally provides most of the energy, is eliminated. An increase in the concentration of ATP and/or creatine phosphate would be of advantage to chronically hypoxic tissue.

Since the glycolytic phase of carbohydrate catabolism is much less efficient than the aerobic, it would also be of advantage to hypoxic tissue to have a more efficient oxygen transferring mechanism. It could thus make better use of the large amount of potential energy present in the lactic acid produced by glycolysis.

With these facts in mind an investigation was made, during chronic anoxia, of the concentrations of the substances containing high-energy phosphate bonds (principally ATP and creatine phosphate) and of two important substances concerned with intracellular oxygen transfer, succinic dehydrogenase and cytochrome c.

Since it was not possible to obtain sufficient tissue from humans at operation, rats made chronically hypoxic by exposure to low barometric pressure for a period of time sufficient to produce a definite polycythemia were used in this part of the study.

PART I

ANOXIA DUE TO CONGENITAL HEART DISEASE IN CHILDREN

EXPERIMENTAL

In order to interpret data obtained from children with congenital heart disease it was necessary to establish values for lactate and pyruvate concentration for normal children, in which this previously had not been done. Since the mean value of the L-P ratio obtained in normal children early in the investigation was apparently significantly higher than that of the most complete reported study in adults (8), L-P ratios were run on a series of normal adults to determine whether or not there is a significant difference between the two groups.

The normal adults were medical students, graduate students and student nurses. Normal children included patients hospitalized at Doernbecher Hospital for conditions other than congenital heart disease. These children were ambulatory and afebrile. Also included were several well children attending the University Tuberculosis Hospital Outpatient Clinic as tuberculosis contacts. Children with congenital heart disease were principally patients hospitalized at Doernbecher Hospital for consideration of surgery, although some private patients hospitalized elsewhere were utilized in addition. The types of congenital heart disease studied were divided into acyanotic and cyanotic groups. The acyanotic group consisted primarily of children with patent ductus arteriosus; in the cyanotic group were mainly children with tetralogy of Fallot, although other types of cyanotic congenital heart disease were studied also.

Blood samples were drawn from an antecubital vein whenever possible. In some of the younger children it was necessary to use

the femoral vein, or, rarely, the external jugular vein. Arterial blood samples were drawn from the femoral artery. Use of a tourniquet was necessary in the children in order to obtain venous samples quickly. Ten ml. syringes were used for sampling. When both the L-P ratio and the blood gases were to be determined, approximately 8 ml. of blood were withdrawn. After removing any bubbles present a portion of the blood was delivered through the needle under 1-2 cm. of mineral oil contained in an oxalate-fluoride treated test tube, stopping exactly at the 5 ml. mark. The remaining 5 ml. were immediately delivered through the needle into 10 ml. of 20% trichloroacetic acid. When only the L-P ratio was to be determined, 5 ml. were drawn and the first delivery, under oil, omitted. Immediate deproteinization was necessary to prevent glycolysis which rapidly alters the values of the lactate and pyruvate of shed blood (9). This method of delivery directly from a syringe has been shown to have an accuracy of $\pm$ 1.2% (10).

The deproteinized sample was centrifuged at 2,000 r.p.m. for 10 minutes. The supernate was used for analysis of lactate and pyruvate.

Lactate concentration was determined in duplicate on 3 ml. aliquots of the supernate by the method of Long (11). The lactate was oxidized to acetaldehyde by ceric sulfate in acid solution at 60° C. The acetaldehyde formed was continuously aerated into a dilute solution of sodium metabisulfite with which it reacts to form an undissociated complex. After removing excess metabisulfite by addition of 0.1 N. iodine solution and carefully adjusting the end-point with starch as indicator, the bound acetaldehyde was liberated by the addition of

disodium phosphate and titrated with 0.005 N. iodine solution to the first blue tinge.

Calculation: ml. 0.005 N. I_2 required x 22.5 =
mg. lactic acid per 100 ml. whole blood.

Standardization of the method using pure lithium lactate prepared by the method of Hillig (12) yielded 96-106% recovery of the lithium lactate as lactic acid.

It was found that a blank, as recommended by Long, was not necessary in the determination. This is probably due to the fact that he used a 30% solution of 35% pure ceric sulfate, which is approximately equivalent to the 10% solution of pure ceric sulfate used here.

Pyruvate concentration was determined on duplicate 1.5 ml. aliquots of the supernate by minor modifications of the method of Friedemann and Haugen (13). Three ml. of 3.3% trichloroacetic acid were mixed with 3 ml. of the clear supernate. The mixture was divided into equal parts, and then treated according to these authors. The diluted supernate was incubated with 2,4-dinitrophenylhydrazine which reacts with pyruvic acid to form the 2,4-dinitrophenylhydrazone. This was successively extracted with benzene and sodium carbonate solution. The characteristic red color was developed by the addition of dilute sodium hydroxide and the extinction measured with the Klett-Summerson photometer, using a 520 millimicron (green) filter. Two blanks treated in exactly the same way as the blood were run with each set of duplicates. The method was standardized by preparing a standard curve with pure sodium pyruvate prepared by the method of Robertson (14). The standard curve followed Beer's law over the range of

concentrations employed.

Calculation: $\frac{\text{reading of unknown}}{\text{reading of 1 mg./100 ml. standard}} \times 0.8 =$

mg. pyruvic acid per 100 ml. whole blood.

The standard deviation from the mean of duplicate determinations performed in this work on 142 samples of human blood was 0.92 mg. for lactate (maximum 2.50 mg.) and 0.05 mg. for pyruvate (maximum 0.10 mg.).

The L-P ratio was calculated by dividing the average value for lactate by the average value for pyruvate.

Carbon dioxide content and oxygen content were determined on single 1 ml. samples of blood by the method of Van Slyke and Neill (15). Oxygen capacity was determined by the tonometric method.

Blood samples were drawn 2-4 hours after the last meal (breakfast or lunch) and analyzed the same day.

Lactic dehydrogenase content of erythrocytes was estimated by quantitative application of the ferricyanide method for the study of dehydrogenase systems of Quastel and Wheatley (16). The principle of the method is that in bicarbonate medium, reduction of 1 mol of ferricyanide by hydrogen atoms released through action of a dehydrogenase system gives rise to 1 mol of carbon dioxide. The use of ferricyanide as a hydrogen acceptor has the advantages of non-toxicity and non-autooxidizability.

The Barcroft-Warburg manometric apparatus with flasks having a center well and one side-arm was used in the determination. The gas phase consisted of a mixture of 90% nitrogen and 10% carbon dioxide. The temperature of the water bath was 38.0° C. and the shaking rate

120 oscillations per minute.

The main vessel of the Barcroft-Warburg flask contained the following solution in a total volume of 3.0 ml.:

Sodium bicarbonate	0.05 M.
Sodium lactate, pH 7.4	0.10 M.
Sodium cyanide, pH 7.4	0.05 M.
Diphosphopyridine nucleotide*	0.0012 M.
Lysed red cells	0.20 ml.

The sodium cyanide was added to fix the pyruvate produced by oxidation of lactate by forming the cyanohydrin. The final pH of this solution at 38.0° C. with the gas mixture used was 7.4.

The lysed red cell solution was prepared as follows. Blood to which not more than 2 mg. of potassium oxalate per ml. had been added was centrifuged and the plasma drawn off. The cells were washed thrice with an equal volume of 0.9% sodium chloride solution by successively mixing, centrifuging and drawing off the supernate. The last centrifugation was carried out at 2,000 r.p.m. for 10 minutes. The cells were then laked in 5 volumes of water.

The side-arm contained 0.2 ml. of 10% sodium ferricyanide in 0.05 M. sodium bicarbonate. A small square of yellow phosphorus was placed in the center well to ensure anaerobiocity.

After gassing, the stopcocks were closed and the closed side of the manometer adjusted to the 150 mm. mark. Readings were taken every

*This was obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio and was stated to be approximately 60% pure by spectrophotometric analysis.

5 minutes until the change in all manometers agreed with the thermobarometer to within 1 mm. The contents of the side-arms were then tipped in and readings taken every 5 minutes for 30 minutes. The total difference in pressure in mm. was multiplied by the flask constant obtained by applying the formula given by Umbreit, Burris and Stauffer (17) after calibration of the flask and manometer to the 150 mm. mark by the method of Schales (18). The figure obtained represents the microliters of carbon dioxide produced in nitrogen per hour ($Q_{CO_2}^{N_2}$). All determinations were run in duplicate.

The stated concentration of the diphosphopyridine nucleotide almost, but not completely, saturated the system. As seen in Figure 4 the rate of carbon dioxide evolution increased rapidly with increasing concentration of the coenzyme at first and then began to level off, but did not reach a maximum with the concentrations employed. This is in agreement with the findings of Green and Brosteaux (19), using an aerobic method for the estimation of animal lactic dehydrogenase.

The rate of carbon dioxide evolution was not linear with respect to time (Green and Brosteaux noted the same finding). At first, this was suspected to be due to insufficient cyanide, but increasing the cyanide concentration did not obviate the condition. Figure 5, taken from data obtained here, illustrates this relation.

Potassium oxalate in the concentration used did not affect the reaction. In higher concentrations, however, the enzyme was apparently inhibited.

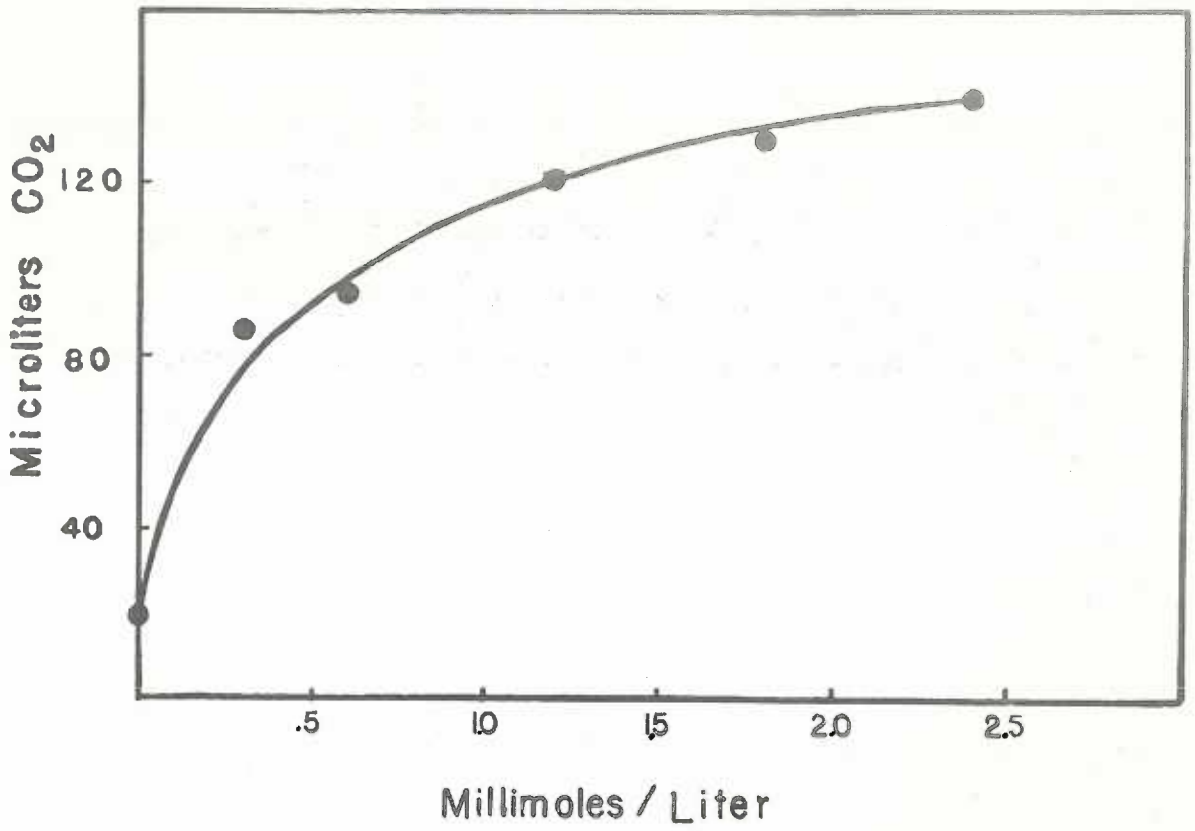
Cell volume of blood samples was determined by centrifuging to

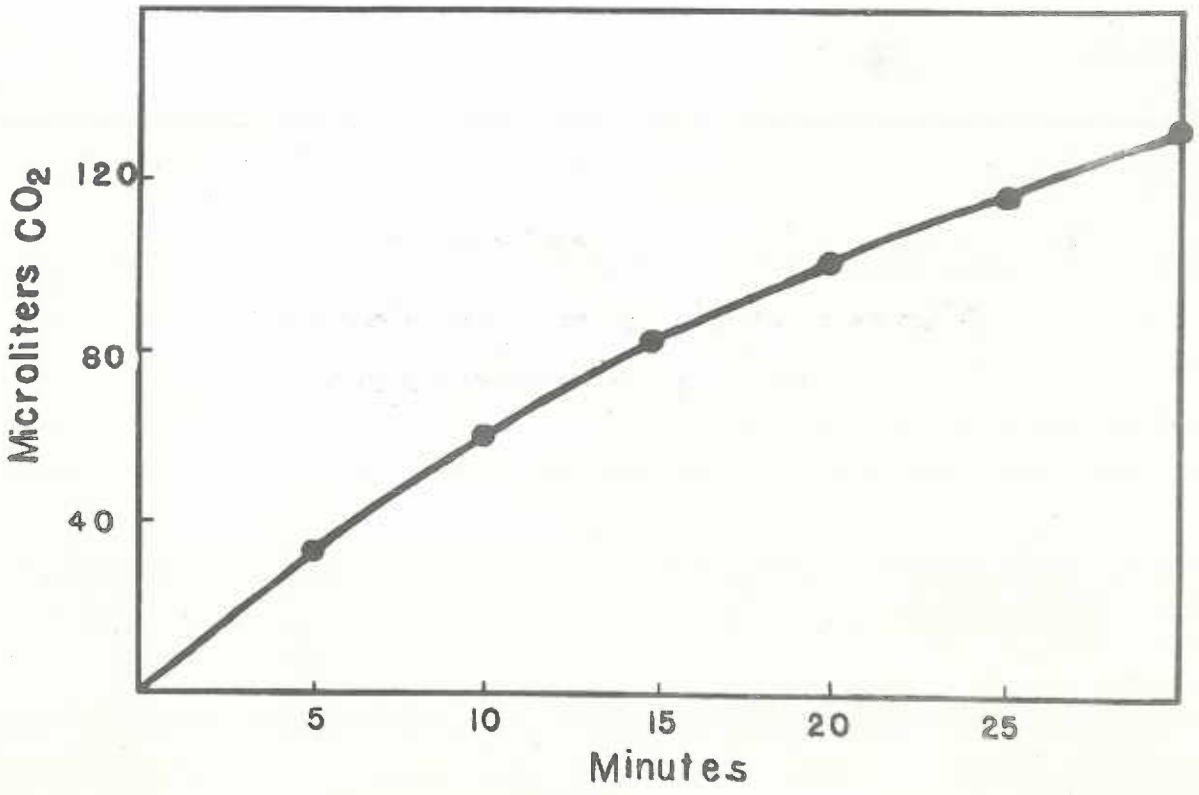
FIGURE 4

Relation of Amount of Carbon Dioxide Evolution After 30 Minutes to Diphosphopyridine Nucleotide Concentration in Lactic Dehydrogenase Assay.

FIGURE 5

Relation of Rate of Carbon Dioxide Evolution to Time in Lactic Dehydrogenase Assay.


Microliters CO_2
120
80
40
.5
1.0
1.5
2.0
2.5
Millimoles / Liter


Microliters CO_2
120
80
40
5
10
15
20
25
Minutes

constant volume of red cells in a Wintrobe blood volume index tube.

Statistical analysis was carried out according to Dunn (20). The following formulae were used.

(1) Mean = $\frac{\Sigma x}{n}$

(2) Standard deviation = $\delta = \frac{\Sigma \bar{x}}{n}$

(3) Standard error of the mean = $S.E._{mean} = \frac{\delta}{(n-1)}$

(4) Standard error of the difference = $S.E._{diff.} = (S.E._x + S.E._y)$

(5) Correlation coefficient = $\frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{((n\Sigma x^2 - (\Sigma x)^2)(n\Sigma y^2 - (\Sigma y)^2))}$

where x or y is the value to be averaged;

$\bar{x}$ is the deviation from the mean of a given value;

n is the number of observations.

(6) Probability ('p') that an observed difference is due to sampling error is inversely proportional to

$$\frac{\text{difference observed}}{S.E._{diff.}}$$

The probability is read from a table relating it to the above quotient. Example: a quotient of 1.95 is equivalent to 'p' of 0.05; this indicates 5 chances per hundred that an observed difference is caused solely by sampling error.

RESULTS

The data obtained relating to blood lactate and pyruvate and the L-P ratio in the first experimental group, consisting of 16 normal children at rest, are tabulated in Table I. As seen from the table no significant difference was found for any of these values between the 8 males and 8 females in this group, or between the 11 children who neither cried nor struggled during the blood sampling and the 5 children who cried and/or struggled to a variable degree.

Table II summarizes similar data obtained in normal adults. Results obtained in a group of 15 medical students only are tabulated, but these are typical of the results obtained in the 42 adults studied. The table demonstrates that no significant difference between the members of the tabulated group was produced by application of a tourniquet. That there was no significant difference between the 32 males and 10 females in the group is also shown.

The differences between the values for children and adults were highly significant for all three of these observations (Table III).

TABLE III

'p' Value for Difference Between Lactate, Pyruvate and L-P Ratio in Normal Children and Normal Adults

	Lactate	Pyruvate	L-P Ratio
'p'	0.000	0.005	0.000

The results relating to blood lactate and pyruvate and the L-P ratio obtained in 7 children with acyanotic congenital heart disease

TABLE I

Lactate, Pyruvate and L-P Ratio in Normal Children

Name	Age	Sex	Cooperation	Lactate	Pyruvate	L-P Ratio
H. O.	7	M.	Good	9.97	0.76	13.0
C. K.	13	F.	Good	14.60	1.24	11.8
L. L.	4	F.	Poor	13.12	1.29	10.2
C. H.	3	F.	Poor	17.55	1.43	12.3
G. R.	6	M.	Good	12.40	1.15	10.8
V. R.	4	F.	Good	20.50	1.67	12.3
E. R.	4	M.	Poor	18.11	1.56	11.6
L. W.	12	M.	Good	11.93	1.04	11.5
H. B.	10	M.	Good	12.04	1.04	11.6
D. E.	6	M.	Poor	12.04	1.18	10.2
R. M.	13	M.	Good	17.64	1.39	12.7
K. C.	6	F.	Good	13.50	0.97	13.9
C. O.	7	F.	Good	11.93	1.04	11.5
W. S.	12	F.	Good	15.19	1.04	14.6
D. L.	11	M.	Poor	15.08	1.22	12.3
D. H.	6	F.	Good	14.30	1.50	9.5
Average and Std. Dev. (16 children)				14.37±2.66	1.22±0.25	11.86±1.24
Average and Std. Dev.						
Males (8 children)				13.65±2.76	1.17±0.23	11.71±1.13
Females (8 children)				15.09±2.56	1.27±0.26	12.01±1.59
'p' Value for Difference				0.32	0.77	0.68
Average and Std. Dev.						
Good Cooperation (11 children)				14.00±2.33	1.17±0.25	12.01±1.55
Poor Cooperation (5 children)				15.38±2.37	1.34±0.14	11.32±0.95
'p' Value for Difference				0.32	0.13	0.25

TABLE II

Lactate, Pyruvate, and L-P Ratio in Normal Adults

Name	Age	Sex	Tourniquet Used	Lactate	Pyruvate	L-P Ratio
D. R.	24	M.	No	11.25	1.08	10.42
V. S.	23	F.	Yes	8.61	1.04	8.27
R. R.	30	M.	No	9.70	1.18	8.22
J. S.	25	M.	Yes	10.56	1.04	8.27
V. S.	28	M.	No	10.78	1.25	8.63
S. T.	28	M.	No	10.12	1.04	9.73
J. W.	27	M.	Yes	10.78	1.08	9.98
M. C.	27	M.	No	9.90	1.11	8.92
N. B.	22	M.	Yes	8.80	0.97	9.07
M. U.	22	M.	Yes	9.88	1.25	7.90
B. M.	33	M.	No	9.90	0.90	11.00
D. M.	24	M.	Yes	10.56	1.25	8.45
B. L.	25	M.	Yes	10.66	1.22	9.56
J. L.	21	M.	No	9.46	1.25	7.56
J. J.	24	M.	Yes	9.90	1.25	7.92
Average and Std. Dev. (42 adults)				9.36±1.61	1.03±0.15	9.10±1.19
Average and Std. Dev.						
Males (32 adults)				9.38±1.74	1.05±0.17	8.93±1.40
Females (10 adults)				9.29±1.67	0.97±0.10	9.48±0.91
'p' Value for Difference				0.90	0.89	0.20
Average and Std. Dev.						
With Tourniquet (8 adults)				10.02±1.02	1.14±0.13	8.91±1.13
Without Tourniquet (7 adults)				10.16±0.57	1.12±0.12	9.17±1.29
'p' Value for Difference				0.75	0.77	0.70

and 11 children with cyanotic congenital heart disease at rest are given in Tables IV and V. The values demonstrate that there is no significant difference between these results and those obtained in normal children.

These changes were studied further by superimposing a state of acute relative anoxia by exercise. The standard exercise test of Cournand and Richards (21) was used. During this test the subject stepped up and down from a stool 20 cm. high 30 times in one minute. Blood samples were drawn from an antecubital vein before, immediately after, and fifteen minutes after completion of the test. Results are summarized in Tables VI and VII. Table VI gives the results of each group of children separately. In Table VII the significance of the difference between corresponding samples from the various groups are compared by use of 'p' values. The findings are depicted graphically in Figure 6.

In order to determine the correlation between the L-P ratio and the degree of oxygenation of tissue, the ratio was compared with mean capillary oxygen tension (pO_2) in normal children and children having patent ductus arteriosus. To approximate mean capillary pO_2 it was necessary to make certain assumptions. When the arterial oxygen saturation of hemoglobin was not determined, a value of 95% had to be assumed. This value has been established in normal individuals using the tonometric method for oxygen capacity. Individuals with patent ductus arteriosus also have 95% saturation of arterial blood at rest by this method, but reversal of flow through the ductus during exercise may produce transient arterial desaturation. For this reason

TABLE IV

Lactate, Pyruvate, and L-P Ratio in Children with Patent Ductus Arteriosus

Name	Age	Sex	Lactate	Pyruvate	L-P Ratio
J. C.	3	M.	16.61	1.22	13.61
S. S.	8	F.	15.86	1.50	10.57
R. C.	4	F.	15.75	1.84	8.56
H. H.	12	F.	15.06	1.25	12.05
A. W.	10	F.	15.53	1.39	11.18
L. C.	5	F.	11.36	1.01	11.25
D. F.	14	F.	11.97	0.97	12.34
J. S.	12	F.	11.00	1.08	10.19
Average and Std. Dev. (8 children)			14.14±2.20	1.28±0.25	11.24±1.47
Average for Normal Children (from Table I)			14.37±2.66	1.22±0.25	11.86±1.24
'p' Value for Difference			0.84	0.66	0.38

TABLE V

Lactate, Pyruvate and L-P Ratio In Children with Cyanotic Congenital Heart Disease

Name	Age	Sex	Diagnosis	Lactate	Pyruvate	L-P Ratio
M. B.	3	M.	Tetralogy of Fallot	20.43	1.56	13.10
C. B.	12	M.	Truncus arteriosus; cor triloculare	10.53	0.94	11.20
T. M.	5	M.	Tetralogy of Fallot	14.62	1.36	10.75
K. C.	1	F.	Probable tetralogy of Fallot	41.65	3.41	12.21
L. K.	4	M.	Probable tetralogy of Fallot	15.08	1.38	10.93
J. S.	6	M.	Tetralogy of Fallot and patent ductus arteriosus	10.35	1.04	9.95
C. L.	4	M.	Unknown	13.28	1.29	10.29
S. S.	5	M.	Tetralogy of Fallot	16.54	1.29	12.82
G. S.	9	M.	Unknown	14.69	1.50	9.80
C. E.	7	M.	Tetralogy of Fallot	15.19	1.18	12.87
Average and Std. Dev. (10 children)				17.24±8.21	1.50±0.64	11.38±1.14
Average for Normal Children (from Table I)				14.37±2.66	1.22±0.25	11.86±1.24
'p' Value for Difference				0.29	0.18	0.34

TABLE VI

Summary of Lactate-Pyruvate and Blood Gas Data Obtained from Standard Exercise Test on Children

	Lactate			Pyruvate			L-P Ratio			CO_2 Content (Vol. %)			O_2 Content (Vol. %)			O_2 Saturation (Vol. %)		
Normal Children	Seven children (2 males and 5 females); age range 6-13 years; mean O_2 capacity = 17.5 vol. % (std. dev. = 1.2); gas values on 4 cases only.																	
	Mean	S.D.[1]	p[2]	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p
Before Exercise	13.51	1.78		1.11	0.21		12.2	1.26		55.2	3.1		9.8	1.6		55.6	1.1	
Immediately After Exercise	17.76	2.71	0.04	1.36	0.12	0.01	13.0	1.59	0.37	54.3	5.2	0.80	10.0	3.5	0.58	56.5	17.6	0.94
15 Minutes After Exercise	15.50	2.15	0.19	1.25	0.19	0.31	12.6	2.03	0.84	53.7	4.3	0.62	11.5	2.2	0.27	66.1	13.6	0.27
Acyanotic Children	Six children (all females); age range 4-12 years; mean O_2 capacity = 18.9 vol. % (std. dev. = 1.9); gas values on 5 cases only.																	
	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p
Before Exercise	13.58	2.16		1.30	0.31		10.8	1.17		40.5	2.6		13.6	0.8		72.7	7.9	
Immediately After Exercise	18.99	4.30	0.02	1.52	0.52	0.40	13.0	1.84	0.02	50.7	2.8	0.09	10.8	3.0	0.06	55.3	8.3	0.00
15 Minutes After Exercise	14.88	3.03	0.42	1.35	0.33	0.80	11.0	0.66	0.74	46.8	1.9	0.27	15.1	2.7	0.27	78.9	13.4	0.37
Cyanotic Children	Three children (all males); age range 6-12 years; mean O_2 capacity = 22.9 vol. % (std. dev. = 1.6); gas values on only one of these on the 15 minute post-exercise sample.																	
	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p
Before Exercise	13.90	2.51		1.20	0.20		11.6	0.92		44.0	2.9		7.1	0.3		31.0	2.8	
Immediately After Exercise	24.59	2.30	0.00	1.79	0.37	0.10	14.1	2.34	0.13	43.6	0.9	0.84	3.9	0.7	0.00	16.9	2.3	0.00
15 Minutes After Exercise	26.73	4.90	0.00	1.83	0.62	0.23	15.5	3.10	0.07	33.7	—	—	12.3	—	—	56.4	—	—

[1]Standard Deviation
[2]Probability

TABLE VII-A

'p' Value for Difference Between Acyanotic and Normal Children During Standard Exercise Test

	Lactate	Pyruvate	L-P Ratio	CO_2 Content	O_2 Content	O_2 Saturation
Before Exercise	0.95	0.23	0.09	0.003	0.000	0.000
Immediately After Exercise	0.58	0.43	1.00	0.29	0.75	0.90
15 Minutes After Exercise	0.71	0.50	0.06	0.01	0.07	0.23

TABLE VII-B

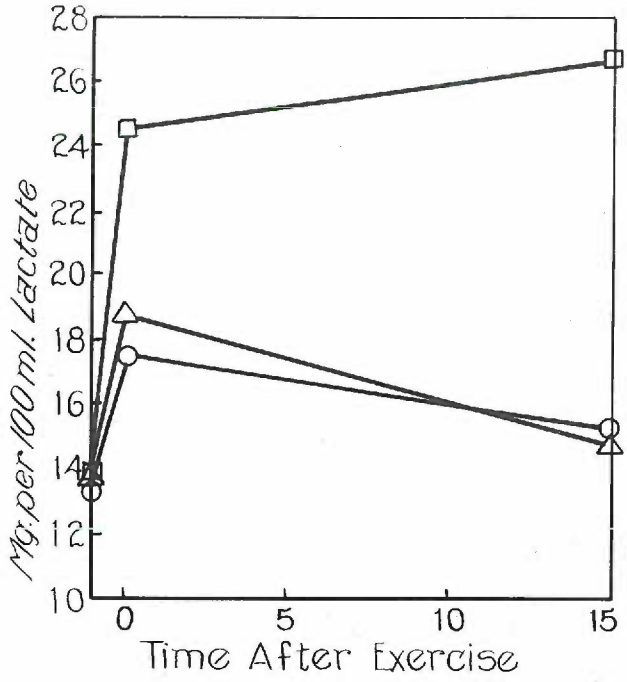
'p' Value for Difference Between Cyanotic and Normal Children During Standard Exercise Test

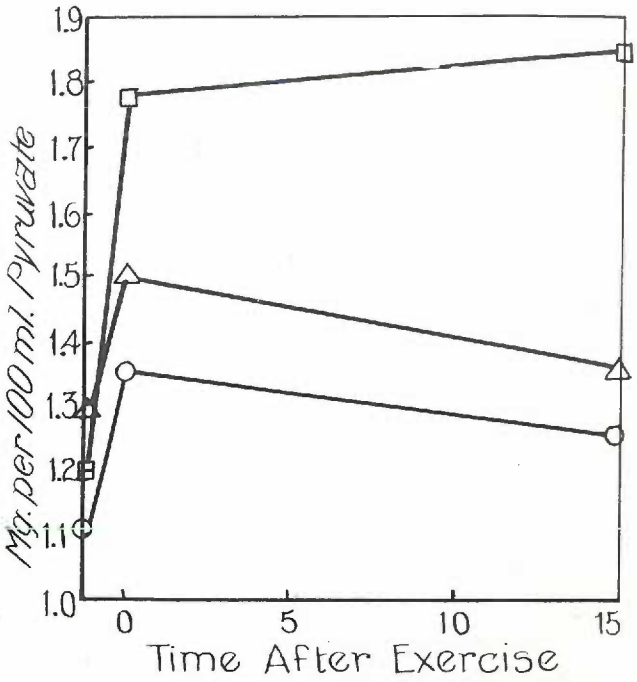
	Lactate	Pyruvate	L-P Ratio	CO_2 Content	O_2 Content	O_2 Saturation
Before Exercise	0.89	0.53	0.38	0.000	0.005	0.000
Immediately After Exercise	0.000	0.09	0.52	0.000	0.000	0.000
15 Minutes After Exercise	0.002	0.19	0.21	—	—	—

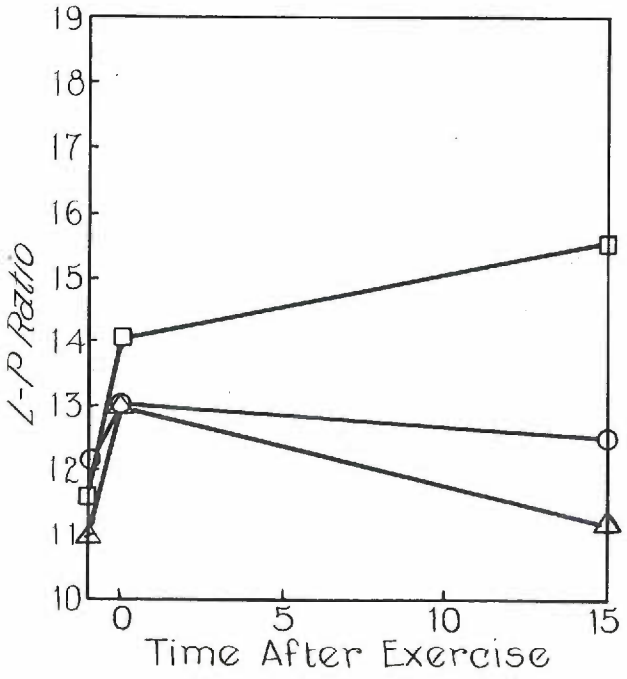
FIGURE 6

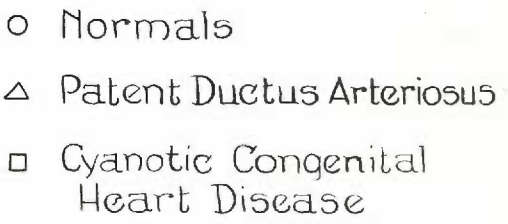
Lactate, Pyruvate and L-P Ratio During

Standard Exercise Test in Children.


Mg. per 100 ml. Lactate
28
26
24
22
20
18
16
14
12
10
0
5
10
15
Time After Exercise


Mg. per 100 ml. Pyruvate
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
5
10
15
Time After Exercise


L-P Ratio
19
18
17
16
15
14
13
12
11
10
0
5
10
15
Time After Exercise


Normals
Patent Ductus Arteriosus
Cyanotic Congenital Heart Disease

samples taken immediately after exercise in patients with patent ductus arteriosus were not utilized in this particular study. Linear reduction of blood oxygen saturation in the capillaries had to be assumed. According to Lundsgaard and Van Slyke (22) this is a reasonable assumption to make. Mean capillary carbon dioxide tension (pCO_2) of 50 mm. Hg. had to be assumed. Although this figure undoubtedly varies among individuals, it probably represents a close approximation to the mean (23). With these assumptions in mind the mean capillary blood oxygen saturation was calculated using the determined venous blood oxygen saturation with the following formula:

$$\text{Mean capillary } O_2 \text{ saturation in } \% = \frac{\text{arterial } O_2 \text{ saturation in } \% - \text{venous } O_2 \text{ saturation in } \%}{2}$$

Mean capillary pO_2 in mm. Hg. was then read from a standard oxygen dissociation curve of hemoglobin at pCO_2 of 50 mm. Hg.

Figure 7 is a graph of the L-P ratio against mean capillary pO_2 in 28 children plotted from the data of Table VIII. The correlation coefficient is minus 0.60 showing a definite inverse relationship between these two variables. Results obtained in 6 children with cyanotic congenital heart disease at rest are also plotted on the graph from the data of Table IX but were omitted from calculation of the correlation coefficient.

The results of the lactic dehydrogenase assay on 30 individuals are listed in Table X. The 'index' ($Q_{CO_2}^{N_2}$ x hematocrit) indicates the relative amount of the enzyme present per unit volume of whole blood. This value is plotted against the hematocrit in Figure 8. As seen in the graph, the amount of lactic dehydrogenase per unit volume of

TABLE VIII

Correlation of L-P Ratio and Mean Capillary pO_2 in Normal Children and Children with Patent Ductus Arteriosus

Name	Age	Sex	Status	L-P Ratio	% Venous O_2 Saturation	Mean Capillary pO_2 in mm. Hg.
Normal Children						
C. O.	7	F.	Resting	11.5	58	53
C. O.	7	F.	Immed. p-Ex.	10.6	74	67
C. O.	7	F.	15 min. p-Ex.	10.6	68	62
W. S.	12	F.	Resting	14.6	49	49
W. S.	12	F.	Immed. p-Ex.	14.8	52	50
D. L.	11	M.	Resting	12.3	47	48
D. L.	11	M.	Immed. p-Ex.	13.2	30	42
D. L.	11	M.	15 min. p-Ex.	13.0	43	47
D. H.	6	F.	Resting	9.5	69	62
D. H.	6	F.	Immed. p-Ex.	11.1	71	64
D. H.	6	F.	15 min. p-Ex.	9.8	75	67
L. L.	4	F.	Resting	10.2	84	80
E. R.	4	M.	Resting	11.6	73	67
D. E.	6	M.	Resting	10.2	57	53
H. B.	10	M.	Resting	11.6	50	50
R. M.	13	M.	Resting	12.7	65	60
G. R.	6	M.	Resting	10.8	54	52
V. R.	4	F.	Resting	12.3	59	55
L. W.	12	M.	Resting	11.5	70	64
S. S.	8	F.	Resting	10.6	77	69

TABLE VIII (cont.)

Correlation of L-P Ratio and Mean Capillary pO_2 in Normal Children and Children with Patent Ductus Arteriosus

Name	Age	Sex	Status	L-P Ratio	% Venous O_2 Saturation	Mean Capillary pO_2 in mm. Hg.
Children with Patent Ductus Arteriosus						
R. C.	4	F.	Resting	8.6	85	80
R. C.	4	F.	15 min. p-Ex.	10.5	94	90
A. W.	10	F.	Resting	11.7	63	58
A. W.	10	F.	15 min. p-Ex.	11.7	81	73
L. C.	5	F.	Resting	11.3	77	69
L. C.	5	F.	15 min. p-Ex.	10.2	65	60
J. S.	12	F.	Resting	10.2	65	60
J. S.	12	F.	15 min. p-Ex.	10.5	61	57

TABLE IX

L-P Ratio and Mean Capillary pO_2 in Resting Children with Cyanotic Congenital Heart Disease

Name	Age	Sex	Diagnosis	L-P Ratio	% Arterial O_2 Saturation	% Venous O_2 Saturation	Mean Capillary pO_2 in mm. Hg.
C. B.	12	M.	Truncus arteriosus; cor triloculare	11.2	67	28	33
T. M.	5	M.	Tetralogy of Fallot	10.8	56	34	31
K. C.	1	F.	Probable tetralogy of Fallot	12.2	50	9	23
L. K.	4	M.	Probable tetralogy of Fallot	10.9	65	53	36
J. S.	6	M.	Tetralogy of Fallot and patent ductus arteriosus	10.0	78	52	43
C. L.	4	M.	Unknown	10.3	83	73	57

FIGURE 7

Correlation of L-P Ratio and Mean

Capillary pO_2 in Children.

○ - Normals at rest.

◐ - Normals immediately after exercise.

● - Normals 15 minutes after exercise.

□ - Patent ductus arteriosus at rest.

■ - Patent ductus arteriosus 15 minutes after exercise.

△ - Cyanotic congenital heart disease at rest.

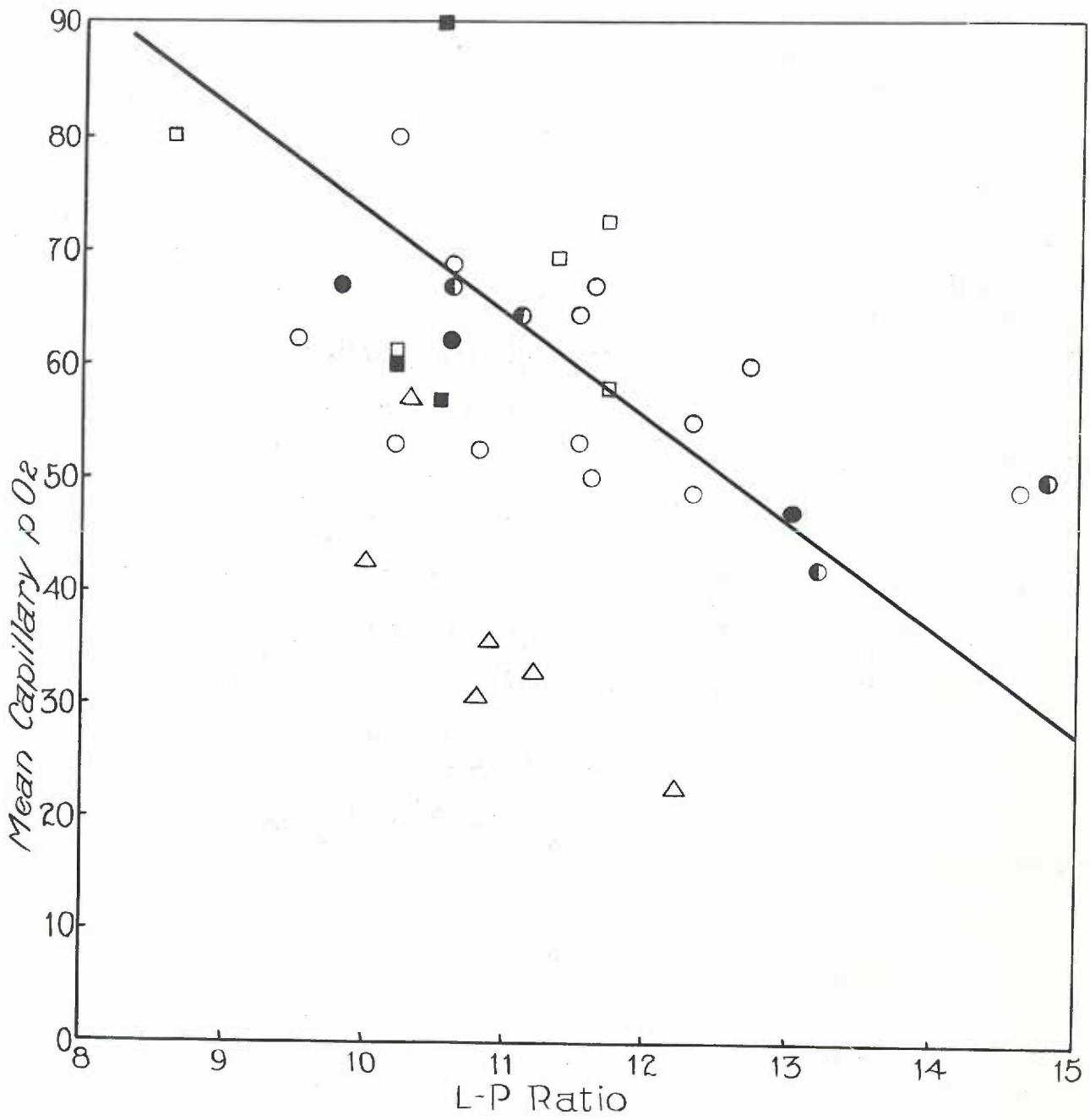

Mean Capillary pO_2
90
80
70
60
50
40
30
20
10
0
8
9
10
11
12
13
14
15
L-P Ratio

TABLE X

Results of Lactic Dehydrogenase Assay on 30 Individuals

Name	Age	Diagnosis	$Q_{CO_2}^{N_2}$	Hematocrit	$(Q_{CO_2}^{N_2}$ x Hematocrit) Index
R. H.	24	Normal	121	49	61
M. P.	24	Tetralogy of Fallot (postoperative)	135	50	68
T. W.	18	Tetralogy of Fallot	158	77	122
P. K.	9 mo.	Tetralogy of Fallot	133	56	75
M. M.	8	Normal	140	38	52
F. G.	6	Normal	144	33	48
N. R.	24	Normal	118	47	55
H. R.	22	Normal	121	50	61
H. S.	23	Normal	149	48	71
C. E.	7	Tetralogy of Fallot	138	61	84
D. B.	30	Normal	142	41	58
S. G.	24	Normal	128	39	50
D. F.	14	Patent ductus arteriosus	132	40	53
T. R.	6	Normal	145	45	65
N. M.	11	Normal	151	36	55
T. D.	9	Normal	180	34	61
W. C.	4	Normal	132	41	54
E. W.	2	Normal	156	43	67
S. K.	4	Tetralogy of Fallot	145	73	106
J. S.	24	Normal	134	48	64
R. P.	32	Normal	138	44	61
N. S.	25	Normal	124	44	55

TABLE X (cont.)

Results of Lactic Dehydrogenase Assay on 30 Individuals

Name	Age	Diagnosis	$Q_{CO_2}^{N_2}$	Hematocrit	($Q_{CO_2}^{N_2}$ x Hematocrit) Index
C. D.	5 mo.	Normal	142	31	44
M. G.	9 mo.	Normal	151	30	45
M. C.	1	Tetralogy of Fallot	154	49	75
K. C.	1	Truncus arteriosus with hypochromic anemia	176	40	70
G. F.	72	Hypochromic anemia	168	21	35
R. M.	71	Hypochromic anemia	183	33	60
M. J.	69	Hypochromic anemia	142	25	36
I. H.	76	Hypochromic anemia	141	30	42

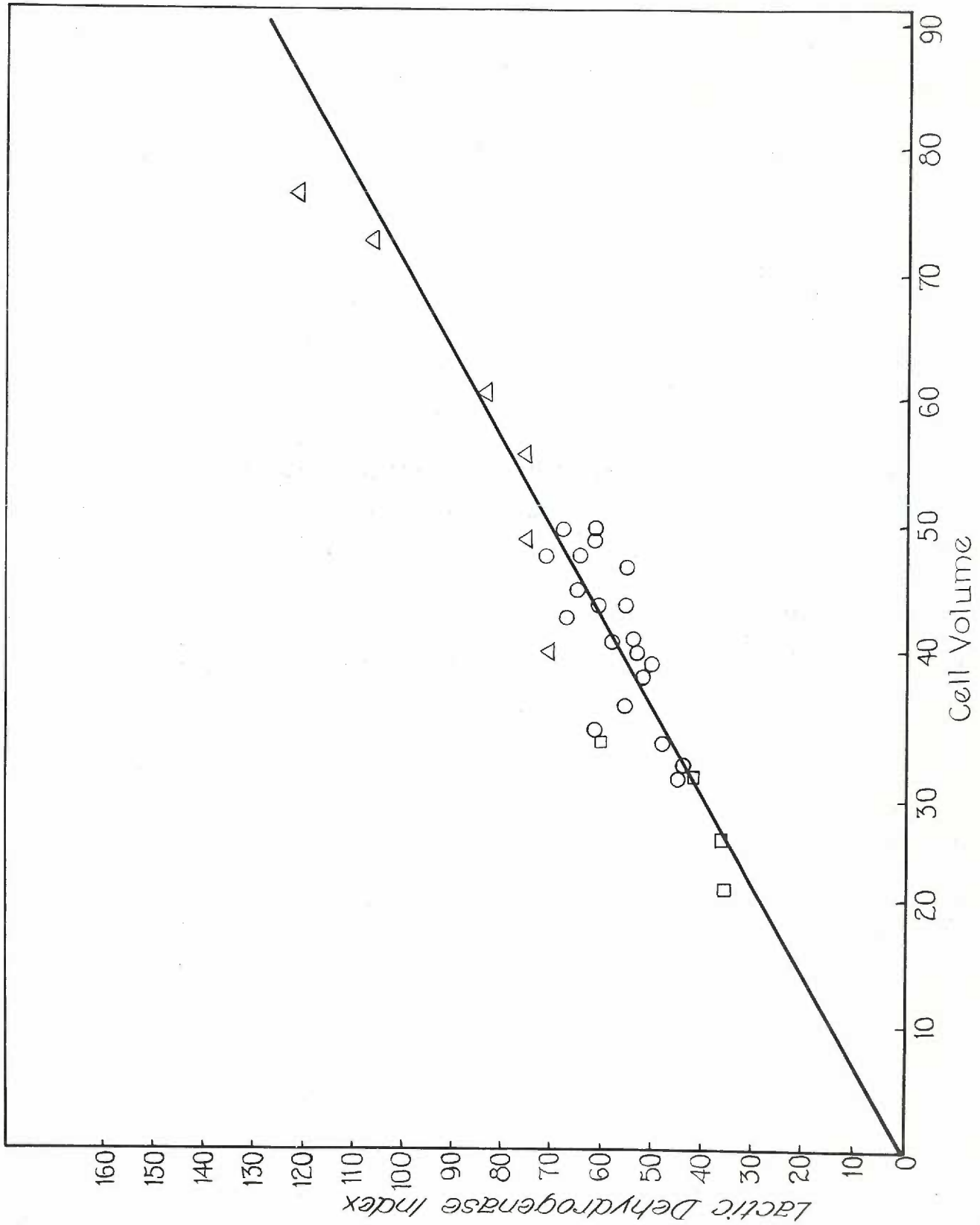
FIGURE 8

Correlation of Lactic Dehydrogenase Index and Cell Volume.

◐ - Normals.

△ - Cyanotic congenital heart disease.

□ - Anemias.


Lactic Dehydrogenase Index
Cell Volume
0
10
20
30
40
50
60
70
80
90
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160

red cells was unaltered in the patients with cyanotic congenital heart disease, the amount of enzyme per unit volume of whole blood being solely a function of the cell volume in all persons studied.

DISCUSSION

The differences observed in the concentrations of blood lactate and pyruvate and the L-P ratio in adults and children are of practical importance as well as of considerable theoretical interest. An L-P ratio of 6 in an adult is considered indicative of moderate to severe thiamin deficiency (8). If this is true, a ratio of 8 would represent an equivalent state of thiamin deficiency in a child. The only other reported factor affecting the ratio is relative hypoxia produced by exercise (6) or acute exposure to low barometric pressure (7). Why age should affect the L-P ratio is not apparent. In attempting to explain this fact it may be pointed out that children have a basal metabolic rate higher than that of adults. In order to quantitate these factors, Boothby and Sandiford's figures (24) for the basal metabolism corresponding to the mean age of the normal children studeid were compared with the basal metabolism corresponding to the mean age of the adults (averaged for males and females). Their results indicated the basal metabolism of children to be increased 26% as compared with the values for adults. The values obtained in this study show that the L-P ratio was increased 29% in the children as compared with the adults. Since lactate and pyruvate are important intermediates in the oxidation of glucose, from which the body normally obtains most of its energy, it is possible that the ratio might be related to the basal metabolism or to factors controlling basal metabolism. This viewpoint is substantiated by comparing the L-P ratio capillary pO_2 correlation in the normal-acyanotic group of children with that of children in the cyanotic group. As seen in

Figure 7 there is very poor correlation between the two groups. The cyanotic children have a normal L-P ratio in the face of a markedly lowered capillary pO_2. The two cyanotic patients who most nearly correlate with the normal-acyanotic group have additional lesions mitigating the severity of tissue anoxia. It is well known that these cyanotic children have a very low basal metabolic rate, averaging about minus 40 (25). The possibility is presented that the normal L-P ratio observed in these children is related to the lowered metabolic rate.

The results obtained from the standard exercise test demonstrate that normal children and children with patent ductus arteriosus behave similarly with respect to exercise, while quite a different picture is seen in children with cyanotic congenital heart disease.

In normal children, the values for lactate and pyruvate are significantly higher immediately following exercise and after 15 minutes return to a value that is not significantly different from the resting sample. Blood carbon dioxide and oxygen contents are not significantly altered at any time.

In children with patent ductus arteriosus the values for lactate and the L-P ratio, but not the pyruvate, are significantly higher immediately after exercise and return to resting limits 15 minutes after exercise as do the normals. Blood carbon dioxide content in the acyanotic group is not significantly changed, but blood oxygen saturation falls significantly below the resting value in the first post-exercise sample and rises significantly above the resting value in the second post-exercise sample (15 minutes). The fall can be

attributed to an increased coefficient of oxygen utilization and possible reversal of shunt flow (to right-left) through the ductus immediately after exercise, while the rise most probably represents an overcompensation of the mechanisms by which oxygen supply to the tissues is maintained.

In the exercised children with cyanotic congenital heart disease, the values for lactate are significantly higher than the resting values both immediately after exercise and fifteen minutes after exercise. The values for pyruvate and the L-P ratio are slightly higher in both of the post-exercise samples. The values for the blood carbon dioxide content are not essentially different, but the blood oxygen content is markedly lower in the immediate post-exercise sample.

Comparison of corresponding samples (Table VII) provides further information about the differences between the various groups. Certain interesting differences are seen between the normal and acyanotic group. Before exercise the L-P ratio of the acyanotic group is slightly lower than that of the normal group, while blood oxygen saturation is significantly higher. Blood carbon dioxide content is significantly lower. Immediately after exercise, these differences have disappeared, and 15 minutes after exercise, the values are similar to those for pre-exercise. The relation of the L-P ratio and blood oxygen saturation in the normal and acyanotic groups reflects the inverse correlation between these two variables already discussed.

The higher oxygen saturation and lower carbon dioxide content of venous blood in patients with patent ductus arteriosus have not been

reported previously. Two possible explanations of this phenomenon are: 1. Increased pO_2 of arterial blood due to recirculation of a large portion of the left ventricular output through the pulmonary capillary bed. 2. A vasomotor alteration resulting in peripheral vasodilatation as a compensatory mechanism whereby systemic blood flow is kept within normal limits in the presence of a 'leak' in the aorta. The former is unlikely since a large increase in the pO_2 of arterial blood would produce only a very small increase in oxygen saturation. The latter is reasonable and helps to explain the normal exercise tolerance found in these individuals.

In the cyanotic group predicted changes are noted. The blood lactate concentration is significantly higher than for the normal group in both post-exercise samples, but not in the pre-exercise sample. Blood pyruvate concentration is slightly higher in both post-exercise samples, while the L-P ratio is slightly higher only in the sample taken 15 minutes after exercise. The blood carbon dioxide content is significantly lower in the two samples in which it was possible to carry out statistical analysis. It is well known that the decreased arterial pO_2 acts as a stimulus to respiration sufficient to reduce blood carbon dioxide concentration in cyanotic congenital heart disease.

It is clear from the results of the lactic dehydrogenase assay that the blood of children with cyanotic congenital heart disease has a higher than normal content of this enzyme by virtue of having more red cells per unit volume. Such a result cannot be considered to indicate a compensative mechanism since the concentration of the enzyme in the red cells remains unchanged. However, the results do

indicate a primary association between lactic dehydrogenase and red blood cell metabolism. One of the most important functions of erythrocytes is that of keeping the iron of hemoglobin in the ferrous state. The mechanism by which this is carried out ("reconversion mechanism" (26)) is not completely understood, but it is an enzymatic process involving a number of compounds, among which are glucose, lactate and diphosphopyridine nucleotide (27, 28, 29). These facts, together with the high concentration of lactic dehydrogenase observed in red cells, suggest that conversion of lactate to pyruvate may be one of the principal mechanisms by which hemoglobin is kept in its active ferrous form.

PART II

ANOXIA IN EXPERIMENTAL ANIMALS

EXPERIMENTAL

In this part of the investigation 10 male rats of the Sprague-Dawley strain weighing 320 to 365 gm. were used. Five animals were kept as controls and a state of chronic anoxia produced in the other 5 by placing them in a sealed chamber at reduced barometric pressure. The apparatus is diagramed in Figure 9. The chamber was partially evacuated by means of a Cenco Hyvac oil pump. An adjustable inlet cock permitted fine control of the pressure within the system. The pressure was read with a mercury manometer and a mercury safety valve prevented the pressure from dropping below a specified limit. The rats were kept in the chamber continuously for 7-11 days except for brief periods on alternate days when they were removed in order to clean the chamber. Ample food (Purina Laboratory Chow) and water were present at all times. The pressure was kept between 440 and 420 mm. Hg. (equivalent to about 15,000 feet altitude). The times of 'ascent' and 'descent' were 15 minutes.

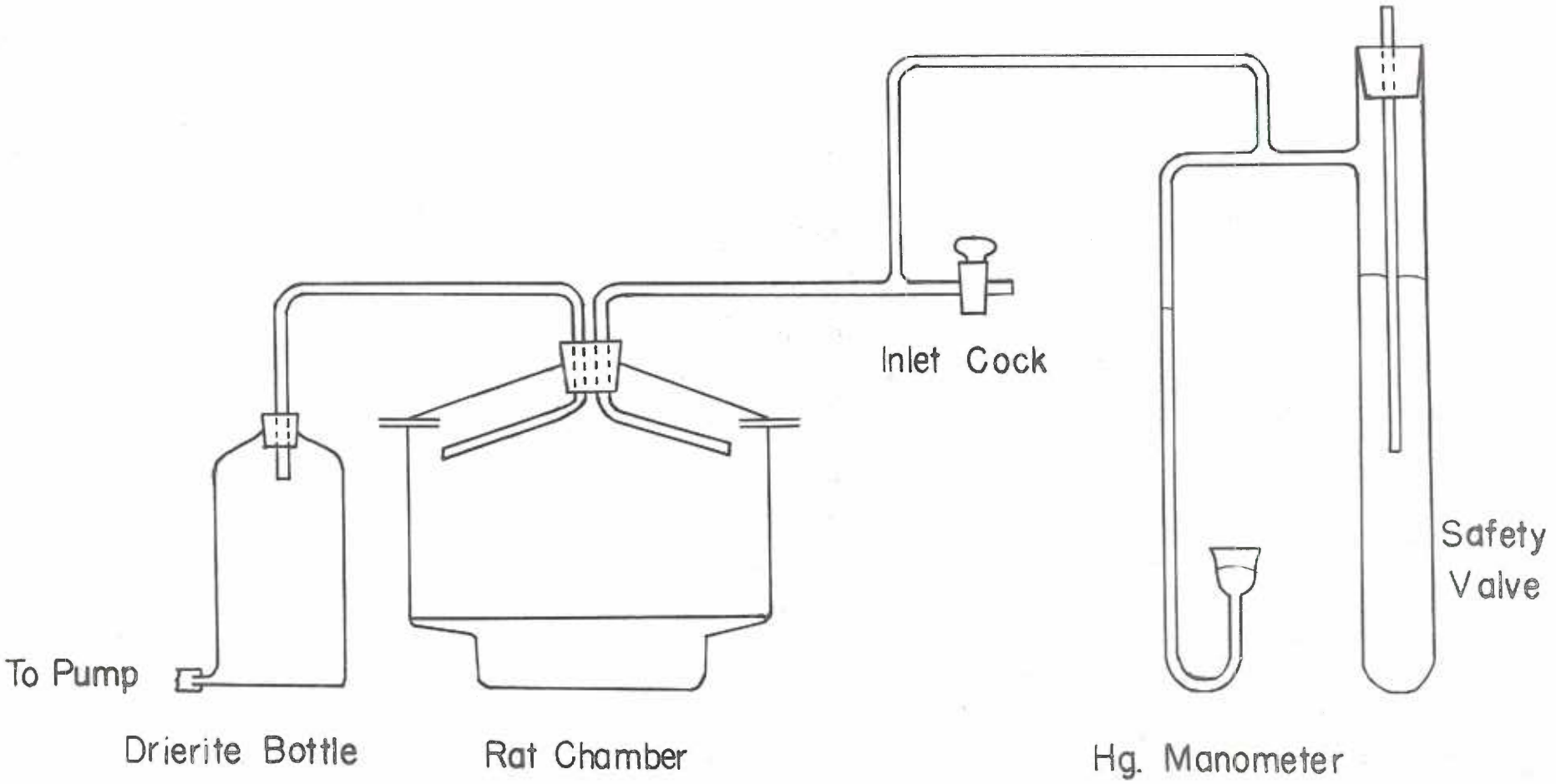
Before placing the animals in the altitude chamber they were weighed and 0.2 ml. of blood withdrawn by cardiac puncture for hemoglobin determination. A modification of the method of Evelyn and Malloy (30) was used. One-tenth ml. of blood was laked in 10.0 ml. of M./60 phosphate buffer, pH 6.6 before clotting occurred. Three ml. of the laked blood solution were delivered into the cuvette of a Beckman Model DU spectrophotometer and 1 drop of 20% potassium ferricyanide added, followed by 1 drop of 5% neutralized sodium cyanide, converting the hemoglobin into cyanmethemoglobin. A water blank was treated in the same way. The instrument was nulled with the blank

FIGURE 9

Diagram of Low Pressure Apparatus

Used to Produce a State of

Chronic Anoxia in Rats.


Inlet Cock
Safety
Valve
To Pump
Drierite Bottle
Rat Chamber
Hg. Manometer

solution at a wave-length of 540 millimicrons and the extinction of the cyanmethemoglobin solution read. The method was standardized against the Van Slyke and Neill oxygen capacity method for hemoglobin (hemoglobin in gm./100 ml. = oxygen capacity in vol. %/1.34). Hemoglobin concentration was again measured before sacrificing the animal in order to determine the degree of polycythemia produced by exposure to reduced barometric pressure.

The animals were fasted for 24 hours prior to tissue analysis. This procedure was necessary to eliminate liver glycogen interference with cytochrome c analysis, and also served as an additional control.

The rats were killed by decapitation and heart, liver and gastrocnemius muscle taken for analysis. The tissues were removed as quickly as possible from the decapitated animal. Approximately 0.5 gm. portions of each were taken for analysis of easy hydrolyzable phosphorus fractions and total pentose, weighed on a torsion balance, placed in an ice-cold mortar and ground simultaneously with 0.5 ml. of quartz sand, 2 ml. of 20% trichloroacetic acid and a liberal quantity of dry ice chips. By this procedure it was possible to precipitate the proteins and freeze the tissue within 5 minutes of sacrificing the animal. The weighed samples were then placed in the freezing compartment of the refrigerator. After taking an approximately 2 gm. portion of liver for analysis of succinic dehydrogenase, the rest of the samples were placed in the freezing compartment of the refrigerator in small stoppered bottles. Succinic dehydrogenase was assayed on duplicate 5-15 mg. slices of liver by the method of Quastel and Wheatley (16). The principle of this method is the same as that of the lactic

dehydrogenase assay outlined previously. In order to expedite the analysis all preliminary preparations were carried out before sacrificing the rat.

After completion of the succinic dehydrogenase assay (about 1 hour) the weighed samples were removed from the refrigerator and allowed to thaw. As soon as possible they were poured into 15 ml. centrifuge tubes and centrifuged. The supernate from each was poured into a 10 ml. volumetric flask and immediately neutralized to phenolphthalein with 2.5 N. NaOH. The precipitate in the centrifuge tube and that remaining in the mortar were washed twice with 2 ml. portions of 20% trichloroacetic acid, the washings added to the original supernate and neutralized as above. The extract was made up to 10 ml. with water. Four 2 ml. portions were analyzed for phosphate fractions by the methods given by LePage and Umbreit (17). Two 2 ml. portions were transferred to 8 inch test tubes. Two ml. of 2 N. HCl were added to one of these which was then placed in a boiling water bath. After 23 minutes the other aliquot was acidified similarly and placed in the boiling water bath. Exactly 7 minutes later both tubes were removed from the bath and cooled in running tap water. Twenty minutes before the end of the boiling period a third aliquot was acidified as above followed by the addition of 10 ml. of a solution of 2.5% ammonium molybdate in 5 N. HCl. To a fourth aliquot was added 0.4 ml. of a 10% solution of calcium chloride saturated with calcium hydroxide at pH 8.8. After 10 minutes the precipitate of calcium phosphate was centrifuged down, washed with a little of the calcium chloride solution diluted 1-5 with water and centrifuged again. The

precipitate was then dissolved in 2 ml. of 2 N. HCl. The above procedures were so timed that all the aliquots were ready for color development at once. Inorganic phosphate was determined on each aliquot by the Fiske and Subbarow method except that the reducing agent described by Allen (31) was used. Ten ml. of the ammonium molybdate solution were added to all aliquots except the third. This was followed by 2 ml. of a solution of 1% amidol (2,4-diaminophenol dihydrochloride) in 20% sodium bisulfite. The solutions were made up to 100 ml. in volumetric flasks and mixed. After 15 minutes the optical density was measured at a wave-length of 600 millimicrons in the Coleman Junior spectrophotometer. A standard containing 0.08 mg. of inorganic phosphorus was run with each determination.

In the above procedure each of the fractions analyzed presumably represents the whole or a portion of certain phosphorus-containing substances present in trichloroacetic acid extracts of tissue. The aliquot precipitated with calcium ion represents 'true' inorganic phosphorus—that is, phosphorus present in inorganic form in the intact animal. The aliquot incubated in the cold for 20 minutes with molybdate reagent represents 'true' inorganic phosphorus plus creatine phosphate (phosphocreatine is rapidly hydrolyzed in acid solution in the presence of molybdate). The phosphorus contained in the aliquot boiled in 1 N. HCl for 7 minutes represents 66% of the 2 pyrophosphates of ATP and 50% of the pyrophosphate of ADP (plus small amounts of phosphate from other esters), in addition to inorganic phosphorus and phosphorus derived from phosphocreatine. The aliquot boiled for 30 minutes contains all the pyrophosphate of ATP and ADP as inorganic

phosphate.

One ml. of the remaining neutralized extract was analyzed for total pentose by the method of Mejbaum (32). The bound pentose was hydrolyzed and converted to furfural by boiling in a strong acid solution containing orcinol and ferric chloride, with which furfural reacts to give a characteristic green color. The pentose was then determined by comparing the photometric density at 660 millimicrons wave-length with that of a standard pentose solution treated similarly. The pentose determined by this method represents principally that contained in adenylic acid and its derivatives, although some of it is derived from pentose phosphate, pyridine nucleotides and other compounds.

The remaining portions of liver and skeletal muscle were removed from the refrigerator. The liver was analyzed for cytochrome c by the method of Prader and Gonella (33). The cytochrome was extracted with acid ammonium sulfate solution and hemoglobin removed from the extract by fractional precipitation with ammonium sulfate. The cytochrome was then precipitated with trichloroacetic acid, centrifuged off and dissolved in dilute alkali. After conversion to ferrocytochrome c by addition of sodium hydrosulfite, the optical densities at 550 and 560 millimicrons wave-length were measured, the difference between them being proportional to the amount of cytochrome c present. The method was standardized against pure cytochrome c prepared from horse heart by the method of Keilin and Hartree (34).

The skeletal muscle was analyzed for total creatinine by the method of Barclay and Kenney (35). The tissue was digested in 1 N. HCl, con-

verting all the creatine into creatinine. The extract was filtered and deproteinized. Creatinine was determined nephelometrically after mixing with a Nessler-type reagent. A standard creatinine solution was run with each determination.

Per cent dry weight was determined on small portions of each tissue by weighing before and after heating overnight in the oven at 90° C.

RESULTS

The animals kept at reduced barometric pressure uniformly lost weight during the 7-11 days of exposure. This loss of weight was not due to dehydration for the per cent dry weight of the heart, liver and skeletal muscle of exposed animals was not materially different than that of the control group (Table XI). This confirmed the impression that the animals ate less at 'altitude' than at the usual elevation. The low barometric pressure produced a definite polycythemia in all exposed animals. The increase in hemoglobin concentration averaged 24% and varied from 15% in one of the animals exposed for 7 days to 39% in the animal exposed for 11 days (Table XI).

No significant change was observed in the concentration of any of the substances analyzed in these experiments. Succinic dehydrogenase activity of liver fell slightly while cytochrome concentration remained practically unchanged (Table XII). Total creatinine of muscle showed no marked change; total pentose of heart fell slightly while that of liver and skeletal muscle rose slightly in the exposed rats as compared with the controls (Table XIII). In the phosphorus fractions the only consistent change was a small elevation of the 'true' inorganic phosphorus and creatinine phosphate fractions (Table XIV and Figure 10). The 7 and 30 minute hydrolysis fractions remained practically unchanged.

In Tables XI-XIV each animal is represented by the same number.

TABLE XI-A

Weight, Hemoglobin, and % Dry Weight of Tissues in Control Animals

Animal	Weight in Gm.	Gm. % Hemoglobin	% Dry Weight		
			Heart	Liver	Muscle
1	330	15.2	23	24	24
2	340	16.0	23	24	27
3	350	15.8	22	25	23
4	330	17.4	24	27	26
5	340	17.6	22	23	24
Average	338	16.4	23	25	25
Std. Dev.	± 7.5	± 0.99	± 0.8	± 1.4	± 1.5

TABLE XI-B

Weight and Hemoglobin Before and After Exposure to Reduced Barometric Pressure and % Dry Weight of Tissues in Experimental Animals

Animal	Wt. in Gm.		Gm. % Hemoglobin		% Dry Weight		
	Before	After	Before	After	Heart	Liver	Muscle
1 (11)*	320	250	16.0	22.2	23	29	27
2 (7)	365	330	16.1	19.2	23	27	21
3 (8)	350	300	16.8	21.0	20	27	24
4 (7)	340	310	17.1	21.0	23	25	24
5 (7)	350	310	17.9	20.5	22	25	23
Average	345	300	16.8	20.8	22	27	24
Std. Dev.	± 11.8	± 26.8	± 0.70	± 0.97	± 1.2	± 1.5	± 1.9

*Days at altitude.

TABLE XII

Succinic Dehydrogenase and Cytochrome c in Control Rats and Rats with Compensatory Polycythemia

Animal	Succinic Dehydrogenase in Liver ($Q_{CO_2}^{N_2}$)		Cytochrome c in Liver (mg. % wet weight)	
	Control	Exptl.	Control	Exptl.
1	77	70	7.1	4.8
2	79	76	5.0	5.5
3	73	73	4.5	6.2
4	87	75	7.0	5.8
5	73	64	7.3	7.7
Average	78	72	6.2	6.0
Std. Dev.	± 5.2	± 4.4	± 1.2	± 1.0

TABLE XIII

Total Creatinine of Muscle and Total Pentose of Heart, Liver and Muscle in Control Rats and Rats with Compensatory Polycythemia

Animal	Total Creatinine of Muscle (mg. % wet wt.)		Total Pentose of Heart, Liver and Muscle (mg. % wet wt.)					
			Heart		Liver		Muscle	
	Control	Exptl.	Control	Exptl.	Control	Exptl.	Control	Exptl.
1	—	—	—	—	—	—	—	—
2	328	313	174	134	176	201	184	201
3	342	306	180	189	171	190	188	201
4	314	376	164	181	139	161	167	186
5	374	340	219	170	186	171	192	185
Average	340	333	184	169	168	181	183	198
Std. Dev.	± 22.2	± 28.3	± 21.0	± 21.0	± 17.6	± 15.7	± 14.0	± 9.1

TABLE XIV

Easy Hydrolyzable Phosphorus Fractions of Rat Tissue
(mg. % wet weight)

	Liver				Cardiac Muscle				Skeletal Muscle			
Control Animal	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
1	—	20	26	30	—	48	61	64	—	78	100	124
2	15	21	30	32	33	49	63	66	47	58	77	88
3	22	28	39	45	33	46	58	62	61	73	93	103
4	30	41	47	52	40	51	65	69	61	76	106	127
5	20	27	42	52	38	53	61	65	61	83	112	127
Average	22	27	37	42	36	49	62	65	58	74	98	114
Std. Dev.	5.4	7.5	7.7	8.3	3.1	2.4	2.4	2.3	6.1	8.5	12.1	15.7
Experimental Animal	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
1	—	30	43	46	—	63	73	77	—	90	115	137
2	17	22	35	38	35	47	57	60	72	88	112	122
3	23	28	38	43	41	49	60	68	55	70	100	113
4	26	31	41	48	35	48	62	68	57	70	96	112
5	28	36	43	46	37	57	72	80	71	90	108	115
Average	24	29	40	44	37	53	65	71	64	82	106	120
Std. Dev.	4.2	4.6	3.2	3.5	2.4	6.2	7.1	4.4	7.8	9.5	7.1	9.3

(1) 'True' inorganic phosphorus
(2) 'True' inorganic phosphorus plus phosphocreatine phosphorus
(3) Inorganic phosphorus after 7 minutes hydrolysis
(4) Inorganic phosphorus after 30 minutes hydrolysis

FIGURE 10

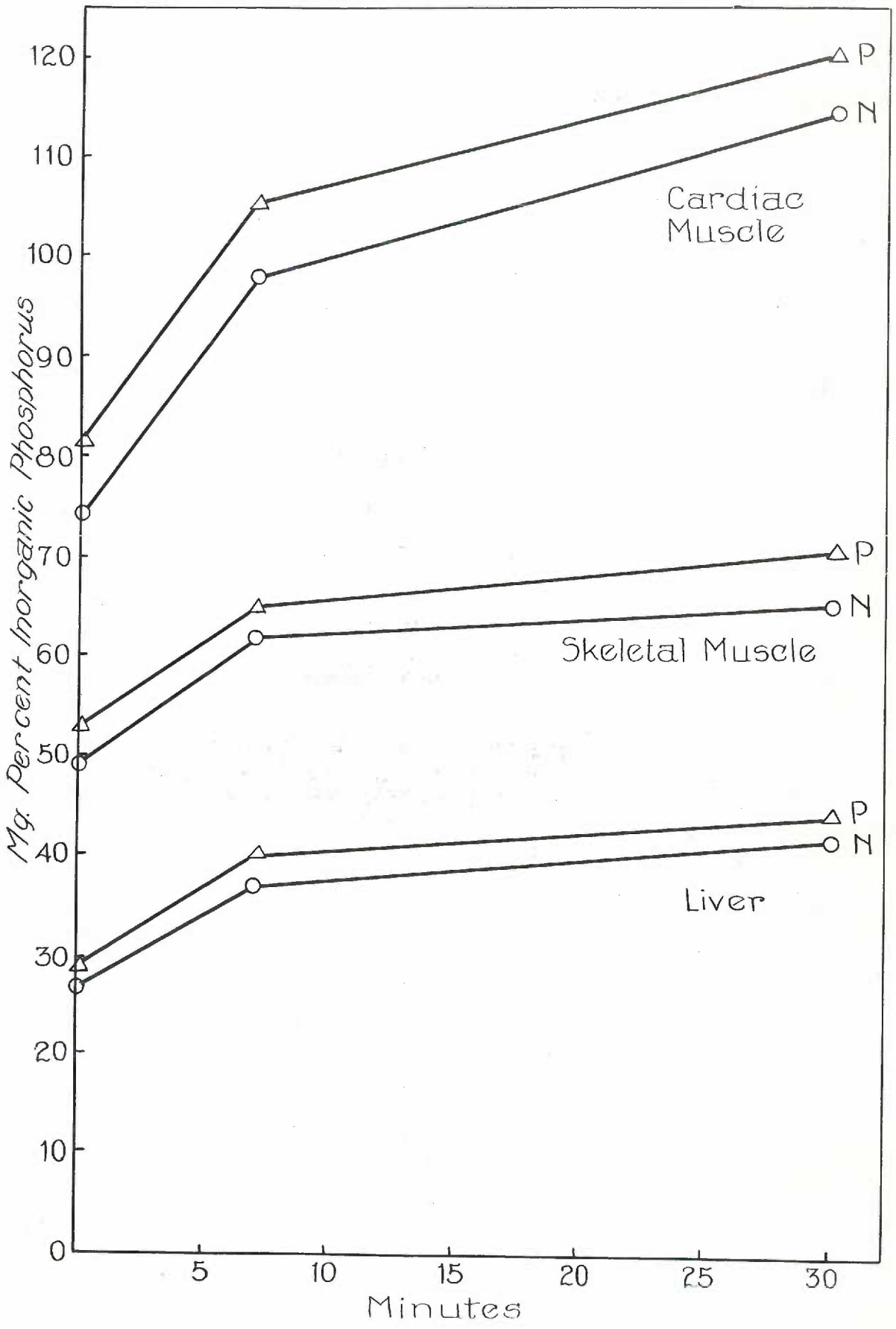
Easy Hydrolyzable Phosphorus

Fractions of Rat Tissues.

N - Normals

P - Polycythemic

(Value at zero time represents
sum of 'true' inorganic phosphorus
and creatine phosphate fractions)


Mg. Per cent Inorganic Phosphorus
120
110
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
Minutes
P
N
Cardiac Muscle
P
N
Skeletal Muscle
P
N
Liver

DISCUSSION

The results obtained in this part of the investigation were for practical purposes entirely negative. The 'true' inorganic phosphorus concentrations given are probably too high and the creatine phosphate concentrations correspondingly low because of the extreme ease of hydrolysis of creatine phosphate both in vivo and in acid solution. For this reason the sum of the inorganic phosphorus and phosphocreatine fractions represents mainly the latter compound. The slight but consistent elevation of these fractions suggests, therefore, that creatine phosphate concentration was slightly increased in the polycythemic rats as compared with the control rats. An increase in the concentration of creatine phosphate would be of value to an animal under conditions of chronic anoxia without an increase in the concentration of ATP because the amount of ATP that can be resynthesized anaerobically depends to a large degree on the amount of creatine phosphate present.

The constancy of cytochrome c concentration in the polycythemic rats as compared with the normal animals confirms Drabkin (36), who found that the rate of appearance of new cytochrome c in regenerating rat liver was unchanged under conditions of chronic anoxia.

From the results of this experiment it cannot be said that there is no tissue response to chronic anoxia through increased concentration of certain substances concerned with energy transformation and oxygen transfer. More severe or prolonged hypoxia might result in alterations in the cell not observed here. However, the results obtained substantiate Drabkin's statement (36) that the primary response

observed in the blood in response to chronic anoxia is separate and distinct from any secondary response occurring in the tissues. Further confirmation of this statement may be found in the recent work of Bowen (37) and Poel (38) who found that myoglobin concentration in skeletal muscle of rats and dogs respectively remains essentially unchanged after several months exposure to barometric pressure reduced sufficiently to produce marked polycythemia.

In view of the results obtained here and elsewhere it can be stated that the only aspect of general tissue metabolism known to be associated with chronic anoxia is a reduced metabolic rate. There is little information in the literature on the relation between pO_2 and respiratory rate in vitro in which oxygen diffusion can be eliminated as a limiting factor. For many years following the classical experiments of Warburg and Kubowitz (39) on the influence of oxygen tension on the rate of respiration of Micrococcus candicans it was generally held that oxygen supply is not a limiting factor in the rate of cellular respiration. However, Kempner (40) has demonstrated that below certain critical limits of oxygen tension oxygen uptake of many tissues in vitro falls off rapidly. Krogh (41) calculated the capillary-cell pO_2 difference necessary to maintain the observed oxygen consumption of mammalian muscle at rest and arrived at figures of 19-49 mm. Hg. The oxygen dissociation curve of myoglobin provides evidence that a marked pO_2 gradient exists between the capillary and muscle cells containing this pigment. Myoglobin, in marked contrast to hemoglobin, is 50% saturated with oxygen at pO_2 of only 3 mm. Hg. (42). Thus, in order for this pigment to function effectively the

pO_2 in muscle cells must be at least this low (43). The average capillary pO_2 found in normal children at rest in this investigation was 60 mm. Hg. Assuming an intracellular pO_2 of 3 mm. Hg. the capillary-cell pO_2 difference in these children averaged 57 mm. Hg., or about 25% more than the maximum amount Krogh calculated to be necessary to maintain a normal rate of oxygen consumption. In the children with cyanotic congenital heart disease the average capillary pO_2 was found to be 37 mm. Hg., so that their capillary-cell pO_2 difference was 34 mm. Hg., or 25% less than the maximum amount Krogh calculated to be necessary to maintain a normal rate of oxygen consumption, other factors being constant. On the basis of these calculations, therefore, the supply of oxygen could be a limiting factor in the rate of oxygen consumption in these children. That such a mechanism actually exists has not been demonstrated in vivo, but the fact that individuals with cyanotic congenital heart disease under conditions of severe chronic anoxia do have a low metabolic rate strongly suggests its presence.

SUMMARY AND CONCLUSIONS

1. Blood lactate and pyruvate concentrations and lactate-pyruvate ratio values were established for normal adults and normal children. The resting values for lactate, pyruvate and the lactate-pyruvate ratio are significantly lower in adults than in children.

2. Children with acyanotic congenital heart disease have a normal lactate-pyruvate ratio at rest. Children with cyanotic congenital heart disease have a normal resting lactate-pyruvate ratio in spite of a markedly lowered mean capillary oxygen tension. The possibility is presented that the normal lactate-pyruvate ratio in these children is related to their low metabolic rate.

3. The lactate-pyruvate ratio varies inversely with mean capillary oxygen tension at rest and after exercise in normal children and children with patent ductus arteriosus.

4. Children with patent ductus arteriosus have a significantly higher oxygen saturation of venous blood at rest than normal children. The existence of a vasomotor mechanism, hitherto undescribed, whereby systemic blood flow is maintained by peripheral vasodilatation in the presence of a 'leak' in the aorta is postulated.

5. Children with acyanotic congenital heart disease tolerate exercise as well as normal children, while children with cyanotic congenital heart disease tolerate exercise less well than normal children as measured by blood lactate and pyruvate, the lactate-pyruvate ratio and the blood gases.

6. The lactic dehydrogenase content of red blood cells is unchanged in individuals with cyanotic congenital heart disease as compared with normals.

7. In rats made polycythemic by exposure to low barometric pressure there is no significant change in the succinic dehydrogenase and cytochrome c contents of liver, in the easy hydrolyzable phosphorus fractions and total pentose concentration of heart, liver and skeletal muscle, or in the total creatinine content of skeletal muscle.

8. In view of the above findings it is strongly believed that the principal compensative mechanism operating in the tissues of children with cyanotic congenital heart disease is a reduced metabolic rate resulting directly from inadequate tissue oxygenation.

BIBLIOGRAPHY

1. Cournand, A., Baldwin, J. and Himmelstein, A. Cardiac Catheterization in Congenital Heart Disease, Commonwealth Fund, New York, 1948.

2. Haldane, J. S. Respiration, Yale University Press, New Haven, 1922.

3. Green, D. E., Needham, D. M. and Dewan, J. G. Dismutations and oxidoreductions, Biochem. J., vol. 31, pp. 2327-2352, 1937.

4. Stotz, E. and Bessey, O. A. The blood lactic-pyruvic relation and its use in experimental thiamine deficiency in pigeons, J. Biol. Chem., vol. 143, pp. 625-631, 1942.

5. Peters, R. A. and Thompson, R. H. S. Pyruvic acid as an intermediary metabolite in the brain tissue of avitaminous and normal pigeons, Biochem. J., vol. 28, pp. 916-925, 1934.

6. Friedemann, T. E. and Barborka, C. J. The significance of the ratio of lactic to pyruvic acid in the blood after exercise, J. Biol. Chem., vol. 141, pp. 993-994, 1941.

7. Tepperman, J. and Tepperman, H. M. On the blood lactic acid response to measured exercise in hypoxic human subjects, J. Clin. Invest., vol. 27, pp. 176-187, 1948.

8. Goldsmith, G. A. The blood lactate-pyruvate relationship in various physiologic and pathologic states, Am. J. Med. Sci., vol. 215, pp. 182-188, 1948.

9. Bueding, E. and Goodhart, R. Removal of pyruvic acid from human blood in vitro, J. Biol. Chem., vol. 141, pp. 931-935, 1941.

10. Friedemann, T. E. and Haugen, G. E. Pyruvic acid. II. The determination of keto acids in blood and urine, J. Biol. Chem., vol. 147, pp. 415-442, 1943.

11. Long, C. The stabilization and estimation of lactic acid in blood samples, Biochem. J., vol. 40, pp. 27-33, 1946.

12. Hillig, F. The colorimetric determination of lactic acid in milk and milk products, J. Assoc. Offic. Agr. Chemists, vol. 20, pp. 130-140, 1937.

13. Friedemann, T. E. and Haugen, G. E. Pyruvic acid. I. Collection of blood for the determination of pyruvic and lactic acids, J. Biol. Chem., vol. 144, pp. 67-77, 1942.

14. Robertson, W. B. The preparation of sodium pyruvate, Science, vol. 96, pp. 93-94, 1942.

15. Van Slyke, D. D. and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement, J. Biol. Chem., vol. 61, pp. 523-573, 1924.

16. Quastel, J. H. and Wheatley, A. H. M. On ferricyanide as a reagent for the manometric investigation of dehydrogenase systems, Biochem. J., vol. 32, pp. 936-943, 1938.

17. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. Manometric Technics and Related Methods for the Study of Tissue Metabolism, Burgess Publishing Co., Minneapolis, 1945.

18. Schales, O. Rapid and accurate calibration of Warburg manometers, Arch. Biochem., vol. 3, pp. 475-476, 1944.

19. Green, D. E. and Brosteaux, J. The lactic acid dehydrogenase system of animal tissues, Biochem. J., vol. 30, pp. 1489-1508, 1936.

20. Dunn, H. L. Application of statistical methods in physiology, Physiol. Reviews, vol. 9, pp. 275-398, 1929.

21. Cournand, A. and Richards, D. W., Jr. Pulmonary insufficiency, Am. Rev. Tuberc., vol. 44, pp. 26-41, 1941.

22. Lundsgaard, C. and Van Slyke, D. D. Cyanosis, Medicine, vol. 2, pp. 1-76, 1923.

23. Peters, J. P. and Van Slyke, D. D. Quantitative Clinical Chemistry, Vol. I., Interpretations, Williams and Wilkins Co., Baltimore, 1931.

24. Boothby, W. M. and Sandiford, I. A comparison of the DuBois and the Harris and Benedict normal standards for the estimation of the basal metabolic rate, J. Biol. Chem., vol. 54, pp. 767-781, 1922.

25. Bing, R. J. Physiological studies in congenital heart disease. II. Results of preoperative studies in patients with tetralogy of Fallot, Bull. Johns Hopkins Hosp., vol. 80, pp. 121-141, 1947.

26. Finch, C. A. Methemoglobinemia and sulfhemoglobinemia, New Engl. J. Med., vol. 239, pp. 470-476, 1948.

27. Wendel, W. B. Oxidations by erythrocytes and catalytic influence of methylene blue. I. Oxidation of lactate to pyruvate, J. Biol. Chem., vol. 102, pp. 373-383, 1933.

28. Gutman, H. R., Jandorf, B. J. and Bodansky, O. Role of pyridine nucleotides in reduction of methemoglobin, J. Biol. Chem., vol. 169, pp. 145-152, 1947.

29. Drabkin, D. L. Maintenance of active hemoglobin--function of erythrocytes, Federation Proc., vol. 5, p. 132, 1946.

30. Evelyn, K. A. and Malloy, H. T. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood, J. Biol. Chem., vol. 126, pp. 655-662, 1938.

31. Allen, R. J. L. The estimation of phosphorus, Biochem. J., vol. 34, pp. 858-865, 1940.

32. Mejbaum, W. Über die bestimmung kleiner pentosemengen, insbesondere in derivaten der adenylsaure, Z. Physiol. Chem., vol. 258, pp. 117-120, 1939.

33. Prader, A. and Gonella, A. Die spectrophotometrische bestimmung von cytochrome c im gewebe, Experientia, vol. 3, pp. 462-464, 1947.

34. Keilin, D. and Hartree, E. F. Purification and properties of cytochrome c, Biochem. J., vol. 39, pp. 289-292, 1945.

35. Barclay, J. A. and Kenney, R. A. A method for the estimation of creatinine, Biochem. J., vol. 41, pp. 586-589, 1947.

36. Drabkin, D. L. Liver regeneration and cytochrome c metabolism. Influence of anoxia and of injection of cytochrome c, J. Biol. Chem., vol. 171, pp. 409-418, 1947.

37. Bowen, W. Hypoxia of 18,000 feet simulated altitude and myoglobin content of dogs, Federation Proc., vol. 8, p. 14, 1949.

38. Poel, W. Effect of anoxic anoxia on myoglobin concentration in striated muscle, Am. J. Physiol., vol. 156, pp. 44-51, 1949.

39. Warburg, O. and Kubowitz, F. Atmung bei sehr sauerstoffdrucken, Biochem. Z., vol. 214, pp. 5-18, 1929.

40. Kempner, W. The role of oxygen tension in biological oxidations, Cold Spring Harbor Symposia Quant. Biol., vol. 7, pp. 269-289, 1939.

41. Krogh, A. The Anatomy and Physiology of Capillaries, Yale University Press, New Haven, 1929.

42. Theorell, H. Krystallinisches myoglobin. V. Metteilung: die sauerstoffbindungskurve des myoglobins, Biochem. Z., vol. 268, pp. 73-82, 1934.

43. Höber, R. Physical Chemistry of Cells and Tissues, The Blakiston Company, Philadelphia, 1945.

This work was done during tenure of a Life Insurance Medical Research Student Fellowship. The work was supported by a grant from the Oregon Heart Association.

The author is indebted to Dr. Elton Watkins for drawing the blood samples from children and for valuable advice; to Miss Shirley Gullixson for performing many of the pyruvate and blood gas analyses; to Dr. E. S. West for numerous suggestions and criticisms, and for the use of Figures 1 and 2; and to other members of the Department of Biochemistry.

Nancy E. Moores
(Typist)