I.   THE ORGANIC ACID-SOLUBLE PHOSPHORUS
            COMPOUNDS OF BLOOD

I I.   THE RATIO OF THE EASILY HYDROLYZABLE
         PHOSPHORUS TO THE NUCLEOTIDE PHOSPHO-
         RUS IN RED, WHITE, AND HEART MUSCLE.

by

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I. THE ORGANIC ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF BLOOD.

CONTENTS

HISTORICAL

The organic acid soluble phosphorus compounds of
blood are found almost completely in the cells. The presence
of some organic phosphorus in the plasma, usually less than
half a milligram per hundred cc., has been demonstrated by
different workers(5,19,20,27,36).

The only organic acid-soluble phosphorus compounds
which have been proven to exist in blood are diphospho - 1 -
glyceric acid and adenylic acid. In addition a compound or
a group of compounds quickly hydrolyzed by hot, normal acid,
and designated "pyro"phosphate was shown to exist in blood
by Lohmann. (33)

Diphospho - 1 - glyceric acid: This compound was
first isolated from pig blood by Greenwald (21) and from
horse blood by Jost (24). It forms the largest fraction of
the organic acid soluble phosphorus of blood.

Adenylic acid: Jackson (22) isolated from human blood
an impure nucleotide which he believed to be a mixture of
adenine and uracil nucleotides. A year later Hoffman(23)
isolated adenine nucleotide in crystalline form from pig blood.
Buell and Perkins (7) developed a micronephelometric method
for the determination of the total nucleotides in blood and
applied it to the blood of different species. In 1929 Moz-
olowski (38) determined the nucleotide content of the blood
of pig, rabbit, man, horse, and ox. The values obtained by

both Buell and Perkins and by Mozolowski are low in comparison to those we obtained using the method of Kerr and Bliah (29) due to the fact that these workers lost some nucleotide during the precipitation of proteins.

Among the phosphorus compounds whose presence or identity in blood have not yet been definitely established are the so-called "pyrophosphate", phosphocreatine, hexosephosphate, mono-phospho glyceric acid, and α keto-trihydroxy-adipic diphosphate.

Pyrophosphate: In 1928, Lohmann (32,33) discovered that fresh muscle contains a phosphorus compound which hydrolyzes during seven minutes at $100^{\circ}C$. in normal hydrochloric acid and which he isolated as pyrophosphate. This compound he found also in the blood of the ox and the pig though his values are too low for the pig blood and too high for the ox as compared with our values.

Later Barrenscheen and Braun (2) attempted to isolate pyrophosphate from blood and obtained a barium salt which, judging from their data for its elementary analysis, may have been an impure salt of adenosine triphosphate.

Fiske (15) obtained from protein-free muscle filtrate a new substance, adenosine triphosphate, containing adenosine, a carbohydrate, and three molecules of phosphoric acid. Two of the three phosphoric acid molecules were easily removed by hydrolysis and these he believed accounted for the easily hydrolyzable phosphorus of Lohmann.

The presence of adenosine triphosphate in muscle as
well as the presence in blood of both adenylic acid and the
easily hydrolyzable phosphorus suggested to us the hypothesis
that adenosine triphosphate might be present in blood. We there-
fore attempted to prove or disprove this hypothesis by a study
of the ratio of the nucleotide phosphorus concentration to that
of the fraction of phosphorus hydrolyzable during seven minutes
in normal hydrochloric acid at 100°C. in the blood of different
species. These determinations were made possible because of
the availability of the simple and reliable method of Kerr
and Blish (29) for nucleotide determinations.

<u>Phosphocreatine</u>. This compound though found in muscle
has been reported absent from the blood of rabbit, dog, cat,
and man (9). On the other hand, Gereb and Laszlo (16) believe
that phosphocreatine exists in blood because they found a
difference between the inorganic phosphate determined by
applying Lohmann's colorimetric method (inorganic plus phos-
phocreatine) to the trichloroacetic acid filtrate and the
value obtained on examining the precipitate thrown down by
magnesia mixture (true inorganic phosphate). Our experiments,
described later, seem to settle this question in favor of the
absence of phosphocreatine from blood.

<u>Hexosephosphate</u>: The interest in the possible presence
of hexosephosphates in blood was aroused by the discovery of
"lactacidogen" or hexosemonophosphate in muscle by Embden and
Zimmermann (11). Its presence in blood has been taken for

granted by some workers who are quoted by Van Slyke and Peters(39).

In 1924, Goodwin and Robison (17) reported the isolation of two barium salts of phosphoric esters from blood. One was practically insoluble in water, only slightly soluble in dilute acids but soluble in warm hydrochloric acid. This salt did not reduce Fehling's solution and was not hydrolyzed by the bone enzyme of Kay and Robison (41). The other salt rotated the plane of polarized light to the left, was readily soluble in water and reduced Fehling's solution. Its reducing power as glucose was similar to that of a hexosemonophosphate but it had only one third the required phosphorus content. It is this preliminary report that is often quoted to prove the presence of hexosephosphates in blood. Robison himself later stated that the above reducing power was probably due to contamination with some other blood compound.*

Roche and Roche (44) postulated the presence of a hexosephosphate ester in blood as an intermediate in the glucolysis of sugar but offered no evidence of its presence. Lawaczeck (31) attempted to determine hexosephosphates in the blood of normal and diabetic individuals by measuring the reducing power of the precipitate obtained on addition of barium hydroxide(added to separate hexosephosphate from free sugar). He does not give the details of the procedure but as the barium salt of hexosephosphate is soluble in water, only a portion of the hexosephosphate could have been precipitated and that only by adsorption or by formation of double salts. Hence his results can not be accepted.

* Personal communication to Dr. Kerr.

Our experiments on hexosephosphates are described later.

In spite of the doubt as to the presence of hexose-phosphate in blood, there are experimental findings in support of the hypothesis that glucolysis and esterfication of glucose with phosphorus go on simultaneously and that the presence of phosphates is a prerequisite for glucolysis. These facts are the following:-

(1) In normal blood the sugar disappears at a fairly constant rate after withdrawal from the body, the concentration of inorganic phosphates either remaining constant or diminishing slightly during glucolysis but increasing rapidly at the end of the glucolytic process (18).

(2) The addition of glucose to blood maintains a vigorous glucolysis accompanied by a decrease in the inorganic phosphates (24).

(3) Glucolysis in erythrocytes is accompanied by the formation of new phosphorus compounds (13).

(4) Sugars which do not promote the disappearance of inorganic phosphates are not glucolysed (46).

(5) In glucolysis, the addition of sugar diminishes the cleavage of the phosphoric esters (46).

(6) The reaction optima of glucolysis and esterfication both lie in the same region of slight alkalinity (24).

(7) In general the glucolytic ability of erythrocytes of different species corresponds to the extent of phosphoric acid cleavage on autolysis, pig blood excepted (13).

(8) The rate of glucose disappearance and lactic acid production in defibrinated blood is increased if inorganic

phosphate is added(1).

(9) All factors which prevent the disappearance of in-
organic phosphates such as acidity, hemolysis, and the presence
of fluorides are also unfavorable for glucolysis (46).

(10) The factors which promote the hydrolysis of or-
ganic phosphates are the same as those which inhibit glucolysis
(hemolysis, ether, and chloroform).(2)

The above facts do not necessarily mean that the reactions
between sugar and phosphates result in a simple hexosephosphate
compound.

Methods used to fractionate the organic phosphorus
compounds of blood; Various methods have been evolved for the
fractional determination of the acid soluble phosphorus compounds
of blood. Kay and Robison (41) separated the organic compounds
into two groups, one hydrolyzable by a bone enzyme* and the
other non-hydrolyzable.

The following is an example of the results they ob-
tained working with rabbit blood.

Kind	Mg. P per 100 gms.	% of organic
T. A. S. P.	32	
Inorg. P	5.1	
Ester P	26.9	
Hydrol. by bone enzyme	8.5	32
Non " " " "	18.4	68

* This bone enzyme was discovered in ossifying cartilage and
studied by Robison and his co-workers (17,25,35,36,37,40,41,
42,43) and has been the basis for Robison's theory of bone formation.

Kay and Robison's division was recently criticised by King (30) who found that both fractions of blood organic esters are almost completely hydrolysed by the bone enzyme, at or near neutrality during two days, at 37.5°C. Bodansky and Bakwin (5A) showed that diphospho - 1 - glyceric acid is not hydrolysed by bone enzyme at pH 9.0 during 300 hours, under the conditions used by Kay and Robison.

Another method used is that of fractional acid hydrolysis which consists in determining the amount of organic phosphate hydrolysed in acid solution for different periods of time. Rona and Iwasaki (45) determined the inorganic phosphate, the fraction hydrolysed during two hours in half normal hydrochloric acid at 100°C., and the total acid soluble phosphate. Bomskov (6) determined the inorganic phosphate, the fraction hydrolysed during seven minutes in normal hydrochloric acid at 100°C., that hydrolysed during three hours and the total acid soluble phosphorus and interpreted his results as follows:-

He assumed that phosphocreatine was present in blood and that his inorganic phosphorus values included the phosphocreatine. The difference between the seven-minute hydrolysis value and the inorganic phosphorus he assumed to be pyrophosphate, that between three hours and seven-minute hydrolysis, the hexosephosphate, and that between the total acid soluble phosphorus and the three hours hydrolysis value as diphospho - 1 - glyceric acid.

Although methods of fractionation either by acid hydrolysis or by enzymes help to increase our knowledge of the phosphorus compounds of blood, such definite naming of the fractions hydrolyzable during different periods can hardly be accepted when the <u>presence</u> of some of these compounds (hexosephosphate for example) has not yet been proved and when the presence of adenylic acid is ignored, as in the case of Bomskov's work.

EXPERIMENTAL

On the Presence of Phosphocreatine in Blood

Three experiments were made on dog blood to determine the presence or absence of phosphocreatine. The blood was taken by heart puncture directly into ice-cold 10% trichloroacetic acid and the amount of added blood determined by weighing. The precipitated proteins were quickly removed by centrifugation and filtration. The filtrate was received in a flask containing sodium hydroxide, thus neutralizing the acid rapidly. To the neutralized filtrate one-fourth volume of 10% calcium chloride

saturated with calcium hydroxide was added to precipitate the
inorganic phosphate, and after 10-30 minutes the mixture was
centrifuged. The supernatant liquid was tested for phospho-
creatine by adding acid molybdate (Fisk's Molybdate I) and
allowing to stand half an hour for the hydrolysis of phospho-
creatine. On addition of the reducer (aminonaphthol sulphonic
acid) no blue color developed, indicating complete absence of
phosphocreatine.

To check the presence of traces of the color undetected
by sight the experiment was repeated as follows. Neutralized
filtrate equivalent to 7 cc. of blood was allowed to stand
thirty minutes after Molybdate I was added, then a known quan-
tity of a standard phosphate solution was added, the color
developed by the reducer, then compared in the colorimeter
against two standards, one containing the same amount of acid,
base, and calcium chloride solution as the unknown, and the
other the usual standard. The colorimetric readings were
exactly the same for the two standards and also for the un-
known, showing that no trace of phosphocreatine was present in
the blood.

Adenosine Triphosphate

As already stated earlier in this paper, the presence
in blood of the so-called "pyrophosphate" and of adenylic acid
suggested that these might be present combined with each other
in the form of adenosine triphosphate. To test this hypothesis
we proposed to determine the ratio of the nucleotide P to the
easily hydrolyzable phosphorus.

Methods:

Blood was drawn from the animals by vein puncture, heart puncture, or decapitation. It was immediately defibrinated with a glass rod or with glass beads. The clotting time of fowl blood was so short that sometimes a clot started to form before the blood was completely drawn; hence in such cases the blood was filtered through glass-wall after defibrination. A few cc. of the well mixed defibrinated blood were kept aside for hematocrit determinations.

A known amount of blood, usually 30 cc. was then pipetted into four volumes of 10% trichloroacetic acid. This precipitation was always done immediately after defibrination even though this required carrying the measured trichloroacetic acid in stoppered flasks to the slaughter house or to the animal.

The hematocrits were done in quadruplicate in special 10 cm. graduated capillary tubes and centrifuged for twenty minutes at the highest speed obtainable with the centrifuge (International centrifuge SB1)

Inorganic and total acid soluble phosphorus were determined by the Fiske and Subbarow method (14). For the determination of the easily hydrolyzable phosphorus, 5 cc. of blood filtrate were pipetted into a test tube graduated at 25 cc. One cc. of six normal hydrochloric acid was added, the tubes covered with tin foil and immersed in boiling water for exactly seven minutes. They were then cooled by immersion in cold water and diluted. After addition of 2.5 cc. of Molybdate II and one cc. of the reducer, the tubes were

filled to the mark with distilled water, mixed thoroughly, and compared with the standard in a colorimeter. The standard was prepared with the same percent of acids and reagents as the unknown.

Each of these determinations was done in duplicate. The inorganic phosphorus and the easily hydrolyzable phosphorus were determined as soon as possible, usually within an hour after the blood filtrate reached the laboratory.

The nucleotide nitrogen was determined by the method of Kerr and Blish (29) in triplicate. 25 cc. of filtrate, equivalent to 5 cc. of blood, were usually used for each determination. A blank determination of nitrogen in the reagents was made every few days.

Results: The results obtained are presented in Table I. The nucleotide phosphorus was calculated from the nucleotide nitrogen by assuming an atom of phosphorus for five atoms of nitrogen since it was shown by Mozolowski (38) that all the nucleotide purine in freshly drawn blood is adenine. The hydrolyzable organic phosphorus is represented by the difference between the inorganic phosphorus before and after the seven-minutes hydrolysis. The corpuscle content was calculated from the value for whole blood and the hematocrit ignoring the traces of organic phosphorus in the plasma. The total organic phosphorus represents the difference between the total acid soluble and the inorganic phosphorus.

It is clear from the tabulated data that the nucleo-

TABLE I

The phosphorus partition in the whole blood and corpuscles of different species
and the ratio of the nucleotide to hydrolysable organic phosphorus.

SPECIES		mg P per 100cc whole blood					Ratio of Nucleotide P to Hydrol.	Hematocrit	mg P per 100cc corpuscles			
		T.A.S.P.	Inorg.P	Nucleotide P	Hydrolys.org.P	Undeter P			Total org.P	Nucleotide P	Hydr org.P	Undeter P
Male	1.	13.92	2.51	0.51	0.89	10.01	1:1.75	30.8	37.0	1.76	2.9	32.4
	2.	12.93	2.02	0.51	1.06	9.54	1:2.08	20.2	54.0	2.50	5.2	46.3
	Ave.	13.42	2.26	0.51	0.97	9.67	1:1.91		45.5	2.13	4.0	39.3
Horse	1.	24.60	2.35	0.62	1.37	20.26	1:2.21	48.8	45.6	1.3	2.8	41.5
	2.	18.04	2.10	0.55	1.08	14.31	1:1.97	33.2	48.0	1.7	3.8	43.0
	Ave.	21.32	2.22	0.58	1.22	17.28	1:2.09		46.8	1.5	3.3	42.2
Ox	1.	9.36	5.53	0.99	1.75	1.09	1:1.77					
	2.	10.00	5.58	0.92	1.92	1.28	1:1.94					
	3.	7.39	3.85	0.90	1.65	0.99	1:1.83					
	4.	9.00	6.30	0.83	1.05	1.02	1:1.67					
	5.	6.61	3.32	0.77	1.16	1.36	1:1.51	42.0	7.8	1.8	2.8	3.2
	6.	10.05	5.18	0.83	1.62	2.40	1:1.96	37.0	13.2	2.2	4.4	6.5
	7.	8.54	5.40	0.57	1.38	1.20	1:2.42	31.2	10.1	1.8	4.4	3.9
	8.	7.77	4.31	0.61	1.48	1.39	1:2.39	39.4	8.7	1.5	3.7	3.5
	Ave.	8.59	4.97	0.78	1.50	1.34	1:1.94		9.4	1.8	3.8	4.5
Buffalo	1.	8.00	4.32	0.52	1.43	1.73	1:2.66					
	2.	7.75	4.67	0.48	1.28	1.32	1:2.66					
	3.	6.97	4.06	0.59	1.11	1.21	1:1.89					
	Ave.	7.57	4.35	0.53	1.27	1.42	1:2.43					
Goat	1.	7.92	4.89	0.70	1.48	0.91	1:2.04					
	2.	8.16	4.20	0.82	1.50	1.64	1:1.83					
	3.	9.38	5.80	0.79	1.18	1.61	1:1.49	25.5	14.0	3.1	4.6	6.3
	4.	7.27	4.10	0.94	1.85	0.78	1:1.54	28.9	10.9	5.0	3.2	2.7
	5.	11.96	8.40	0.94	1.65	0.97	1:1.74	27.6	12.6	6.0	3.2	3.6
	Ave.	8.94	5.48	0.84	1.52	1.18	1:1.73		12.6	4.7	3.7	4.2
Cat	1.	12.44	6.53	0.78	1.53	3.60	1:1.98	26.0	22.7	3.0	5.9	13.8
	2.	11.31	6.86	0.67	1.69	2.09	1:2.50	22.9	19.4	2.9	7.4	9.1
	3.	12.46	5.80	1.02	2.04	4.20	1:2.00	36.4	19.9	2.8	5.6	11.5
	Ave.	12.07	6.20	0.82	1.75	3.30	1:2.16		20.7	2.9	6.3	11.5
Sheep	1.	9.78	6.00	1.21	2.13	1.65	1:1.77					
	2.	9.46	5.03	1.12	1.87	1.44	1:1.68	32.6	13.6	3.4	5.1	5.1
	3.	9.24	5.06	1.18	1.85	1.15	1:1.57	29.5	14.5	4.0	6.2	4.3
	Ave.	9.49	5.36	1.17	1.95	1.41	1:1.67		14.0	3.7	5.6	4.7

| SPECIES | | mg P per 100cc whole blood | | | | | Ratio of Nucleotide P to Hydrol. | Hematocrit. | mg P per 100cc corpuscles. | | | |
		T.A.S.P.	Inorg.P	Nucleotide P	Hydrolys. org. P	Undetex P			Total org. P	Nucleotide P	Hydrorg. P	Undeter. P
Camel	1.	19.85	5.46	1.08	1.85	11.66	1:1.54	23.2	68.0	4.7	7.1	50.2
	2.	21.42	4.09	1.24	1.84	14.25	1:1.49	24.5	70.7	5.1	7.5	58.1
	3.	17.90	4.86	1.32	1.96	9.76	1:1.54	24.1	54.1	5.4	8.1	40.5
	4.	20.20	4.94	1.67	2.86	10.73	1:1.71	22.5	67.8	7.4	12.7	47.7
	5.	22.80	4.68	1.67	3.12	13.33	1:1.90	26.0	69.7	6.4	12.0	51.3
	Ave.	20.43	4.81	1.40	2.29	11.95	1:1.64		64.9	5.8	9.5	50.0
Dog	1.	24.13	4.28	1.27	2.24	16.34	1:1.76					
	2.	24.84	4.92	1.28	2.40	16.24	1:1.69					
	3.	24.65	4.50	1.21	2.09	16.05	1:1.76					
	4.	20.43	3.85	0.87	1.55	14.16	1:1.79					
	5.	28.67	4.45	1.67	3.05	19.50	1:1.82	42.3	57.2	3.9	7.2	46.1
	6.	16.84	2.34	0.91	1.82	11.77	1:2.00	26.0	55.7	3.5	7.0	45.2
	Ave.	23.29	4.06	1.20	2.19	15.68	1:1.85		56.5	3.7	7.1	45.6
Guinea Pig		26.31	3.54	1.56	2.90	19.87	1:1.87					
Man	1.	20.76	4.02	2.24	3.75	10.75	1:1.68					
	2.	25.52	3.67	2.81	5.37	13.67	1:1.94					
	3.	25.84	3.24	2.75	5.39	14.46	1:1.98					
	4.	21.28	3.75	2.18	3.41	11.94	1:1.57	35.7	49.1	6.1	9.6	33.4
	5.	24.08	2.90	2.83	5.76	12.59	1:2.04	45.0	47.1	6.0	12.8	28.3
	Ave.	23.50	3.52	2.56	4.74	12.68	1:1.84		48.5	6.0	11.2	30.8
Rabbit	1.	37.00	3.68	3.76	6.63	23.35	1:1.66					
	2.	49.60	5.45	4.15	7.22	32.78	1:1.71					
	3.	37.85	4.19	3.06	5.48	24.52	1:1.60	42.0	84.3	7.7	15.1	61.5
	4.	39.06	4.65	3.25	6.34	24.82	1:1.96	41.3	93.7	7.6	16.8	69.3
	5.	43.38	4.65	3.16	6.97	28.60	1:2.17					
	Ave.	41.26	4.51	3.48	6.53	26.81	1:1.86		89.0	7.6	16.0	65.4
Pig	1.	43.19	6.08	4.01	7.84	25.26	1:1.96					
	2.	40.40	4.93	3.41	7.49	24.57	1:2.17					
	3.	52.49	6.91	4.82	8.47	37.11	1:1.76	41.4	110	11.6	20.5	77.9
	4.	40.73	4.58	4.42	9.20	22.53	1:2.10	37.9	95.4	14.0	24.0	57.4
	5.	37.95	4.27	3.71	7.67	22.30	1:2.08	36.0	93.5	10.3	21.3	61.9
	Ave.	42.95	5.35	4.07	8.13	26.35	1:2.01		99.6	12.0	21.9	65.7
Chicken	1.	25.50	3.24	1.60	2.59	17.64	1:1.62	22.0	101.2	7.3	11.8	82.1
	2.	39.40	2.81	1.62	3.85	29.79	1:2.38	36.2	95.8	4.2	10.1	81.5
	Ave.	32.45	3.02	1.61	3.22	23.66	1:2.00		98.5	5.7	11.0	81.8

SPECIES		mg P per 100cc whole blood					Ratio of Nucleotide P to Hydrol.	Hema-to-crit.	mg P per 100cc corpuscles.			
		T.A.S.P.	Inorg.P	Nucle-otide P	Hydr-olys. org. P	Un-doter P			Tot-al org. P	Nucl-eo-tide P	Hydr-org. P	Un-de-ter. P
Turkey	1.	38.77	3.32	3.68	5.95	25.82	1:1.63	37.2	95.5	9.9	16.0	69.4
	2.	43.15	3.41	4.38	6.98	28.56	1:1.61	43.3	91.8	10.2	16.1	65.5
	Ave.	40.96	3.36	4.03	6.46	27.10	1:1.62		93.5	10.0	16.1	67.5
Duck	1.	45.48	6.09	4.65	8.19	26.35	1:1.68	36.2	106.8	13.4	23.6	72.8
	2.	44.10	7.25	4.82	7.81	24.22	1:1.60	32.8	112	14.7	23.8	73.9
	Ave.	44.79	6.67	4.63	8.00	25.28	1:1.64		110	14.0	23.2	73.5
Goose	1.	52.08	5.06	5.18	8.88	32.96	1:1.71	44.5	103	11.7	19.9	71.6
	2.	49.81	5.41	5.23	9.23	29.84	1:1.74	40.0	111	13.2	23.1	74.7
	Ave.	50.99	5.23	5.21	9.06	31.40	1:1.73		107.1	12.4	21.5	73.1

tide phosphorus rises and falls with the easily hydrolyzable
phosphorus whether in the different species or in animals of
the same species. The theoretical ratio would be 1:2 if all
the nucleotide present in blood were present as adenosinetri-
phosphate. Our figures approach the theoretical values fairly
well considering all possible sources of error in the deter-
minations.

In most cases the hydrolyzable phosphorus is less than
theoretical. This could be caused by (a) incomplete hydrolysis
during the seven minutes, (b) hydrolysis in the acid filtrate
before the phosphorus determinations, (c) autolytic breakdown
of the triphosphate during defibrination, and (d), the presence
of adenosine polyphosphates other than the triphosphate (e.g.
adenosine diphosphate or a compound of the type of the "Herz
nucleotide", (8A) which has a N:P ratio of 10:5.

Fiske states that with pure solutions of adenosine
triphosphate exactly two-thirds of the phosphorus is hydrolyzed
in 15 minutes and not seven.(Personal communication to Dr. Kerr)

Hydrolysis curves performed on blood filtrates show
only slight differences between the seven and fifteen minute
values. Table II shows the effect of the time of hydrolysis
on the values of the easily hydrolyzable phosphorus of the
same blood. Moreover other compounds in the blood are being

Table II.

Species	mg. P per 100 cc. Blood	
	7 min.	15 min.
Cow 1	1.58	1.48
" 2	1.46	1.57
Camel 1	1.96	2.48
" 2	2.86	3.01
Chicken 1	2.69	2.92
" 2	3.85	4.04

hydrolyzed to a slight extent at the same time, hence the 15-minute value would be somewhat too high, and the 7-minute value a little low.

Kay (26) studied the hydrolysis in the trichloroacetic acid filtrate and showed that a slight hydrolysis does take place in the first day. In our experiments the determinations were made as quickly as possible after the precipitation of the proteins but in some cases the blood filtrate had to be carried a long distance in warm weather. In one experiment with donkey blood the acid filtrate was kept overnight and gave a ratio of 1:1.23 showing that a great part of the easily hydrolyzable phosphorus had been lost.

The experimental work included in this paper was already completed when the work of Barrenscheen and Fils (5) came to our attention. These authors isolated adenosine triphosphate from human blood, although their yield was very low.

Keeping the above considerations in mind, our study of the ratio of nucleotide phosphorus to the easily hydrolyzable phosphorus leads us to conclude that the adenylic acid of blood is linked to two easily hydrolyzable phosphorus groups in the form of adenosine triphosphate.

Bone-enzyme Hydrolyzable Phosphorus. Below in Table III a comparison is made between the organic phosphorus in the corpuscles of different species hydrolyzed by the bone and kidney phosphatases according to Kay (24A, 24B) and our values for the sum of the nucleotide plus the easily hydrolyzable phos-

phorus for the same species. The agreement between the two values suggests that adenosine triphosphate may have been the substrate for the bone and kidney phosphotases in Kay's *in vitro* experiments.

Table III

| Species | mg. P per 100 cc. corpuscles | | |
	Nucleotide plus Hydrol. P	Bone-enzyme Hydrol.P	Kidney-enzyme Hydrol.P
Pig	33.6		26
Rabbit	23.7	26.4	24
Man	18.3	14.9	16
Dog	10.9		10
Horse	4.5		7
Cat	9.4		12.2
Sheep	9.9	8.2	9.2
Ox	5.4	5.9	6.4
Goat	8.9	8.1	8.5

<u>Organic Phosphorus and Glycolysis</u>. Our results shed some light
on the problem of glycolysis in blood. Barrenscheen and
Vasarhelyi (4), from a study of the hydrolyzable phosphorus
content and the glycolysis of the blood of a few species suggested
that there seemed to be a relationship between the glycolytic
power and the "pyrophosphate" content. In a study of the fac-
tors which inhibit glycolysis, Barrenscheen and Braun (2) came
to the conclusion that the "pyrophosphate fraction" is essential
for glycolysis. Engelhardt and Ljubimowa (13) studied the
relation of glycolytic ability to the amount of autolysis of
the phosphoric esters. According to them the extent of auto-
lysis of organic phosphorus varies in different species as follows:-

   cow   goat   horse   dog, pig   man, guinea pig   rabbit

while the order of glycolytic power was:-

   ox   goat   dog   horse   guinea pig   man   rabbit.

     Barrenscheen and Vasarhelyi found the pyrophosphate
content to vary from one species to the other as follows:-
   horse   ox   dog   rabbit   guinea pig   man   pig.

     Our figures for the sum of nucleotide and hydrolyzable
phosphorus content for the same species varied as follows:-
   horse   ox   goat   dog   guinea pig   man   rabbit   pig.

     It is evident that our results correspond with those of
Englehardt and Ljubimowa for glycolysis with the exception of
the horse, which is only an apparent exception as can be seen
by a study of the individual experiments of Englehardt rather
than from his averages. The following figures quoted from the

paper of these authors shows the decrease in the sugar content
of the blood cells of the ox, goat, and horse during four hours.

	Ox	Goat	Horse
	0	75	73
	45	90	74
	19	98	149
	51		99
Average	38	88	99

The decrease in the sugar content of horse blood is
less than in ox blood if we neglect "149" which varies so
far from the mean of the other three figures. This places the
horse between the goat and ox in the order of glycolytic power
and makes the series correspond closely to our results.

As for the relative position of ox and horse an
examination of our values for the nucleotide P content of the
horse (and mule) with the ox shows that individual variations
in the two groups fluctuate above and below both averages
showing that horse and ox are very nearly related with respect
to their blood adenosine triphosphate.

Species Differences.   Table I shows that the blood of
different species vary greatly in their phosphorus content.
The different phosphorus fractions do not vary in amount
directly with the total acid soluble phosphorus. For example,
the inorganic phosphorus and the nucleotide phosphorus are
higher in ox blood than in horse or mule blood, yet the total
acid soluble phosphorus is higher in the latter than in the
former.

The undetermined phosphorus, which contains the diphosphoglyceric acid and possibly other unidentified compounds varies exceedingly in the corpuscles of different species, ox, sheep and goat, having only about three to six milligrams per hundred cc. of corpuscles while rabbit, pig, and fowl contain sixty milligrams or more.

Hexosephosphate

As evidence received under the discussion of glucolysis suggests the possible presence of a hexose phosphate in blood, attempts were made to isolate or detect such a compound. For isolation experiments pig blood was chosen because it contains more organic phosphorus than the blood of other mammals, but the wisdom of the choice seems doubtful since pig blood is reported different from other blood with respect to its carbohydrate metabolism (4). Glycolysis does not take place in pig erythrocytes (13). Warburg and Christian (47) found two substances in the blood of horse and rat which activate methemoglobin and cause it to become an oxygen carrier in the respiration of blood __in vitro__ provided hexose-monophosphate is present. These two activators they failed to find in pig blood. Since the presence of hexosephosphate was originally postulated (44) as an intermediate in glucose degradation, the results obtained with pig blood cannot be applied to other bloods without caution.

Attempts to isolate Hexose-monophosphate from blood:
Although these experiments are inconclusive, a record of them may be of value in clearing the way for future attempts to isolate hexosephosphate from blood.

(a) In the first experiment the procedure followed
was suggested by the method of Embden and Zimmerman (11) for
the isolation of hexose-monophosphate from muscle. Pig blood
(1.6 liters) was taken at the slaughter house and there immediate-
ly added to four liters of twelve percent trichloroacetic acid.
The filtrate was made normal with respect to hydrochloric acid,
brought to a boil to hydrolyze the adenosine triphosphate to
adenylic acid, then cooled. The solution was next neutralized
with sodium hydroxide and acidified slightly with acetic acid.
At this point the work had to be interrupted and the solution
was kept on ice for twenty-six days. Some hydrolysis of the
esters may have occurred but hexosephosphate requires more heat
and greater acidity than was available for its complete
hydrolysis according to Lohmann.

The inorganic phosphates were precipitated by alkali-
nization with powdered magnesium oxide. A saturated solution
of lead acetate was added in excess to precipitate the organic
phosphorus. Hexosemonophosphate forms a soluble lead salt
but in the presence of excess lead acetate it seems to be
adsorbed on the other simultaneously precipitated phosphorus
compounds. The washed precipitate was decomposed with hydro-
gen sulfide and filtered. The aerated filtrate containing the
free phosphoric esters was made alkaline with a hot saturated
solution of barium hydroxide. The precipitate contained the
barium salt of Greenwald's diphospho - 1 - glyceric acid (21).
The filtrate contained adenylic acid and any other compounds
which form soluble barium salts, including the supposed
hexosephosphate. Barium was exactly removed with sulfuric
acid. In order to remove the nucleotides a solution of phos-

photungstic acid(10) was added in small quantities until no
more precipitate was formed. After removal of the excess phos-
photungstic and sulfuric acids with barium hydroxide, and the
barium with the exact amount of sulfuric acid, the resulting
filtrate (which should contain hexosephosphate if present in
blood) contained only a negligible trace of phosphorus. The
failure to find hexosephosphate in this experiment is not
proof of its absence. Possible reasons for this failure may be:-
(1) adsorption on the precipitated barium salts, especially on
barium sulfate, (2) chemical changes in the hexosephosphate
on long standing, and (3) the solubility of its lead salt.

(b) The second experiment differed from the one pre-
ceeding in that the insoluble barium salts were removed before
the lead precipitation. Seven liters of pig blood were added
directly to eight and a half liters of 10% trichloroacetic acid.
The filtrate was nearly neutralized with concentrated sodium
hydroxide, then made slightly alkaline with barium hydroxide.
This procedure should precipitate inorganic phosphorus, diphos-
phoglyceric acid, and adenosine triphosphate. No inorganic
phosphorus, but 19% of the organic phosphorus was left in the
filtrate. Most of this phosphorus was precipitated with lead
acetate in neutral solution. The small amount of phosphorus
remaining in the lead filtrate was discarded since it amounted
to only 6 mg. for the entire seven liters of blood, or less
than 0.1 mg. per 100 cc.

The washed lead precipitate was decomposed with hydro-
gen sulfide. To the aerated filtrate a methyl alcohol solution

of brucine hydrochloride was added until blue litmus paper was
scarcely turned red. The filtrate was evaporated in vocue at
35-40°C. to a volume of 60 cc. To this concentrated solution
acetone was added until cloudiness appeared. On standing on
ice , a substance started forming in clumps on the sides of the
flask. This was filtered off and more acetone was added with
more formation of the same substance. Three fractions were
formed in this way amounting to about two grams. This substance
accounted for the greater part of the phosphorus in the solu-
tion. It was yellowish in color and hygroscopic. No definite
crystalline structure could be observed under the microscope.
A solution of this precipitate gave a strong orcine test for
pentoses but did not reduce Fehling's solution indicating the
absence of hexosephosphate. Free adenylic acid if originally
present in the blood uncombined with "pyrophosphate" would
separate at this point.

(c) No fundamentally different procedure was used
in the third experiment. Sixteen liters of pig blood were
added to fourteen liters of 13.5% trichloroacetic acid in the
slaughter house. The filtrate was nearly neutralized with
sodium hydroxide and then made alkaline with ammonium hydroxide.
Lead acetate solution was added to precipitate the phosphorus and
the trace of phosphorus left in the supernatant liquid was
precipitated with basic lead acetate.

The combined lead precipitates were suspended in water
and decomposed with hydrogen sulfide. The aerated filtrate
was made alkaline with a hot saturated solution of barium

hydroxide. The filtrate from the barium precipitation contained only 85 milligrams of phosphorus equivalent to 0.5 milligrams per 100 cc. blood. The purine nucleotide content of the filtrate was determined and it was calculated that about 50% of the phosphorus present could be accounted for if the nucleotide were adenylic acid or 63% if inosinic acid.

Lead acetate was added to precipitate the phosphorus compounds in the solution but again a trace (7 mgms., or 0.04 mg. per 100 cc. blood) was not precipitated. Lead was removed with hydrogen sulfide and the volume of the filtrate was reduced to 4 cc. by evaporation in vacuo at 40-55°C. This viscous, yellowish solution gave a positive orcin test for pentoses but did not reduce Benedict's solution indicating the absence of hexosemonophosphate.

The solution was kept on ice and acetone was added at intervals. A small precipitate formed but the hexosephosphate if present should have remained in the supernatant liquid. This contained 6.2 milligrams inorganic phosphorus and 7.8 milligrams of organic phosphorus and gave a positive orcine test. Again the presence of the nucleotide made impossible any attempt to separate a hexosephosphate if present.

(d) In the fourth experiment the Schenck method was used to precipitate the blood proteins. To fifteen liters of pig blood mercuricchloride and hydrochloric acid were added. To the protein-free filtrate lead acetate was added and the

solution made alkaline with ammonium hydroxide. Lead chloride, lead hydroxide, and all the phosphorus compounds were precipitated. Lead was removed with hydrogen sulfide. After the precipitation of the barium insoluble salts, the volume of the filtrate was reduced in vacuo. On the addition of acetone only a small precipitate formed, due possibly to nearly complete removal of adenosine triphosphate with barium. Acetone was distilled off from the supernatant liquid in vacuo. The solution contained adenylic acid, did not reduce Benedict's solution (indicating absence of hexosephosphate) had no rotatory power, contained eighty milligrams of organic phosphorus and no inorganic phosphorus.

An attempt to remove the adenylic acid present with mercuric acetate after addition of sodium acetate and acetic acid was unsuccessful. The mercury was therefore removed with hydrogen sulfide and the solution neutralized with sodium hydroxide.

A solution of brucine in methyl alcohol was added to part of the filtrate which was then made neutral with acetic acid. It was evaporated in vacuo to a volume of thirty cc. Acetone was added until a cloud formed, then more at intervals for a few days. One cc. of a yellowish, heavy liquid separated. All the phosphorus was in this liquid except for a trace in the acetone. Nearly half the phosphorus was in the inorganic form showing that hydrolysis had taken place.

Two-hundredth of a cc. of this liquid gave a green cloudiness to Benedict's solution and three-hundredth of a cc. a strong positive pentose test with orcine solution.

The presence of nucleotide again prevented the detection of hexosephosphate if the latter were present.

Discussion and Conclusions: The above experiments do not prove conclusively that hexosephosphate is absent from blood since not all traces of phosphorus were accounted for, but they show that hexosephosphate could not be present in appreciable amounts and therefore could not account for a large fraction of the undetermined phosphorus in blood. For example, in Experiment (c) the barium soluble phosphorus represented only 0.5 mg. per 100 cc. of blood.

Unless the solution was made alkaline lead precipitation did not bring down all the phosphorus, suggesting the presence in blood of a phosphorus compound which forms lead soluble salts.

The greater fraction of the organic phosphorus compounds in pig blood form barium insoluble salts. At least half of the smaller fraction which was not precipitated by barium hydroxide (0.5 mg. P per 100 cc. in Exper. C) was composed of a nucleotide, leaving about 0.25 mg. P unaccounted for.

Attempts to Detect Hexosephosphate Indirectly. Two different types of experiments were tried to detect hexosephosphate in blood. The first was by applying to blood the method of Cori and Cori (8) for determining hexosephosphate in muscle, and the second by the determination of the silver soluble phosphorus compounds in the trichloroacetic filtrate of blood after separating the other phosphorus compounds.

(a) Normal and diabetic human blood were used for this experiment. The blood was defibrinated and the proteins precipitated with trichloroacetic acid. The method of Cori and Cori (8) was followed. The general outline of the procedure involves removal of inorganic phosphorus, adenosine triphosphate and diphosphoglyceric acid with hot saturated solution of barium hydroxide (adenylic acid is not removed), precipitation of the soluble barium salts in 80% alcohol, and the removal of barium from the alcohol precipitate by sulfuric acid. In the resulting solution hexosephosphate is determined by measuring the reducing power (using the method of Hagedorn and Jensen) and the total acid soluble phosphorus (method of Fiske and Subbarow (14)).

The results obtained with human blood, normal and diabetic, are given below expressed as milligrams of phosphorus per 100 cc. of blood.

	Normal	Diabetic
Total P in Ba-Alc. prec.	7.0	3.3
Hexosephosphate P (Calc.from reducing power)	0.0	0.94

The big difference between the phosphorus values obtained by direct ashing and by calculation from the reducing power point to the presence of phosphorus compounds other than hexose phosphate which form barium soluble salts. Among these would be adenylic acid which might have been formed by the hydrolysis of adenosine triphosphate. Cori and Cori (8) showed that glutothione is precipitated together with the barium

hexosephosphate and interferes by reducing the Hagedorn - Jensen reagents. In view of the presence of interfering substances, and the small amount of hexosephosphate in blood, if present at all, this method cannot be applied to blood.

(b) The silver salt of hexose-monophosphate is soluble while all known phosphorus compounds of blood form silver salts which are insoluble at pH 6.8*. This fact was used as a basis for detecting hexosephosphate in blood.

The proteins of the blood were precipitated with 10% trichloroacetic acid. The filtrate was neutralized with sodium hydroxide till very faintly pink to phenolphthalein. One-tenth volume of 20% silver nitrate solution was added. The precipi-tate was centrifuged and the supernatant liquid filtered. Silver was removed from the filtrate with hydrogen sulfide. The aerated filtrate contained quantities of organic phospho-rus as listed below:-

Dog blood	1.1 mg.	P per 100 cc. blood
Pig "	1.0 "	P " " " "
Turkey"	1.1 "	P " " " "

From these experiments it is clear that not more than one milligram of phosphorus per hundred cc. of blood could be due to hexosephosphate.

* A personal communication to Dr. Kerr from Dr. Fiske.

S U M M A R Y

1.   Phosphocreatine was proved absent from dog blood.

2.   The ratio of the nucleotide phosphorus to the organic phos-
phorus hydrolyzed by normal HCl in seven minutes at 100°C. in
the blood of different species approached the theoretical value
of 1:2 for adenosine triphosphate. This constitutes indirect
evidence of the presence of adenosine triphosphate in blood.

3.   Attempts to isolate hexosephosphate from pig blood were
unsuccessful. The fractions in which the compound was expected
gave negative tests with Benedict's solution. Quantitative
experiments prove that hexosephosphate, if present in blood,
could not account for more than one milligram of the phospho-
rus of dog, pig, human, and turkey blood.

4.   The sum of the hydrolyzable and nucleotide phosphorus
(which our experiments indicate to be adenosine triphosphate)
and the undetermined fraction of the organic phosphorus vary
greately from one species to the other.

5.   The suggestion is made that adenosine triphosphate is the
substrate for Kay's bone enzyme in his in vitro experiments.

6.   Glycolytic power is directly related to the adenosine
triphosphate content of the erythrocytes of different species.

A C K N O W L E D G E M E N T S

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II. THE RATIO OF THE EASILY HYDROLYZABLE PHOSPHORUS TO THE NUCLEOTIDE PHOSPHO-RUS IN RED, WHITE, AND HEART MUSCLE.

As different kinds of muscle have been known to vary in their chemical composition (4) it was considered worth while to study the relation of the easily hydrolyzable phosphorus to the nucleotide phosphorus in different tissues. Our experiments on red, white, and heart muscle are recorded in this paper.

Experimental. The animal, dog or rabbit, was anesthetized with ether and the required muscle was exposed, removed, and plunged immediately in liquid air. In the case of the rabbit the whole group of calf muscles (gastrocnemius, plantaris, soleus) was removed, while in the case of the dog tissue only a piece of the required tissue was cut off. The frozen tissue was ground in a mortar chilled with liquid air, transferred to a weighed quantity of 5% trichloroacetic acid and the weight of the tissue sample determined by reweighing. Enough trichloroacetic acid was then added to make a definite dilution, usually 1:10.

The inorganic phosphorus, the phosphorus hydrolyzable during seven minutes at $100^{\circ}C$. in N hydrochloric acid, the total acid soluble phosphorus, and the nucleotide nitrogen were determined as described in the previous paper. The nucleotide phosphorus was calculated on the assumption that each atom of phosphorus corresponds to five atoms of nitrogen.

Results and Discussion. The results of the experiments

for rabbit gastrocnemius and dog heart and Sartorius are given
in Table I. The ratio of the nucleotide phosphorus to the easily
hydrolyzable phosphorus in red muscle was of the same magnitude
as the ratio in blood, the average for the three determinations
being 1:1.77. Heart muscle on the other hand showed a consistently
lower ratio with an average of 1:1.37. This difference suggests
two different explanations. First, that heart muscle contains
the same adenosine triphosphate as red muscle but contains in
addition substances that can hydrolyze the latter more quickly,
so that part of the phosphorus is hydrolyzed in the interval
between the removal of the muscle and its complete fixation in
liquid air, and second that heart muscle contains a different
adenosine polyphosphate. This explanation seems to be supported
by the recent findings of other workers. Deutiche (3) refers
to the isolation by Embden of a "Herzmucleotid" from heart
muscle. the constitution of which is not established, but which
contains phosphorus and nitrogen in the ratio of 5:10 suggesting
a dinucleotide made up of a molecule of adenosine triphosphate
in combination with a molecule of adenosine diphosphate, or
possibly two molecules of adenosine triphosphate with one phos-
phoric acid shared by the two nucleotides. The theoretical
ratio of the nucleotide phosphorus to the hydrolyzable organic
phosphorus of such a compound would be 2:3 or 1:1.5. Our results
for heart muscle come much nearer this ratio than that of 1:2.

An adenosine polyphosphoric acid found in heart distinct
from the one found in muscle has also been mentioned by Loh-
mann (1).

Our figures for rabbit muscle show no uniformity for the

ratio of the easily hydrolyzable phosphorus to the nucleotide phosphorus. This may be due to the fact that post mortem changes take place much more rapidly in white than in red muscle(4), and some of the easily hydrolyzable phosphorus may have been hydrolyzed during the few seconds between the isolation of the muscle and its complete freezing in liquid air. Cori and Cori(2) found it difficult to obtain resting values for hexosephosphate in rabbit's muscle because of the twitching accompanying exposure and excision.

Adenine nucleotide nitrogen was determined in the skeletal muscle of the rabbit by Ostern and Parnas (5). Their results give about one half of the nucleotide nitrogen found by our method.

SUMMARY

The ratio of the nucleotide phosphorus to the easily hydrolyzable phosphorus in the red muscle of dog, white muscle of rabbit and heart muscle has been determined. Heart muscle seems to contain a different adenosine polyphosphate than blood and red muscle. The results obtained for rabbit muscle were not uniform, but the ratio of hydrolyzable phosphorus to nucleotide was distinctly lower than with red muscle and in no case was over 1:1.6.

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TABLE I.

Tissue	mg. P per 100 gms. tissue				Ratio of Nucleotide P to hydrol.P.
	T.A.S.P.	Inorg. plus P.C.	Nucleo-tide P.	Hydrol. Org. P.	
Dog	141.8	68.5	21.5	39.3	1:1.83
Sartorius	151.9	91.2	20.6	36.9	1:1.79
"	151.5	90.0	21.0	34.5	1:1.69
Ave.	148.4	83.2	20.0	36.9	1:1.77
Dog heart	110.3	44.7	20.1	27.0	1:1.34
" "	118.5	57.5	21.1	29.6	1:1.40
"	112.0	49.6	21.7	29.5	1:1.36
Ave.	113.6	50.5	21.0	28.7	1:1.37
Rabbit	191.1	89.4	29.9	46.5	1:1.55
Calf mus-cles	-	75.1	24.4	31.0	1:1.27
"	-	79.6	26.0	27.9	1:1.07
"	179.9	72.8	26.7	41.8	1:1.57
"	-	79.0	29.9	43.0	1:1.44
Ave.	185.5	79.2	27.3	38.0	1:1.38