CL. THE PHOSPHORIC-ESTERASE OF BLOOD.

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In a previous communication [Martland and Robison, 1924] it was shown that after blood has been laked with distilled water a rapid increase in the amount of inorganic phosphate occurs even at room temperature. The change is still more rapid at 37° but is entirely inhibited when the blood is laked with 1 % trichloroacetic acid, which indicates that it is due to the enzymic hydrolysis of phosphoric esters in the blood. The possibility that in these esters the phosphoric acid is combined with sugar or a sugar derivative suggested that this enzyme (both in its hydrolytic and synthetic function) might play a part in carbohydrate metabolism. We have therefore continued the investigation of its properties and have also sought for any changes in the blood sugar which might be correlated with the action of this enzyme on the organic phosphates.

In the first place our plan was to find the optimum conditions for the hydrolysis and if possible also for synthesis of the blood phosphoric esters by the enzyme, the location of the enzyme (whether in plasma or corpuscles) and the type of phosphoric ester which it can hydrolyse. It was also of interest to find whether the organic phosphate of blood, which is hydrolysed by this enzyme, is the same as that which is acted on by bone and whether any increase occurs in the reducing sugar of the blood as the result of the hydrolysis. The presence of this phosphoric esterase in blood has been also noticed by Lawaczeck [1924] whose paper did not come to our notice until most of our experiments were completed. With regard to certain of the conditions affecting these enzymic reactions his results are similar to those which we have obtained using different methods. He also gives interesting data with regard to the influence of different ions and of CO_2 on the action of the enzyme. Some of these experiments we have repeated and confirmed. His paper does not deal with the question of glycolysis.

Hydrolysis of the phosphoric esters in laked blood.

The course of the hydrolysis in laked blood was studied over periods of several days in order to find whether the whole of the acid-soluble phosphoric esters were decomposed by the blood enzymes.

A quantity of human venous blood (containing oxalate) was laked with distilled water and the volume made up to five times that of the blood. 5 cc. of the laked blood and 0·1 cc. chloroform were pipetted into a number of test tubes, which were closed with rubber stoppers and kept at 38°. At intervals the amount of hydrolysis was measured by adding 5 cc. of a solution of 5 % trichloroacetic acid to one of the tubes to precipitate the proteins and estimating the inorganic phosphate (by Briggs' method) in the filtrate. The total P in this filtrate was also estimated in order to find whether any acid-soluble P (glycerophosphate) was being produced from the lipins by enzymic removal of the fatty acid groups.

Another portion of the same blood was centrifuged and the plasma and corpuscles, each diluted five times with distilled water, were examined in the same way as the whole blood. In each case the initial amount of phosphate was estimated separately by laking 1 cc. whole blood etc. in presence of trichloroacetic acid to prevent enzyme action. The results are shown in Table I.

Table I. *Hydrolysis of phosphoric esters in whole blood, corpuscles and plasma at* 38°.

	mg. P per 100 cc.								
Time in hrs....	0	3	6	22	30	46	72	96	120
Whole blood:									
Inorganic phosphate	3·02	8·05	11·7	22·0	22·2	26·1	—	27·7	28·2
Total acid-soluble P	—	30·1	29·2	30·4	—	30·7	30·7	29·8	30·2
Plasma:									
Inorganic phosphate	3·00	—	—	3·17	—	3·38	—	—	—
Total acid-soluble P	3·50	3·52	—	—	—	3·57	—	—	—
Corpuscles:									
Inorganic phosphate	2·97	10·8	15·7	25·6	28·4	37·6	46·1	49·3	—
Total acid-soluble P	52·2	52·3	54·3(?)	52·4	52·6	52·1	52·1	52·6	—

The constancy of the figures for total acid-soluble P (within the experimental error) is evidence of the absence of any lecithinase from blood—or at least of its inactivity under these conditions. The inorganic phosphate in the laked whole blood and in laked corpuscles increases rapidly at first and then more slowly, until after 4 days it amounts to 93 % of the organic acid-soluble P in both cases. Hydrolysis was at this stage proceeding with extreme slowness.

The small amount of organic acid-soluble P in the plasma is also hydrolysed with extreme slowness, suggesting that it is of the same type as that forming the last 7 % of the phosphoric esters in the corpuscles or else that the enzyme is absent from the plasma. The former alternative is probably the correct one.

Between 22 and 30 hours there appears to be a temporary slowing down in the rate of hydrolysis. We have also noticed this in other experiments, but do not know what significance it may possess.

The optimum p_H *of the enzyme.*

In order to obtain some idea of the optimum p_H for this phosphoric esterase we prepared a series of tubes containing 5 cc. of laked whole blood (1–4 H_2O), and to each added one or more drops of 0·5 *N* H_2SO_4, or NaOH, or H_2O. The

amount of hydrolysis was measured after 2 hours at 38°. The values of p_H shown in Table II are only approximate, but it is obvious that the rate of hydrolysis is greatest at a p_H slightly on the acid side of the normal blood reaction and close to the neutral point. The phosphoric esterase of the blood is thus markedly different from that of the bone, which has an optimum p_H of 8·4–9·4 [Robison and Soames, 1924].

Table II.

Approximate p_H	Amount of hydrolysis after 2 hrs. at 38° mg. P per 100 cc. blood
5·8	1·4
6·2	2·3
6·4	4·0
7·0	6·3
7·4	4·8
7·8	4·9
8·2	1·8
8·6	1·4

The location of the enzyme.

The absence of any rapid hydrolysis in unlaked blood and its onset after rupture of the corpuscles by laking suggested that the enzyme might occur only in the plasma. Against this was to be placed the fact that in the experiment (Table I) the rate of hydrolysis in the laked corpuscles was even higher than that in the whole blood, although the amount of adherent plasma pipetted with the corpuscles must have been relatively small. Experiments were carried out to obtain more information on this point. Since the plasma contains only negligible amounts of the esters it was necessary to provide additional substrate in the form of a neutralised trichloroacetic acid filtrate from the whole blood. The experiment was made with mixed arterial and venous blood (containing oxalate) from a rabbit, part being used for the preparation of the protein-free filtrate (F) and part being centrifuged for the preparation of the laked corpuscles (C) and diluted plasma (P) (both 1 in 5) as in the first experiment. The corpuscles were not washed.

A number of tubes was prepared, each containing:

2 cc. of C or P (equivalent to 0·4 cc. corpuscles or plasma);

{ 5 cc. of F (equivalent to 0·455 cc. whole blood); CF, PF,
or 5 cc. H_2O; CO, PO;

0·1 cc. chloroform.

They were stoppered and kept at 38°, tubes from each series being removed at intervals for estimation of inorganic phosphate.

The results, shown in Table III, are expressed as mg. P per 100 cc. corpuscles and plasma respectively. On this basis the organic P in 5 cc. of the blood filtrate, F, represented an additional 34·7 mg. P, but even this did not bring the amount of substrate in PF up to half of that present in the corpuscles alone (CO).

The rate of hydrolysis is almost identical in CO and CF as would be expected

from the large excess of substrate present in both. The fact that in both cases the rate drops considerably and equally after 24 hours would seem to indicate failure of the enzyme rather than the completion of hydrolysis of one of two or more substrates.

In PF the initial rate of hydrolysis was much slower than with the corpuscles but after 72 hours the difference in the rates had almost disappeared. One must conclude therefore that the enzyme is present in both plasma and corpuscles or else that, being in colloidal solution in the plasma it becomes adsorbed on to the surface of the corpuscles either in the body or during centrifugalisation.

Table III.

	Total organic acid soluble mg. P	Amount of hydrolysis expressed as inorganic phosphate mg. P per 100 cc. corpuscles or plasma			
No. of hours...	0	24	48	72	144
CO. Laked corpuscles + water	83·7	25·0	29·4	33·1	40·5
CF. Laked corpuscles + blood filtrate	118·4	25·2	31·2	34·9	41·7
PO. Diluted plasma + water	0·7	0·4	0·6	0·7	0·8
PF. Diluted plasma + blood filtrate	35·4	6·5	10·3	11·8	16·7

Action of the blood enzyme on various phosphoric esters.

The action of the enzyme was tested on a number of naturally occurring phosphoric esters. Human blood (venous) was laked by dilution with distilled water (1 in 5) and 2 cc. of this fluid was at once pipetted into a number of tubes each containing 1 cc. of the ester solution + 0·1 cc. chloroform. These solutions were all brought to p_H 7·0. Into one tube of each series 1 cc. of a 10 % solution of trichloroacetic acid was pipetted before adding the diluted blood, and these tubes provided the figures for zero time. The remainder were kept at 38° for the specified time, after which 1 cc. of the trichloroacetic solution was added. Control tubes containing 2 cc. water + 1 cc. ester solution were also kept at 38° for the same time. The amount of inorganic phosphate in 1 cc. of the filtrates after subtracting the amount at zero time, and allowing for any chemical hydrolysis as shown by the controls, gave the amount of ester hydrolysed by 0·1 cc. blood (0·5 cc. diluted blood).

Table IV. *Hydrolysis of various phosphoric esters by the blood enzyme.*

Ester	Total organic P mg.	Net amount of hydrolysis (mg. P) effected by 100 cc. human blood	
		20 hours	40 hours
Sodium glycerophosphate	780	—	14·2
Potassium hexosemonophosphate	780	11·4	15·4
Potassium hexosediphosphate	1160	144	303
Sodium nucleate	780	0	0
Sodium caseinogenate	780	0	0
Phytin	780	0	0
		Rabbit's blood	
		24 hours	48 hours
Mixed nucleotides from yeast nucleic acid	310	34	54

The results shown in Table IV have been multiplied by 1000 and therefore give the amounts hydrolysed by 100 cc. blood. In the case of sodium nucleate difficulty was caused in estimating the phosphate by the formation of a precipitate and the results are a little doubtful. The action of the enzyme on nucleotides was tested by a separate experiment using rabbit's blood. The mixture of nucleotides was prepared from yeast nucleic acid by the method of Jones and Perkins [1923]. In this experiment the reducing sugar was also estimated, but no evidence of the liberation of any reducing compound was found.

It is seen that the phosphoric esterase of the blood resembles that of muscle in its ability to hydrolyse hexosediphosphoric ester, and in its relative inactivity towards hexosemonophosphoric and glycerophosphoric esters, which were hydrolysed at only 1/20 the rate of the diphosphate and only 1/300 the rate of hydrolysis of the monophosphate by the bone enzyme (from the same weight of bone). It is differentiated both from muscle and bone enzymes by its ability to hydrolyse the unknown phosphoric esters of the blood. It has no action on caseinogen, phytin or nucleic acid but is able to hydrolyse the individual nucleotides.

Comparison of the effect of the blood and bone enzymes on the phosphoric esters of the blood.

It was of interest to find what action the phosphoric esterase of the blood has on that portion of the organic acid-soluble compounds which are hydrolysed by bone extracts. This was done by allowing laked whole blood to autolyse for a certain time and then adding trichloroacetic acid to destroy the enzyme and precipitate the proteins. The neutralised filtrate was examined with bone enzyme by the method described by Kay and Robison [1924, 1]. The protein-free filtrate from another sample of the original blood—laked with trichloroacetic acid—was also treated with bone enzyme in the same way. Table V gives the results of two experiments on human blood (from different subjects), one after 20 hours' and the other after 140 hours' autolysis.

Table V. *Hydrolysis by bone extract of laked human blood before and after autolysis.*

	mg. P per 100 cc.			
	Inorganic	Organic acid-soluble	Hydrolysed by bone	% of organic P hydrolysed by bone
I. Blood as drawn (venous)	2·64	24·0	6·6	27
Do. after 20 hrs. autolysis at 38° (laked)	10·7	15·7	3·4	22
II. Blood as drawn (venous)	3·0	23·0	6·3	27
Do. after 140 hrs. autolysis at 38° (laked)	22·7	3·5	2·6	74

Apparently a portion of the esters hydrolysable by bone is also hydrolysed by the blood enzyme during the early stages of autolysis, but the remaining

portion is very resistant to the action of the latter, and constitutes that residue of organic phosphorus compounds which was found in all experiments after prolonged autolysis. This seems to confirm the results obtained by Kay and Robison [1924, 2] using the muscle enzyme, and to suggest the presence of compounds of both the hexosemonophosphate and hexosediphosphate type.

Changes in the sugar content of the blood during hydrolysis of the phosphoric ester.

The possibility that the hydrolysis of the phosphoric esters might be accompanied by an increase in the amount of reducing sugar in the blood was investigated in a number of experiments, the sugar being estimated in the neutralised trichloroacetic acid filtrates by the method of Hagedorn and Jensen [1923].

Some of the results are shown in Table VI.

Table VI. *Changes in the amount of reducing sugar during autolysis of laked blood.*

Exp. 1. Human blood laked with distilled water (1 in 5).

Time (hrs.)...	0	20	40	64	112	160	184
Inorganic PO_4 (mg. P)	2·64	11·6	16·3	16·3	18·0	20·0	22·0
Sugar (mg.)	118	116	116	116	120	128	150

Exp. 2. Human blood laked with distilled water (1 in 5).

Time (hrs.)...	0	24	44	92	116	142	166
Inorganic PO_4 (mg. P)	3·0	10·6	13·9	17·1	17·8	19·2	20·8
Sugar (mg.)	128	128	150	173	—	173	173

Exp. 3. Rabbit's blood laked with distilled water (1 in 5).

Time (hrs.)...	0	24	48	144	216'
Whole blood:					
Inorganic PO_4 (mg. P)	5	11·1	21·8	32·0	33·3
Sugar (mg.)	126	124	147	(99)?	143
Corpuscles:					
Inorganic PO_4 (mg. P)	(5·4)	45·4	86·7	89·3	—
Sugar (mg.)	53	44	47	68	—
Plasma:					
Inorganic PO_4 (mg. P)	5·4	5·4	5·8	—	—
Sugar (mg.)	128	128	128	—	—

In general it was found that no significant change in the reducing power occurred during the early stages of the autolysis in laked blood or corpuscles, although 50 % of the phosphoric esters might be hydrolysed. In the later stages of the autolysis a rise of about 30 % in the sugar value was usually observed. In one experiment with diluted plasma no change in the sugar value was found after 48 hours at 38°.

An increased sugar content could be explained by the hydrolysis of hexosephosphoric esters which possess a lower reducing power than that of the equivalent amount of free hexose, but the matter is not so simple as this. The reducing group is not liberated simultaneously with the phosphate and

may be derived from an entirely different compound by a reaction having no connection with the other. The presence in the blood of "combined sugar" (or "protein sugar") which can be set free by acid hydrolysis has been noted by many workers [cf. Condorelli, 1924]. For the present we retain an opinion that this sugar is combined on the one hand with phosphoric acid and on the other—through the potential aldehydic or ketonic group—with some other molecule, these bonds being hydrolysed by different enzymes at different rates. The evidence for this is admittedly slight and further investigation is desirable.

Possible changes of the sugar content were also looked for during the hydrolysis of blood filtrates by bone extracts. Since hexosemonophosphoric ester possesses 66 % of the reducing power of its equivalent in hexose, the liberation of inorganic phosphate equivalent to 6 mg. P from this compound would give an increased reducing power equal to 12 mg. glucose. Such increases were actually observed in one or two experiments, but in others no significant change was found and we concluded that under the conditions of the experiments the possible changes were too small to be satisfactorily demonstrated.

Changes in unlaked blood.

The laking of whole blood or corpuscles with distilled water produces two marked results, the rapid hydrolysis of the phosphoric esters and the inhibition of glycolysis, followed after 24–48 hours by an increase in the reducing sugar. In unlaked blood very little change occurs in the inorganic phosphate but the sugar rapidly disappears. In order to obtain more evidence as to the possible connection between these two reactions we investigated the behaviour of unlaked blood under slightly varying conditions of p_H.

Exp. 1 was carried out with rabbit's blood (mixed arterial and venous, containing oxalate); 2 cc. of whole blood, corpuscles or plasma were measured into tubes which were kept at 38° for 3 hours without antiseptic, the inorganic phosphate and sugar being thus estimated. The results are given in mg. per 100 cc.

Exp. 1.

	0 hours		3 hours	
	H_3PO_4 mg. P	Sugar mg.	H_3PO_4 mg. P	Sugar mg.
Whole blood	5·46	119	6·36	57
Do. shaken out with air	5·46	119	5·24	70
Do. + ·01 cc. NaOH	5·46	119	5·40	70
Corpuscles	5·36	52	16·7	21
Plasma	5·54	128	5·54	132

In this experiment an appreciable hydrolysis of organic phosphate occurred in the whole blood as taken from the animal but was entirely inhibited by the addition of a very small amount of alkali, or by shaking out with air, which would likewise raise the p_H. In the latter case a small decrease in free phosphate was noted. Glycolysis occurred in both whole blood and corpuscles but not in the plasma.

The effect of small additions of alkali and of acid was further tested in the following two experiments, in which changes in unlaked and laked blood were simultaneously investigated. Defibrinated rabbit's blood was used. Results are given in mg. per 100 cc. blood.

Exp. 2.

	Hours at 38°	Unlaked blood: Inorganic phosphate mg. P	Unlaked blood: Sugar mg.	Blood laked with distilled water (1 in 5): Inorganic phosphate mg. P	Blood laked with distilled water (1 in 5): Sugar mg.
0. Whole blood	0	4·79	116	—	—
1. Defibrinated blood (D)	0	4·54	115	—	—
2. 2 cc. D + ·045 cc. *N* H_2SO_4	2	7·10	98	8·6	(99)?
3. „ + ·030 cc. „	2	5·83	94	10·1	105
4. „ + ·015 cc. „	2	4·16	77	10·1	105
5. „ + ·005 cc. „	2	4·06	73	—	—
6. „ + ·005 cc. *N* NaOH	2	3·56	75	8·4	95
7. „ + ·015 cc. „	2	3·62	73	8·4	96
8. „ + ·030 cc. „	2	3·80	61	6·4	88
9. „ + ·045 cc. „	2	5·03	69	6·0	88

Exp. 3.

0. Whole blood	0	5·98	116	10·0	—
1. Defibrinated blood (D)	0	5·70	118	—	—
6. 2 cc. D + ·005 cc. *N* NaOH	2	4·94	72	—	—
6 *a*. „ + ·005 cc. „	3	5·47	68	—	—
8. „ + ·030 cc. „	2	5·23	74	—	—
8 *a*. „ + ·030 cc. „	3	5·70	79	—	—
10. „ + ·04 cc. „	2	—	—	9·43	103
11. „ + ·07 cc. „	2	—	—	6·71	104
12. „ + ·10 cc. „	2	—	—	6·71	104
13. Laked blood without acid or alkali	2	—	—	10·06	107

In both experiments there was a definite reduction in the amount of inorganic phosphate on defibrination, which was also noted by Lawaczeck. A further marked reduction occurs when the blood is kept during 2 hours at 38°, the amount of this reduction being greatest in the presence of small amounts of added alkali. (The defibrinated blood, which has been shaken with air, with a low CO_2 content, is itself more alkaline than normal blood.) This decrease in free phosphate must indicate a synthesis of organic phosphorus compounds, taking place in unlaked whole blood most readily when the p_H is above the normal. Exp. 3 shows that this synthetic power is only retained for a limited period after withdrawal of the blood from the body, the decrease in free phosphate being less after 3 hours than after 2 hours. This also is in agreement with Lawaczeck's findings.

When the p_H rises above or falls below certain limits, the rate of hydrolysis of the phosphoric esters becomes greater than the rate of synthesis, and the inorganic phosphate increases. At a p_H somewhat lower than 7·0 the rate of hydrolysis may approach that in laked blood.

Lawaczeck's interesting findings on the effect of passing carbon dioxide or pure air through the blood would seem to be due to the alteration of p_H thus brought about.

We have obtained similar results with blood shaken up several times with carbon dioxide or with air free from carbon dioxide and then kept in a corked flask at 38° for 2 hours.

		Inorganic phosphate mg. P	Sugar mg.
0.	Whole blood	5·65	112
1.	Defibrinated blood	5·41	112
2.	„ „ shaken with pure air	5·44	63
3.	„ „ „ „ CO_2	12·82	106
4.	„ „ „ „ CO_2 and kept for 1 hour at 38° ...	9·05	107
5.	„ „ „ „ CO_2 „ „ 38° and then shaken with pure air and kept for a further 1 hour at 38° ...	6·25	95

The variations in the amount of reducing sugar under the above conditions are also very interesting. When the synthesis of phosphoric esters is greatest considerable glycolysis occurs, although the converse is not necessarily true. On the other hand, when rapid hydrolysis is taking place the glycolysis is much less rapid or may be entirely absent. One cannot, however, draw the conclusion that the two reactions—esterification of phosphoric acid and synthesis of non-reducing compounds from sugar—are necessarily connected, since two independent enzyme reactions, both affected similarly by the p_H, might well show parallel variations of this kind. Recent work by Kay [1924] has shown that during acidosis produced in a human subject by the ingestion of ammonium chloride the amount of phosphoric esters in the venous blood was considerably diminished. It seems possible that *in vivo* as *in vitro* a delicate equilibrium exists between the synthetic and the hydrolytic activities of the blood enzyme and that this is markedly affected by very slight changes in the reaction of the blood.

The whole of the evidence of our experiments is not sufficient to prove that the phosphoric esters and esterase of the blood actually play a part in carbohydrate metabolism, but we think that the experimental facts are very suggestive and are worth further investigation and confirmation.

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