CXLIX. THE ROLE OF PHOSPHATES IN CARBOHYDRATE METABOLISM.

I. THE ACTION OF THE MUSCLE ENZYME ON THE ORGANIC PHOSPHORUS COMPOUNDS OF BLOOD.

II. THE EFFECT OF INSULIN ADMINISTRATION ON THE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN BLOOD AND MUSCLE.

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DURING the fermentation of sugar by yeast juice in presence of phosphates at least two compounds of carbohydrate with phosphoric acid are produced, a hexosediphosphoric ester $C_6H_{10}O_4(PO_4H_2)_2$ and a hexosemonophosphoric ester $C_6H_{11}O_5PO_4H_2$, the position of the acid group in the latter compound being almost certainly different from that of either of the groups in the former.

The interest, which has long surrounded hexosediphosphoric ester and the problem of its rôle in the carbohydrate metabolism of the yeast cell, has been extended by the work of Embden and his collaborators, who have brought forward evidence of its occurrence in muscle and of its probable identity with the precursor of lactic acid. The more recently discovered monophosphoric ester may also have its counterpart in animal physiology. It has been shown [Robison, 1923; Robison and Soames, 1924] that whereas this compound is scarcely acted on by the enzymes of muscle it is rapidly hydrolysed by an enzyme which is secreted in bones and ossifying cartilage and in teeth, and further [Kay and Robison, 1924] that blood contains appreciable amounts of a phosphoric ester which is hydrolysed by this bone enzyme. The light which these facts may throw on the hitherto unexplained process of ossification has been discussed in the papers referred to above, but the exact composition of the blood ester has yet to be determined. The possibility that it consists wholly or in part of a hexosediphosphate was from the first of twofold interest to us. If this could be demonstrated it would provide another clue to the problem of carbohydrate metabolism and a link with Embden's "lactacidogen," but for the suggested scheme of ossification the diphosphoric ester would not furnish a satisfactory substrate, since it is readily hydrolysed by muscle and

non-ossifying cartilage as well as by bone. We therefore planned to investigate the action of both bone and muscle enzymes on the phosphorus compounds in the blood of man and of animals under different conditions of nutrition, using these enzymes as tools for the discovery and quantitative estimation of compounds of the type of hexosemonophosphoric ester and hexosediphosphoric ester respectively. Only the results of the experiments with the bone enzyme, carried out chiefly during 1923, have so far been published [Kay and Robison, 1924] but with most of these samples of blood the action of muscle extracts was simultaneously investigated, adopting the same technique as described for bone extracts. The results were neither so consistent nor so easy to interpret as those obtained with the bone enzyme and they were therefore held back in the hope that further work might enable us to draw more definite conclusions. The difficulty was caused partly by the fact that the amounts of inorganic and of hydrolysable organic phosphates in the muscle extracts were relatively large, while the amounts of hydrolysis observed were small, and partly by the fact that the muscle enzyme is not entirely without action on the monophosphoric ester, although its power is very slight in comparison with that of the bone enzyme.

Although, therefore, some of the results pointed to the presence of a compound of the hexosediphosphate type, it is not possible to state definitely that such a compound is a normal constituent of blood.

It is, however, certain that a large proportion of the phosphoric ester of blood which is hydrolysable by bone is not readily hydrolysed by muscle, and that the requirements of a specific substrate for the scheme of ossification are thus satisfied.

It is true that, so far, no proof has been given that this phosphoric ester is a derivative of a hexose or of any carbohydrate, but the isolation of its barium salt, although still in impure condition [Goodwin and Robison, 1924] provides strong evidence that the ester at least possesses reducing properties and is optically active.

Before proceeding to deal with the second part of this investigation, the effect of insulin administration on the distribution of phosphorus compounds in the body, the results obtained up to this point will be briefly set out.

ACTION OF THE MUSCLE ENZYMES ON HEXOSEPHOSPHORIC ESTERS AND ON THE PHOSPHORIC ESTERS IN BLOOD.

From previous experiments [Robison, 1923] it was known that while the bone enzyme hydrolyses hexosemonophosphoric ester and both groups of the diphosphoric ester, the muscle extracts have little or no action on the former but readily hydrolyse at least one of the phosphoric groups of the latter. They appeared to possess less power towards the second group. For the correct interpretation of our results it was necessary to have more precise information on the relative activity of the muscle enzymes with respect to these three ester linkages, under the conditions of our experiments. Table I shows the

rate of hydrolysis of hexosediphosphoric ester by (a) 5 % bone extract prepared by macerating the bones of young rats with 20 times their weight of chloroform water, (b) 10 % muscle extract prepared by macerating rabbit muscle with 10 times its weight of chloroform water.

The proportion of tissue extract to ester solution and the other experimental details were the same as those given in our previous paper (p. 758), and the concentration of combined phosphorus was of the same order as that of the organic acid-soluble phosphorus in the blood experiments.

Table I. *Hydrolysis of potassium hexosediphosphate at 37°; p_H 8.4.*

Time, minutes	5 % bone extract		10 % muscle extract	
	% of combined P hydrolysed	k $\left(\frac{1}{t_1 - t_2} \log \frac{a - x_1}{a - x_2}\right)$	% of combined P hydrolysed	k $\left(\frac{1}{t_1 - t_2} \log \frac{a - x_1}{a - x_2}\right)$
100	64	·0044	13	·0006
200	85	·0038	25	·0006
300	92	·0027	33	·0005
600	96	·0010	50	·0005
1400	98	·0004	68	·0003
2800	100	—	82	·0002
4200	—	—	87	·0001

These results show that the second phosphoric group of hexosediphosphoric ester is also hydrolysed by muscle extract although much more slowly than the first, as can be seen from the sharp fall in the value of k (in the last column) after 50 % of the combined P has been hydrolysed. The values of k for the bone enzyme may also be different with respect to the two groups, but this cannot be so clearly deduced from the figures. They are in any case much greater than the values for the muscle enzyme.

The action of muscle extract on the monophosphoric ester is shown in Table II in which the results of a typical blood experiment are set out in detail. This experiment is the same as that for which the results with the bone enzyme were given in our last paper (p. 759). The action of each enzyme was in this case tested in duplicate, at different p_H values. The bone extract at p_H 9.3 and 8.4 gave almost identical figures and only one series was therefore quoted. The muscle extract was tested at p_H 8.4 and 7.3 and here also the results were in close agreement, which proves that the muscle enzyme does not possess the marked high optimum p_H (8.4–9.4) of the bone enzyme. After deducting the amount of the increase in inorganic phosphate due to hydrolysis of phosphoric esters in the muscle extract (OM) it is seen that a very slight hydrolysis of the hexosemonophosphate (MM) was effected by the enzyme in 10 hours. The increase of inorganic phosphate in the case of the blood filtrate (FM) is slightly greater still, from which the presence of a compound of the hexosediphosphate type, in very small amount, might be inferred. The figures are, however, very near to the limit of experimental error, and deductions must be made with caution. Similar results were obtained in most of our experiments—slight hydrolysis, generally a little more than that found in the controls. A summary of these results is given in Table III. Only in one

experiment (no. 17) did we obtain a considerable amount of hydrolysis, quite outside the range of ordinary experimental error, and largely occurring in the first hour. We can find no reason for doubting this result but we have not been able to repeat it. It may be that the particular nutritive condition of the animals was favourable to the accumulation of this ester in the blood. In another experiment (no. 26) on human blood a slow hydrolysis was observed throughout 44 hours—amounting to 1.4 mg. P per 100 g. blood in 6 hours, 2.0 mg. in 20 hours and 3.2 mg. in 44 hours. Almost identical figures were however in this case obtained for a parallel experiment using sodium glycerophosphate with the same muscle extract, so that one must conclude that this blood contained no ester of the diphosphate type.

Table II. *Exp. no. 33. Whole blood of an adult rabbit taken from carotid and jugular.*

Hydrolysis by 10 % muscle extract at 37.5°.									
Inorganic phosphate (mg. P) in 5 cc. final filtrate—equivalent to 0.5 g. blood.									
	<i>p</i> _H	Time in hours						Increase after hydrolysis	Net amount of hydrolysis
		0	1	2	3	5	10		
FM. Blood filtrate + muscle extract	8.4	·058	·058	·062	·063	·064	·070	·012	·0055
	7.3	·0585	·059	·061	·062	—	·070	·0115	·006
FO. Blood filtrate + water	8.4	·024	—	—	—	·025	·024	—	—
OM. Water + muscle extract	8.4	·036	—	—	—	·040	·0425	·0065	—
	7.3	·0365	—	—	—	·0395	·042	·0055	—
MM. Sodium hexosemono-phosphate + muscle extract	8.4	·0375	·038	—	—	·042	·046	·0085	·002
	7.3	·0375	·038	—	·039	·043	·047	·0095	·004
DM. Sodium hexosediphosphate + muscle extract	8.4	·045	·057	·062	—	·083	·103	·058	·0515
	7.3	·0475	·058	·063	0.69	·082	·102	·0545	·049
Mg. P per 100 g. blood									
Total acid soluble phosphorus	...	...	...	...	...	...	...	= 31.0	
Inorganic phosphate	...	...	...	...	...	...	...	= 4.8	
Phosphoric esters	...	...	...	...	...	...	...	= 26.2	
of which (a) esters hydrolysed by bone enzyme	...	...	...	...	...	...	...	= 6.5	
(b) esters hydrolysed by muscle enzyme	...	...	...	...	...	...	...	= 1.2	
Hexosemonophosphoric ester hydrolysed under similar conditions by muscle enzyme	...	...	...	...	...	...	...	= 0.6 (Total organic P = 21.6)	
Hexosediphosphoric ester hydrolysed under similar conditions by muscle enzyme	...	...	...	...	...	...	...	= 10.0 (Total organic P = 23.0)	

THE EFFECT OF INSULIN ADMINISTRATION ON THE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN BLOOD AND MUSCLE.

The evidence at this stage appeared to show that a hexosediphosphoric ester may occur in blood, but that under the conditions of carbohydrate metabolism usual in our experiments the amounts are too small to be satisfactorily demonstrated by our method. By disturbing the normal carbohydrate metabolism the necessary conditions might be obtained whereby the amount of this substance could be increased.

The possibility presented itself of realising these conditions by the use of insulin, particularly in view of the recent work which had shown that the

Table III.

(For other data of these experiments see Kay and Robison [1924, Table I¹].)

No. of Exp.	Animal	Age	Type of blood	Mg. P per 100 g. blood			
				Hydrolysis by muscle enzyme			Hydrolysed by bone enzyme
				Strength of muscle extract %	Duration of experi- ment hrs.	Amount hydro- lysed	
9	Cockerel	6 months	Mixed	10	18	1.0	3.2
8	Rats	3 "	"	10	18	2.1	7.4
11	"	3 "	"	2½	22	2.4	7.4
12	"	2½ "	"	5	26	1.2	4.7
	"	9 "	"	5	26	2.2	5.4
17	Rabbit	3½ "	"	10	{ 1 24	4.6 7.8	— 9.9
	"	18 "	"	10	{ 1 24	2.5 3.9	— 6.5
33	"	adult	"	10	10	1.2	6.5
40-49	"	young adults	average of 10	10	18	2.8	8.7
27	"	adult	venous	10	3½	0.6	8.5
	"	"	arterial	10	3½	0.7	8.7
28	{ Child ♀ " ♂	{ 10½ years 11 "	venous	10	8	1.7	5.7
26	Man	adult	"	10	6	1.4	6.0

¹ Correction. In this table (p. 760) line 13 should read: "Exps. 40-49. Rabbits—Young adults—Average of 10—Total acid-soluble, 34.5; Inorganic, 5.7; Organic, 28.8; Hydrolysed by bone, 8.7; Not hydrolysed, 20.1; Percentage of organic P hydrolysed by bone, 30."

inorganic phosphate decreases as well as the reducing substance. The decrease is greater than can be explained solely by the increase in blood volume which occurs after insulin administration [Haldane, Kay and Smith, 1924]. Thus in Table IV the first line A shows the figures obtained in some experiments of our own on seven young adult rabbits; line B shows those obtained by Wigglesworth, Woodrow, Winter and Smith [1923] on adult rabbits (average of 6 taken from their protocols); line C shows the serum phosphate results of Harrop and Benedict [1924].

Table IV.

	Inorganic phosphate (mg. P) per 100 cc. blood		Percentage diminution
	Before insulin	After insulin	
A	6.0	4.5	25
B	5.6	3.8	32
C	6.4	3.9	38
Average	6.0	4.1	32

Under the conditions of our experiments (line A) the blood volume increases some 15 %, so that the diminution in inorganic P is a real one, and indicates that inorganic phosphate is actively taken out of the plasma by the body cells after insulin administration. In the tissues, which include the red blood corpuscles, there is a "phosphate vacuum" no less than a "glucose vacuum."

By taking into account these blood volume changes we can examine the hexosephosphate hypothesis on a quantitative basis.

If we take the original blood volume of the 2 kg. rabbit as 110 cc. the increase in blood volume between the administration of insulin and the onset of convulsions (15 %) brings the final blood volume to 126 cc. Taking 6.6 mg. P as the total original inorganic P in 110 cc. blood, and 5.1 as the total inorganic P in 126 cc. blood at the convulsive stage, we see that 1.5 mg. P disappears into the tissues. Assuming that a hexosemonophosphate is synthesised and is taken out of the blood, this amount of P would account for 9 mg. glucose or for 4.5 mg. if a hexosediphosphate were formed. The reducing power of these esters is respectively about 66 % and 33 % of their sugar equivalent so that if they remained in the blood the apparent loss of glucose would be correspondingly less.

Actually the amount of sugar which is taken out of the blood is much greater than this, being very approximately the difference between [0.11 % of 110] g. and [0.045 % of 126] g. Assuming for the time being that there is no mobilisation of sugar from glycogen or other reserves in the other tissues, this gives 65 mg. glucose as the least amount leaving the blood. It is evident that the amount of inorganic phosphate leaving the blood is only sufficient to account, even on the hexosemonophosphate basis, for a maximum of some 14 % of the glucose which is simultaneously being taken out of the blood stream. If an intermediate hexosephosphoric acid complex is to account for the whole of this glucose, phosphate must be provided by some other tissue of the body, in which case an increase in organic at the expense of inorganic phosphate (possibly in the same tissue) may be found, or a change in the state of combination of part of the organic phosphate originally present. Some evidence as to the truth or otherwise of this hypothesis is given below.

Possible changes in the blood.

Although the drop in inorganic phosphate in the blood was insufficient to account for more than 14 % of the fall in glucose, it was possible that the phosphoric esters might have increased in amount. As these are present, both before and after insulin, almost entirely in the corpuscles, the relative amounts of corpuscles and plasma which are altered by the administration of insulin, must be known if the results are to have any value.

The distribution of phosphorus compounds in blood after insulin was investigated by means of the bone and muscle enzymes as already described. In all the determinations shown below (except those of "lecithin" P) two strictly comparable animals, usually from the same litter, were used, the first for the control figures being given saline and treated to the same operative technique as the second animal which received the same volume of insulin solution. The insulin animal was killed by bleeding either just before or actually at the onset of convulsions and the mixed arterial and venous blood used for our determinations. The control was treated in the same way, in some cases just before, in others shortly after the death of the insulin animal.

The results of a series of experiments are summarised as follows:

Table V¹.

Type of phosphorus compound	Control mg. P per 100 cc.	After insulin mg. P per 100 cc.	Decrease %	Remarks
1. Lecithin	10.8	9.1	16	Average of 7 exps.
2. Total acid-soluble	36.3	34.6	5	Average of 11 exps.
3. Inorganic	6.0	4.5	25	" "
4. Organic	30.3	30.1	1	" "
			Increase	
5. Hydrolysable (bone enzyme)	9.2	9.8	6	" "
			Decrease	
6. Hydrolysable (muscle enzyme)	2.9	2.7	6	Average of 9 exps.

In considering the figures for the percentage decrease in the concentration of the various phosphorus compounds we must bear in mind that the blood volume is increased by about 15 % after insulin, and the corpuscles, which contain practically the whole of the organic acid-soluble phosphorus compounds, are diluted by this amount.

We may therefore draw the following tentative conclusions:

(1) The decrease in the lecithin P (16 %) is due entirely to the increase in the blood volume, i.e. no lecithin P enters the blood with the infiltrating fluid (or else the amounts of lecithin entering and leaving the blood during the experimental period balance).

(2) The apparent decrease in total acid-soluble P (5 %) corresponds with an increase in absolute amount equivalent to about 9 %, or 3.5 mg. P per 100 cc. of the original blood volume. This extra phosphate as well as that required to compensate for losses through the kidneys, must be drawn from the other tissues and may be partly derived from the breakdown of phosphoric esters or in the last resort from bone.

(3) The inorganic phosphate suffers a considerable decrease both in percentage (25 %) and in absolute amount (about 0.8 mg. P per 100 cc. of the original blood volume).

(4) The organic acid-soluble P thus undergoes an increase in absolute amount equivalent to nearly 14 % or 4.3 mg. P per 100 cc. of the original blood volume.

(5) The esters hydrolysable by the bone enzyme are also increased but the absolute amount of this increase (2.1 mg. P per 100 cc.) is less than that of the total organic acid-soluble P.

(6) It would seem most probable therefore that a synthesis of organic phosphorus compounds from the inorganic phosphates takes place in the corpuscles after insulin, and it is reasonable to assume that in this synthesis the sugar which simultaneously disappears is also concerned. This does not necessarily imply that the phosphoric ester thus formed is a simple hexose derivative. It may be of a more complex nature—possibly a nucleotide [cf. Jackson, 1923].

¹ Our previous results have been given as mg. P per 100 g. For the insulin experiments the blood was usually measured and the results are given per 100 cc. in order to simplify the correction for blood volume changes.

(7) On the basis of one phosphoric group per molecule of glucose 4.3 mg. P would account for 25 mg. glucose or 39 % of the sugar disappearing from the blood.

In such an investigation as this in which different animals must necessarily serve as controls it is obviously wise to draw conclusions only from the averages of as large a number of individual experiments as possible. The above conclusions may however be illustrated by taking the results of a single experiment on three young rabbits (12 weeks old) from the same litter.

A. Wt 755 g. Control.

B. Wt 735 g. Received 1.5 cc. insulin. Killed 2 hours later.

C. Wt 860 g. " " " 3 "

All three animals fasted during 24 hours before the experiment. Convulsions had not set in at death of either animal. The results obtained with the whole blood and plasma are shown in Table VI.

Table VI. *Whole blood.*

	A (control) %	B (insulin) %	C (insulin) %
Hb before injection	—	69	72
Hb at death... ..	69	56	58
Sugar	.138	.048	.048
Mg. P per 100 cc.			
Inorganic PO ₄	8.2	6.5	6.6
Total acid-soluble P	38.0	34.4 [42.2]*	36.7 [45.6]
Organic acid-soluble P	29.8	27.9 [34.4]	30.1 [37.5]
Ester hydrolysable by bone	6.5	6.4 [7.9]	5.0 [6.2]
Increase in absolute amounts of organic P	—	[4.6]	[7.7]
Glucose equivalent to above (1 mol. glucose:1mol. H ₃ PO ₄)	—	27 mg. = 33 % of sugar disappearing	45 = 54.7 % of sugar disappearing

Plasma.

Inorganic PO ₄	10.2	6.7	6.7
Total acid-soluble P	10.2	6.7	6.7
Organic acid-soluble P	0	0	0

* The figures in brackets show the amounts of P corrected for blood dilution as shown by the change in Hb $\left(\text{i.e.} \times \frac{\text{Hb before injection}}{\text{Hb at death}} \right)$.

Some further light is thrown on this question by the results of recent experiments of Lawaczek [1924], Martland and Robison [1924], Martland, Hansman and Robison [1924], who have shown that blood contains an enzyme which can effect both the hydrolysis and synthesis of phosphoric esters *in vitro*, the direction of the reaction depending on the conditions as regards p_{H} , etc.

It is likely that the blood corpuscles play a more important rôle in sugar metabolism than is usually attributed to them. When we consider that in the yeast cell a relatively small amount of phosphate through a continuous

cycle of reactions, effects the metabolism of large amounts of carbohydrate the significance of the figures in Tables V and VI becomes emphasised. It must be remembered also that although the greater part of the phosphoric esters in the corpuscles are apparently not simple hexose derivatives and are non-reducing (cf. Goodwin and Robison) it is quite possible and even likely that a carbohydrate nucleus is present in their molecules, as for example in the nucleotides whose occurrence in blood has been confirmed by Jackson [1924]. It is an interesting calculation that if each atom of organic acid-soluble P in the corpuscles is associated with one molecule of sugar there is in 100 cc. of blood no less than 160–200 mg. of hidden sugar thus bound up, i.e. considerably more than there is reducing sugar in normal blood.

Changes in the muscle.

The changes in the phosphorus partition in the muscle were examined at the same time, and were found to be as definite as in the blood. Considerable increases were found in the "lactacidogen" phosphoric acid of muscle after insulin whilst the inorganic phosphate and the total acid-soluble phosphorus remained practically unchanged.

The method employed was as follows. Two comparable rabbits usually from the same litter were given the same diet for a preliminary period of several days. They were then starved for about 18 hours and one was given an injection of sufficient insulin to produce convulsions in about 3 hours, the other being given the corresponding quantity of saline. The two animals were then watched until signs of the approach of convulsions were noticed in the insulin animal, when it was killed by bleeding (without anaesthetic). The gastrocnemius and soleus muscles free from connective tissue were immediately removed, weighed and divided exactly into halves, one half being immediately macerated with an ice-cold 2.5 % solution of trichloroacetic acid and sand in an ice-cold mortar. The time from the death of the animal to the complete maceration of the muscle was usually from 3 to 5 minutes. The volume of the mixture (M.T.) was made up to 40 cc. per g. of muscle. The other half of the muscle was ground up with sand and ice-cold water and made up to the same volume (M.A.). In some of the experiments a 0.25 % solution of NaHCO_3 was used instead of distilled water. When this was not done the mixture was neutralised with NaOH (to phenolphthalein) either immediately or after 3 hours at 38° .

The control animal was treated in exactly the same way within a short time of the death of the insulin animal, and the autolysis mixtures (M.A.) from both were then kept at 38° in presence of chloroform.

The trichloroacetic acid mixtures (M.T.), after 3 hours at room temperature with occasional shaking, were filtered and neutralised, and the inorganic phosphate then estimated by the Briggs modification of the Bell-Doisy method. They were also treated with the bone and muscle enzyme preparations according to the technique already described for blood filtrates, and the amount of organic phosphate hydrolysed was determined after 18 hours at 38° and p_{H} 8.4.

The earlier experiments showed somewhat greater amounts of hydrolysis in the muscle extracts from the animals which had received insulin than in those from the controls, but later experiments have rendered this less certain, and the average of eight experiments (Table VII) shows little difference in the amounts of hydrolysis under parallel conditions either by the bone or the muscle enzyme. In the case of the bone enzyme the hydrolysis at this stage was practically complete, only a very slight further increase being obtained after another 18 hours at 38°.

Table VII.

	Amount of hydrolysable acid-sol. P in mg. per 100 g. muscle		Average of 8 exps. Average of 6 exps.
	Control	After insulin	
Bone enzyme, 18 hours	74	76	
Muscle enzyme, 18 "	11	12	
" " 36 "	19	18	" "

With the muscle enzyme, however, slow hydrolysis was still proceeding at the end of 36 hours, and the results are only of significance in suggesting that the greater portion of the phosphoric ester extracted from muscle by trichloroacetic acid is not identical with the hexosediphosphate obtained by the action of yeast juice on sugar, since this ester would have been much more rapidly hydrolysed by the muscle extract used.

Although the use of the bone and muscle enzymes on trichloroacetic extracts yielded these equivocal results, the data obtained by the autolysis of the other halves of the muscle, macerated with water or NaHCO₃ solution, gave more definite evidence of changes in the nature and amounts of the phosphoric esters present at the convulsive stage following insulin injection.

Samples were taken from the muscle autolysis mixtures (M.A.) at intervals, usually after 3 hours, 8 hours, and at 60 hours, when the inorganic phosphate and total phosphorus were determined in aliquot portions of the mixture after precipitation of proteins with trichloroacetic acid and filtration.

As a result of ten experiments the following average figures were obtained:

Table VIII.

		Inorganic phosphate (mg. P) per 100 g. muscle					Total P in solution at 60 hrs.
		0	3	8	12	60	
Control animal	...	95	140	144	*	172	194
Increase	...		45	49		77	
Insulin animal	...	92	161	164		179	196
Increase	...		69	73		87	
Difference (Insulin-Control)		-3	+21	+20	+16	+7	+2

* A few determinations at 12 hrs. gave an average difference of 16 mg.

It will be seen from these figures that:

(1) The amount of inorganic phosphate in rabbit muscle is not appreciably changed after the administration of insulin (but before the onset of convulsions).

(2) The amount of total acid-soluble phosphorus (present after 60 hours' autolysis) is also not changed by insulin.

(3) The amount of combined phosphoric acid which is rapidly set free during autolysis is considerably increased (nearly 50 %) after insulin.

(4) After more prolonged autolysis of the macerated muscle, the difference between the amounts of inorganic phosphate set free in both series (insulin and control) becomes less, and approaches zero after 60 hours at 37°.

During the long-continued autolysis, phosphoric acid may be liberated by the slow action of other enzymes on a variety of organic phosphorus compounds so that the chief significance of our results seems to us to lie in the marked difference between the amounts of phosphate liberated during the first few hours (the "lactacidogen" P).

The increase in rapidity of hydrolysis of the organic phosphate in the macerated muscle aroused the suspicion that insulin might possibly act as a co-enzyme to the phosphoric esterase of muscle, assisting in the synthesis of the ester *in vivo* and in its hydrolysis under the conditions of our experiments *in vitro*. This attractive hypothesis was tested in two experiments with potassium hexosediphosphate solution and muscle enzyme (derived from normal muscle) but the results gave not the slightest indication that insulin had any co-enzymic action. The possibility that the phosphoric esterase of muscle might be otherwise more active after the injection of insulin was also investigated, but no appreciable difference was found, using potassium hexosediphosphate as substrate.

According to Embden the lactacidogen is either hexosediphosphate or a very closely related compound. The rise in rapidly hydrolysable phosphate we may interpret as indicating that the lactacidogen is increased in muscle after the administration of insulin. The source of the phosphorus for the increased amount of this substance appears to be not the blood phosphate but the other phosphoric acid complexes (possibly nucleic acid) in the muscle which are much less readily autolysed than is lactacidogen by the muscle enzymes.

We have no evidence that the carbohydrate portion of the lactacidogen was not also present in the muscle before the administration of insulin, e.g. such a compound of sugar and phosphoric acid might conceivably be formed from a nucleotide by the removal of the purine or pyrimidine group. If, however, the sugar for the increased synthesis of this substance is taken up from the blood the rise in lactacidogen would account quantitatively for much more than the whole of the sugar which thus disappears.

If we assume that the muscles of a 2 kg. rabbit together weigh 800 g. and that the percentage increase of lactacidogen is the same throughout, an increase of 8×21 mg. combined P is found in the muscles, using the 3-hour figure from Table VIII.

If this phosphate is combined with sugar in the molecular ratio of 1 : 1 (hexosemonophosphate) this would account for 980 mg. glucose, or if the

ratio is 2 : 1 (hexosediphosphate) for 490 mg. The actual amount of sugar which disappears from the blood of a 2 kg. rabbit, previously calculated, is only 65 mg. but this does not allow for mobilisation of sugar from glycogen or other reserves¹.

The evidence afforded by our experiments is insufficient to enable us to come to any definite conclusion as to the correctness of this hypothesis but the recent experiments of Dale [1924], Dudley and Marrian [1923] and of Winter and Smith [1924] and others have shown that the sugar which disappears from the blood is neither oxidised nor excreted nor found as increased glycogen nor as fat in the tissues, even when isolated perfused organs are used, but is stored in some unknown form.

We think that the results shown in this paper strengthen the probability that in the mechanism of this storage the phosphoric group plays an important part and that the stored compound itself may prove to be a phosphoric ester.

While these experiments were in progress a short preliminary notice was published by Audova and Wagner [1924, 1, 2] outlining the results of five experiments done in Lemberg in which similar findings had been made as far as the readily autolysable organic phosphoric acid complex in muscle was concerned. The increase in lactacidogen P was of the same order as in our experiments. Their technique was different from our own, and their phosphate determinations seem uniformly a little higher.

Approaching the subject from a different side Harrop and Benedict [1924] have still more recently found similar results to follow insulin injection, though using a technique which might be expected to cause serious changes in the blood phosphate as the effect of shock [cf. Martland and Robison, 1924].

(A decimal point appears to have gone astray in all the muscle phosphate figures given in their Table III.)

SUMMARY.

The phosphoric esters of blood have been investigated by means of the muscle enzyme which hydrolyses hexosediphosphoric esters but has scarcely any action on hexosemonophosphoric ester. While the results indicate that an ester of the hexosediphosphoric type may occur in blood the evidence on this point is not entirely conclusive.

It is, however, certain that the greater part of the organic phosphate which is hydrolysable by the bone enzyme is not hydrolysed by muscle. In this respect the ester therefore satisfies the important condition of a specific substrate in the scheme of ossification.

Changes in the distribution of phosphorus compounds in blood and in muscle after the administration of insulin have also been investigated.

¹ The effect of blood volume on these quantitative relationships is small. If the volume of blood increases from 110 to 126 cc. and if this fluid comes entirely (though this is most unlikely) from the muscular tissue the maximum increase in the concentration of indiffusible substances in the muscle would not exceed 2 %, which is within the experimental errors of the method.

From the average results of 11 experiments on rabbits and taking into account the increase in the blood volume, evidence is produced that after the administration of insulin, organic phosphoric esters are synthesised in the corpuscles at the expense of the inorganic phosphate originally present in the blood plus additional phosphate drawn from the other tissues.

These esters are chiefly of the type not hydrolysed by the bone enzyme but it is none the less possible and is even probable that sugar or a sugar derivative enters into their molecule.

On the basis of one molecule glucose per atom of P the phosphoric acid thus esterified would account for nearly 40 % of the sugar simultaneously disappearing from the blood.

No changes were found in the amount of inorganic phosphate or total acid-soluble phosphorus in muscle, following insulin administration, but there was a marked increase (50 %) in the amount of phosphoric esters ("lactacidogen" P) hydrolysed during 3 hours' autolysis of the macerated muscle at 38°.

The phosphate for this increased "lactacidogen" is apparently drawn from the other phosphorus compounds in the muscle, but we have no evidence as to the origin of the carbohydrate portion of its molecule.

Assuming that lactacidogen is a hexosediphosphate the increased amount found in muscle after insulin would, if the hexose were derived from the blood sugar, account for more than seven times the amount of glucose which disappears from the blood.

REFERENCES.

- Audova and Wagner (1924, 1). *Klin. Wochensch.* **3**, No. 6.
 — (1924, 2). *Compt. Rend. Soc. Biol.* **90**, 308.
 Dale (1924). Communication to Physiological Soc., Feb. 16.
 Dudley and Marrian (1923). *Biochem. J.* **17**, 435.
 Goodwin and Robison (1924). *Biochem. J.* **18**, 1161.
 Haldane, Kay and Smith (1924). *J. Physiol.* In press.
 Harrop and Benedict (1924). *J. Biol. Chem.* **59**, 683.
 Jackson (1923). *J. Biol. Chem.* **57**, 121.
 — (1924). *J. Biol. Chem.* **59**, 529.
 Kay and Robison (1924). *Biochem. J.* **18**, 755.
 Lawaczek (1924). *Biochem. Z.* **145**, 351.
 Martland and Robison (1924). *Biochem. J.* **18**, 765.
 Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
 Robison (1923). *Biochem. J.* **17**, 286.
 Robison and Soames (1924). *Biochem. J.* **18**, 740.
 Wigglesworth, Woodrow, Winter and Smith (1923). *J. Physiol.* **57**, 447.
 Winter and Smith (1924). *J. Physiol.* **57**, Proc. xxxi.