CIX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

I. THE RELATIONSHIP BETWEEN PHOSPHORIC ESTER ACCUMULATION AND PHOSPHORIC ESTER BREAK-DOWN AND LACTIC ACID FORMATION FROM GLYCOGEN.

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THE experiments recorded in this communication are part of an investigation into lactic acid and phosphoric ester formation from carbohydrates in muscle extract. The particular experiments recorded below deal with the supposed relationship of a quantitative nature between the lactic acid formation and the phosphoric ester accumulation and breakdown that accompany that process. Gottschalk [1926] gives a résumé of this subject and sets forth a series of reactions to explain the experimental results of Meyerhof. These results showed that in lactic acid formation from glycogen in muscle extract there are two distinct periods. In the first period there occurs a high rate of lactic acid formation and an accumulation of phosphoric ester. The extent of this accumulation is conditioned by the amount of carbohydrate or the amount of available phosphate. In the second period this accumulated ester is broken down and the rate of lactic acid formation diminishes. It is postulated that in the first period, for every two molecules of diphosphoric ester formed, one is immediately broken down to the end-product, lactic acid, while the other is "stabilised" and accumulates. This implies that for every two molecules of lactic acid formed, two molecules of inorganic phosphate are retained as ester, giving a molar ratio of lactic acid to phosphate retained as ester of unity. Since it is also put forward that the lactic acid formed in the second period originates from the ester accumulated in the first period, the molar ratio of lactic acid to increase in inorganic phosphate should again be unity.

It is to this aspect of the problem that the following experiments apply. Attention is drawn to the fact that the extracts employed here are, relatively speaking, fresh and concentrated. These extracts show different phenomena from those that have been kept for a short time at 37° prior to the addition of glycogen. This will be made the subject of a subsequent communication.

EXPERIMENTAL PROCEDURE.

Cats were killed by a blow on the back of the head and were immediately perfused until free from blood, first with warm and then with cold Ringer's solution. The excised muscles were minced and then put through a grinding mill. The muscle pulp was ground up with silver sand in a mechanical mortar in lots of 350 g. A solution containing sodium bicarbonate and sodium chloride, both to the extent of 0.5 % and cooled to freezing point, was added in portions until about 350 cc. had been added in all. From the mortar the mixture was poured into a sheet of muslin and the extract was squeezed out in a hand-press. The combined extract was then frozen with stirring and filtered through a Büchner funnel without paper, thus separating the extract from the ice crystals. Repetition of this process soon reduces the volume to any desired extent. As a rule it took about 90 minutes to get the extract ready for incubation but every precaution was taken to keep the temperature low.

Before incubation more phosphate was added to the extent of 5 cc. of a 3 % solution of NaH_2PO_4 per 100 cc. extract. The reaction was adjusted if necessary to about p_{H} 7.2 by the addition of a saturated solution of sodium bicarbonate. In the experiments described below, the extract was divided up into lots of 160 cc. and placed in the incubation flasks. As 5 cc. were required for phosphate estimation and 10 cc. for the lactic acid estimation, one required for every withdrawal, at the appointed intervals, about 20 cc. The requisite volume of a freshly prepared 10 % solution of glycogen was measured out into very small flasks, water being added in the cases of the lower concentrations in order that the volume of fluid added might be the same in every case. The contents of the incubation flask were rapidly warmed to 37° , the glycogen solution was added and the mixture thoroughly shaken up. First, 5 cc. were pipetted out into 25 cc. of 2 % trichloroacetic acid and the mixture was at once frozen solid. It was kept in this condition until the phosphate analysis was done. Immediately after this 10 cc. were pipetted into a dry flask and 30 cc. of a tungstic acid solution added. (This tungstic acid was prepared just before the precipitation by mixing 10 cc. of a 5 % solution of sodium tungstate and 25 cc. of 0.5 *N* sulphuric acid.) The time was noted by an observer and the incubation flask immersed in a large water-bath at 37° . Each flask was fitted with a stopper holding three tubes. One reached to the bottom of the flask and through this was passed a stream of nitrogen, previously bubbled through a pyrogallol-potassium hydroxide solution. The second tube was a short elbow tube, not dipping into the extract, and served as a gas outlet. The third tube, like the first, dipped well under the extract; by closing the gas inlet by compressing the rubber connection and blowing gently through the gas outlet the extract could be siphoned out of the flask when it was desired to take a sample. If the second sample was to be taken 10 minutes after the start of the incubation only four incubation flasks could be used simultaneously with comfort, allowing for the time required for warming up the extract and for the pipetting. The

times of withdrawal were indicated by an assistant and were correct to a quarter of a minute.

The inorganic phosphorus was estimated by the method of Fiske and Subbarow [1925]. The colour was allowed to develop for 20 minutes in order to convert into inorganic phosphorus the whole of the labile phosphorus of phosphagen which was therefore included in the determinations of "inorganic phosphorus." The term "inorganic" phosphorus is thus really a misnomer, but as it was intended to investigate lactacidogen formation and breakdown, decrease and increase of "inorganic" phosphorus as estimated by the above method can be taken as indication of lactacidogen accumulation and breakdown. Under the above conditions lactacidogen and hexosediphosphoric acid are stable.

The lactic acid was estimated by the method of Clausen [1922], taking advantage of the improvements suggested by Shaffer [1927]. Interfering carbohydrates were removed by the usual copper-lime method. 20 cc. of the tungstic acid filtrate were accurately pipetted into a 50 cc. volumetric flask, 10 cc. of a 7 % lime suspension added, followed by 6 cc. of a 7 % copper sulphate solution. After shaking at intervals for half an hour, 2 cc. more of the lime suspension were added and the volume made up to 50 cc. This gives a final dilution of the extract of 1 in 10. 10 cc. of the filtrate of this were used for the oxidation by 0.005 *N* KMnO_4 . The aldehyde was aerated into two tubes, the first holding 30 cc. of 0.5 *N* bisulphite and the second 20 cc. The excess of the unbound sulphite was titrated first with 0.1 *N* iodine and finally with 0.005 *N* iodine until just blue. Clausen underestimates the error incurred by overstepping this first end-point. The bound sulphite was titrated, with the gradual addition of saturated sodium bicarbonate solution, with accurate 0.005 *N* iodine. The results are based on a 95 % yield of acetaldehyde.

EXPERIMENTAL RESULTS.

The values for lactic acid and for "inorganic" phosphorus are expressed in the following tables as milligram-molecules or milligram-atoms respectively per 100 cc. extract multiplied by 100. Thus a concentration of 90 mg. lactic acid is represented by 100 as is also a concentration of "inorganic" phosphorus of 30.96 mg. per 100 cc. extract. A change of one unit in the tables indicates, for lactic acid, a change in concentration of 0.9 mg. and for phosphorus 0.3 mg. per 100 cc. extract. The figures can thus be used as molar quantities in any calculations.

Exp. 237.

Time	A. Glycogen 0.4 %		B. Glycogen 1.0 %	
	Extract	200 cc.	Extract	200 cc.
	10 % glycogen	10	10 % glycogen	25
	Water	15		
	Lactic acid	Phosphorus	Lactic acid	Phosphorus
Start	139	163	139	165
10 mins.	212	143	244	100
20 "	275	113	304	45
30 "	319	43	337	18
40 "	335	41	354	24
50 "	349	49	373	35
60 "	373	67	397	50
90 "	402	102	447	97

Time	A. Glycogen 0.3 %			B. Glycogen 0.6 %			C. Glycogen 0.9 %		
	Extract	10 % glycogen	Water	Extract	10 % glycogen	Water	Extract	10 % glycogen	Water
	160 cc.	5	19	160 cc.	10	14	160 cc.	15	9
	Lactic acid		Phosphorus	Lactic acid		Phosphorus	Lactic acid		Phosphorus
Start	214		176	214		177	221		177
10 mins.	264		124	267		98	273		70
20 "	293		113	283		24	291		17
30 "	317		107	299		28	304		25
40 "	340		122	315		48	321		43
50 "	363		148	333		68	329		69
60 "	371		165	344		83	351		91
90 "	381		200	356		164	366		171

Time	A. Glycogen 0.4 %			B. Glycogen 1.0 %		
	Extract	10 % glycogen	Water	Extract	10 % glycogen	Water
	160 cc.	8	24	160 cc.	20	12
	Lactic acid		Phosphorus	Lactic acid		Phosphorus
Start	222		180	220		190
10 mins.	289		122	309		86
20 "	330		150	370		93
30 "	373		152	421		116
40 "	412		156	464		132
50 "	452		169	518		145
60 "	473		191	577		149
90 "	480		218	707		154

Period of ester accumulation.

As the results show, the period of ester accumulation is a very variable quantity both in extent and duration. Exps. 237, 240 and 241 are considered in detail. The increase in lactic acid and the decrease in phosphorus are given for each interval examined and by dividing the former by the latter, the molar ratio of lactic acid formed to organic phosphorus accumulated as ester at the end of each period is obtained.

Time interval (mins.)	Glycogen 0.4 %			Glycogen 1.0 %			L.A.	Phos.	L.A. Phos.
	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.			
0-10	+73	-20	3.65	+105	-64	1.61			
10-20	+63	-30	2.10	+60	-55	1.09			
20-30	+44	-70	0.51	+33	-27	1.22			
0-30	+180	-120	1.50	+198	-147	1.35			

Time interval (mins.)	Glycogen 0.3 %			Glycogen 0.6 %			Glycogen 0.9 %		
	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.
0-10	+50	-52	0.96	+53	-79	0.68	+52	-107	0.49
10-20	+29	-11	2.63	+16	-74	0.22	+18	-53	0.34
0-20	+79	-63	1.25	+69	-93	0.74	+70	-160	0.44

Time interval (mins.)	Glycogen 0.4 %			Glycogen 1.0 %		
	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.
0-10	+67	-58	1.16	+89	-104	0.86

These three experiments were made with extracts prepared in exactly the same manner from three different animals. The comparison between the low and the high concentrations of glycogen with the same extract indicates that

above a certain concentration of glycogen, ester accumulation is more powerfully influenced by the glycogen concentration than is the lactic acid formation. In Exp. 240 this is particularly so. In the first 10 minutes the lactic acid formation is practically the same for concentrations of 0.3, 0.6 and 0.9 % of glycogen, while, on the other hand, the ester accumulation with 0.9 % glycogen is double that with 0.3 %. With one exception, increasing the glycogen concentration leads to a decrease of the ratio of lactic acid to phosphorus accumulated as ester, by increasing the latter quantity out of proportion to the former.

Period of breakdown of the accumulated ester.

Analyses as above are now given for the second period with the same three experiments, the lactic acid formation and the phosphorus increase for every interval being compared.

Exp. 237.	Glycogen 0.4 %			Glycogen 1.0 %					
Time interval (mins.)	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.
40-50	+14	+ 8	1.75	+19	+11	1.73			
50-60	+24	+18	1.33	+24	+15	1.80			
60-90	+29	+35	0.83	+50	+47	1.06			
40-90	+67	+61	1.09	+93	+73	1.27			
Exp. 240.	Glycogen 0.3 %			Glycogen 0.6 %			Glycogen 0.9 %		
30-40	+23	+15	1.53	+16	+20	0.80	+17	+18	0.94
40-50	+23	+26	0.80	+18	+20	0.90	+ 8	+26	0.30
50-60	+ 8	+17	0.47	+11	+15	0.73	+22	+22	1.00
60-90	+10	+35	0.28	+12	+81	0.15	+15	+80	0.19
30-90	+64	+93	0.69	+57	+136	0.42	+62	+146	0.42
Exp. 241.	Glycogen 0.4 %			Glycogen 1.0 %					
10-20	+41	+28	1.48	+61	+ 7	8.71			
20-30	+43	+ 2	21.5	+51	+23	2.21			
30-40	+39	+ 4	9.9	+43	+16	2.69			
40-50	+40	+13	3.07	+54	+13	4.15			
50-60	+21	+22	0.95	+59	+ 4	14.7			
60-90	+ 7	+27	0.26	+130	+ 5	26.0			
10-90	+191	+96	2.0	+398	+68	5.85			

In this period the above ratio is a very variable quantity. The onset of the breakdown of the ester accumulated in the first period is not in every case due to lack of glycogen but occurs in the presence of excess of glycogen. As a general rule, well demonstrated in Exp. 241, the value of the ratio diminishes as the glycogen concentration falls. When all the glycogen has been used up the ratio is invariably less than unity.

Two special cases are now given. The results of Exp. 239 are given in the accompanying graphs. Fig. 1 indicates the changes in "inorganic" phosphorus and Fig. 2 the changes in lactic acid both at intervals of 10 minutes, expressed in the same units as above. The peculiar feature here is the very slight accumulation of ester in the first 10 minutes with all three concentrations of glycogen but followed in the case of 1.0 % glycogen by a second

Fig. 1. Exp. 239. Phosphorus changes with glycogen concentrations of 0.25 %, 0.5 % and 1.0 %.

Fig. 2. Exp. 239. Lactic acid changes with glycogen concentrations of 0.25 %, 0.5 % and 1.0 %.

Fig. 3. Phosphorus changes of Exp. 243. No. 2 extract more concentrated than No. 1.

Fig. 4. Lactic acid changes of Exp. No. 243.

period of extensive ester accumulation. Contrasting 0.5 % and 1.0 % glycogen, it is shown that despite the great difference in behaviour of the phosphorus curves in the last 50 minutes, the lactic acid curves in these two cases are not very markedly different and the great ester accumulation with 1.0 % glycogen is not reflected in the corresponding lactic acid curve as it would be if these two processes were intimately related. Again, the difference between 0.25 % and 0.5 % glycogen is not so great in the phosphorus curves as it is in the lactic acid curves.

This same result was produced in Exp. 243 in the following manner. The muscles from two cats were mixed. Of the combined weight, 500 g. were extracted and gave 650 cc. extract, which was reduced by freezing to 450 cc. This is extract No. 1. The remaining 1200 g. muscle were extracted with a relatively smaller volume of extracting solution and yielded a litre of extract. This extract, No. 2, was not frozen at all. The p_H of both was adjusted to 7.3. The glycogen concentration in both cases was 0.3 %. The results are shown in Figs. 3 and 4. The outstanding feature here is the absence of ester accumulation in No. 2 extract. In No. 1 extract, on the other hand, ester accumulation is quite decided. On comparing the lactic acid curves it is seen that a high rate of lactic acid formation is not conditioned by, or related to, ester accumulation. This experiment demonstrates the danger of generalising from the results of extract No. 1, when by a slight alteration in the method of preparing the extract from the same batch of muscle, the results of No. 2 can be produced.

SUMMARY.

In the type of extract of cat muscle used in this investigation it has been found that phosphoric ester accumulation is not an essential accompaniment of lactic acid formation from glycogen. When ester accumulation does occur, there is no definite constant ratio of the molar amount of lactic acid produced to the molar amount of phosphorus that accumulates as ester. In the second period, when the ester that accumulates in the first period is broken down, the molar ratio of lactic acid produced to phosphorus set free is also very variable.

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