LI. THE NATURE OF THE ACTION OF PANCREATIC EXTRACT ON THE INHIBITION OF LACTIC ACID FORMATION IN MUSCLE.

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THE fact that the formation of lactic acid in muscle is inhibited by an extract from the pancreas was first observed by Winfield and Hopkins [1915]. Foster and Woodrow [1924] pointed out that the factor responsible for the inhibition was neither trypsin, insulin, nor Cohnheim's [1904] glycolytic factor; and they also suggested that it was not the antiglyoxalase of Dakin and Dudley [1913]. Foster [1925], after further work, which was however criticised by Dudley [1926], definitely concluded that the pancreatic factor was not antiglyoxalase. Since that time research in this laboratory by other workers [Reay, 1927; Downes, 1927] has made it seem desirable to add confirmation to portions of Foster's work.

Meyerhof [1926, 1] has recently demonstrated that under proper conditions the muscle enzymes responsible for the formation of lactic acid can be removed from the muscle in aqueous or saline solutions. This discovery opened new paths for the study of the pancreatic factor of Winfield and Hopkins. During the course of the research recorded in this paper Ronzoni [1927] published a preliminary report concerning the efficacy of the pancreatic factor in its action on the soluble enzyme system. Her results are in agreement with the preliminary results included in this paper, except that she states that there is no change in the inorganic phosphate when the formation of lactic acid by the enzymes from muscle is inhibited by the pancreatic factor. Her protocol, however, does not entirely bear out this statement.

METHODS.

Preparation of the soluble lactic acid-forming enzymes from rabbit muscle is carried out as follows. The animal is anaesthetised by an intraperitoneal injection of amytal (75 mg. per kg. body weight). The hind legs are cooled in an ice-bath before killing the animal by bleeding. The muscles of the hind legs are then removed rapidly in a chamber at 0°, and minced in an ice-cold mincing machine. They are then extracted for half an hour with three times their weight of distilled water previously cooled to 0°. The extract is filtered through muslin and the filtrate used without further treatment. Fresh muscle extract was prepared immediately before use.

The following pancreas preparations are used.

(*a*) Desiccated pancreas as described by Foster and Woodrow [1924].

(*b*) One gram of (*a*) extracted for one hour at room temperature with 13·2 cc. of distilled water. This is made up to a total volume of 50 cc. with 95 % alcohol. The preparation is then completed according to Foster's directions, the final volume being 25 cc.

The following buffers are used.

(*a*) Phosphate buffer:

$NaHCO_3$	...	...	0·12 *M.*
K_2HPO_4	...	...	0·04 *M.*
NaCl	...	...	0·02 *M.*

(*b*) Fluoride buffer: the same as the above with the addition of NaF ... 0·10 *M.*

Methods of estimation. 25 cc. of muscle extract are placed in a flask together with 10 cc. of 2 % Lintner's soluble starch, 10 cc. buffer (phosphate unless otherwise stated), and 5 cc. pancreatic extract (or 5 cc. distilled water for the control flasks). After mixing, a sample of 15 cc. is removed at once and delivered into 20 cc. of 6 % hydrochloric acid, and a sample of 1 cc. delivered into 10 cc. of 10 % hydrochloric acid. The mixture is then incubated for 4 hours in a water-bath at 30°, and again sampled in the same fashion.

Proteins are removed from the 15 cc. sample by the addition of 25 cc. of 5 % mercuric chloride, allowing to stand over night before filtering. Saturated potassium hydroxide solution is added to the filtrate until a faint yellow permanent precipitate is obtained (usually 2·0 to 2·5 cc.). The mercury is removed with hydrogen sulphide and the latter by a current of moist air. Phosphates are estimated by the Briggs [1922] modification of the Bell-Doisy method, results being expressed as mg. of phosphorus in 15 cc. of the original mixture.

Sugars are removed from 30 cc. of the hydrogen sulphide-free filtrate by the addition of 10 cc. of 10 % calcium hydroxide suspension and 5 cc. of 10 % copper sulphate. Lactic acid is determined in the filtrate by oxidising with potassium permanganate, by the method of Meyerhof [1920]. Results are expressed as mg. of lactic acid in 15 cc. of the incubation mixture. In calculating the lactic acid and phosphate content no correction has been applied for the change in volume introduced during neutralisation with potassium hydroxide. This introduces a small error (2 cc. added to 60) which is partially compensated for by evaporation during the removal of hydrogen sulphide. This error, of course, is constant and occurs in every determination.

The 1 cc. sample is used for the determination of total carbohydrate as it was found that the starch present does not go through the filter quantitatively during the protein precipitation of the larger sample. The carbohydrates are hydrolysed by boiling on a water-bath for 2½ hours, filtered and neutralised and the reducing power is estimated by the method of Hagedorn and Jensen [1923]. Results are expressed as mg. of glucose in 15 cc. of the incubation mixture.

Experimental.

The first portion of the investigation was conducted in order to confirm the fact that the inhibiting factor is not trypsin. Earlier workers, using muscle hash, caused 40 to 60 % inhibition in the production of lactic acid by the addition of pancreas. As can be seen from the tables below, when pancreas is added to the soluble enzyme system, formation of lactic acid is completely prevented. The difference is undoubtedly due to the elimination of factors such as permeability. The fact that the inhibition is complete and instantaneous would indicate a probability that the action is not due to tryptic proteolysis.

A 70 % alcoholic extract of the desiccated gland prepared carefully according to the instructions of Foster contained no trypsin but caused no inhibition. Dakin and Dudley [1913] stated that incubation for 4 hours at 37° destroyed trypsin. As pointed out by Foster [1925], this is not so; moreover, it was found that the inhibitor was partially destroyed during this treatment. However, preparation (*b*) above showed no tryptic activity during the course of an incubation at 30° for 4 hours and was quite active as an inhibitor as is shown by the Tables. Tryptic activity was tested for (*a*) by the biuret test on the protein-free filtrate after incubation, and (*b*) by the ability of the preparation to change dilute caseinogen solution to compounds not precipitated by acetic acid. The same method of preparation was used throughout.

That this factor does not act merely as an antiglyoxalase is shown by the following work which demonstrates that the inhibition in the formation of lactic acid is due to the failure of the system to form hexosephosphate. Mr J. B. S. Haldane has suggested the possibility that the same factor might prevent the formation of hexosephosphate and also act as an antiglyoxalase. If the mechanism is that of inactivating the co-enzymes, this dual action is quite possible.

During preliminary work it was noted that when the formation of lactic acid was prevented by the use of the pancreatic factor the total reducing power was not changed. Table I contains some typical results.

Table I.

	mg. lactic acid in 15 cc.			mg. total carbohydrates in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	10·6	22·1	+11·5	68·0	50·0	−18·0
Pancreas (*b*)	9·7	10·2	+ 0·5	70·0	70·7	+ 0·7
2. Control	6·8	19·1	+12·3	70·6	54·2	−16·4
Pancreas (*b*)	7·3	7·1	− 0·2	71·9	71·9	0·0
3. Control	6·1	11·9	+ 5·8	69·7	59·5	−10·2
Pancreas (*b*)	5·8	6·6	+ 0·8	67·9	67·3	− 0·6

The hexosephosphates differ from glucose in their reducing power and some of them are sufficiently resistant to mineral acid hydrolysis to withstand the treatment employed for the estimation of total carbohydrates. These results,

therefore, suggested that the hexosephosphates were not being formed in the presence of pancreatic extract. A quantitative study of the phosphate changes showed this to be true.

As pointed out by Meyerhof [1926, 2], during the formation of lactic acid by the soluble enzyme system in phosphate buffer, the free phosphate usually decreases. The experiments in Table II demonstrate this, and also show that when the formation of lactic acid is prevented by the pancreatic factor there is a slight increase in free phosphate.

Table II.

	mg. lactic acid in 15 cc.			mg. P as free phosphate in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	10·2	25·9	+15·7	5·81	5·38	−0·43
Pancreas (*b*)	9·7	9·9	+ 0·2	5·81	6·67	+0·86
2. Control	8·2	18·2	+10·0	5·78	3·83	−1·95
Pancreas (*b*)	8·4	8·4	0·0	5·85	7·14	+1·29
3. Control	8·5	16·4	+ 7·9	5·95	5·03	−0·92
Pancreas (*b*)	10·4	9·7	− 0·7	5·81	7·01	+1·30

These results indicate that in the control flasks hexosephosphates are formed more rapidly than they undergo lactic fermentation, whereas in the presence of pancreas hexosephosphates present in the muscle extract are broken down. Eggleton and Eggleton [1927] have shown that the Briggs modification of the Bell-Doisy methods for the estimation of phosphates determines also the phosphate present as phosphagen. Any possibility that the increase in the free phosphate, shown in these experiments, is caused by phosphagen hydrolysis, is eliminated since the method of estimation includes the phosphagen phosphate both before and after incubation.

Embden and Hayman [1924] showed that the addition of sodium fluoride to hashed muscle caused a marked decrease in free phosphate which was apparently transformed into hexosephosphate. Davenport and Cotonio [1927] demonstrated that the same thing occurred when sodium fluoride was added to the soluble enzyme system of Meyerhof. The results in Table III confirm those of Davenport and Cotonio and in addition show that in the presence of pancreas there is no disappearance of free phosphate.

Table III.

	mg. lactic acid in 15 cc.			mg. P as free phosphate in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	8·6	22·0	+13·4	5·9	3·6	−2·3
Fluoride	7·2	9·3	+ 2·1	5·9	1·3	−4·6
Fl. + pan. (*b*)	8·3	7·5	− 0·8	5·9	6·2	+0·3
2. Control	8·3	22·1	+11·8	5·9	3·5	−2·4
Fluoride	7·7	9·2	+ 1·5	5·6	1·2	−4·4
Fl. + pan. (*b*)	9·2	6·8	− 2·4	5·9	6·4	+0·5

SUMMARY.

(1) The result of former workers in regard to the presence in the pancreas of a substance which inhibits the formation of lactic acid by muscle enzymes has been confirmed.

(2) When the pancreatic factor is added to an extract of muscle, containing lactic acid-forming enzymes, it (*a*) prevents the formation of lactic acid; (*b*) prevents the disappearance of carbohydrate; (*c*) causes an increase in free phosphate content; (*d*) prevents the esterification of phosphate even in presence of fluoride. This demonstrates that the action of the pancreatic factor in inhibiting the formation of lactic acid is that of preventing the formation of hexosephosphates and not in preventing their fermentation.

Research concerning the physical, chemical and physiological properties of the pancreatic factor is in progress. Present results seem to indicate that this substance is present in traces in many other tissues from various species of mammals.

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