LXXVI. THE RELATION BETWEEN THE PANCREAS AND THE CARBOHYDRATE METABOLISM OF MUSCLE.

I. THE EFFECT OF EXTRACTS FROM THE GLAND ON LACTIC ACID PRODUCTION *IN VITRO*.

BY DOROTHY LILIAN FOSTER
AND CYRIL ERSKINE WOODROW.

(Frank Smart Student, Gonville and Caius College.)

From the Biochemical Laboratory, Cambridge.

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THE first chemical research of importance which was inspired by Minkowski's classical discovery concerning the pancreatic function in metabolism was that of Otto Cohnheim [1904] who showed that the combined action of muscle tissue and a pancreatic extract on glucose leads to a fall in the reducing power. This result, which was confirmed and extended by the work of Hall [1907] was for some time interpreted as showing that a pancreatic factor determines the breakdown of sugar in the muscle. As is well known, however, Levene and Meyer [1911] showed that the reducing sugar disappears in these circumstances, as the result, not of a destruction, but of a synthetic process. They were able to demonstrate that a disaccharide is formed, and found that the original reducing power of the added sugar was restored after acid hydrolysis.

A somewhat different (though, almost certainly, related) effect of a pancreatic factor was later demonstrated by Winfield and Hopkins [1915] who showed that pancreatic preparations can inhibit the formation of lactic acid in chopped muscle. Owing to the circumstances of the time, this work could not then be continued. At Professor Hopkins' suggestion, we have now studied the phenomenon more closely.

The source of the pancreas as used by Winfield and Hopkins was found to be immaterial; the maximum effect was obtained by using a commercial preparation of pancreatin. The maximum inhibition of lactic acid formation that they report is one of 30 %, and the average is only 17 %, but it must be remembered that the only method of estimation then available was that devised by Fletcher and Hopkins. This requires relatively large quantities of muscle, and it is impossible during the removal and manipulation of so much mammalian muscle to prevent the development of considerable quantities of

lactic acid. There is no conclusive evidence given in their report that the inhibition of the lactic acid formation was not due to the simple digestion of the muscle by trypsin added with the pancreatic preparation, except that they state that the factor is heat-stable. An examination of their figures, however, shows that there was a considerable diminution of inhibitory power after boiling. In one case only a 2 % inhibition was found, and it has been shown by Mellanby and Woolley [1913], that in the presence of protecting proteins, trypsin can resist boiling for some time. It was to confirm the existence of this inhibitory factor, to show that it is not trypsin, and also to see if the inhibitor and insulin are one and the same substance, that this present work was undertaken.

PREPARATION OF THE GLAND EXTRACT.

It was considered advisable to use the same pancreatic preparation throughout the whole series of experiments. With this end in view the Hardy-Gardiner [1910] technique as modified by Young [1922] for obtaining a dry, unchanged, and soluble protein was applied to the pancreas. About 2 lbs. of pig's pancreas obtained from a commercial firm was freed from fat, minced, and placed in cold store at -6° overnight. The next day it was placed in a stoppered jar and covered with three to four times its volume of 97 % alcohol previously cooled to the same temperature. It was allowed to stand for two days with occasional shaking and the alcohol then changed. This was repeated once more with 97 % alcohol; then twice with an equal volume of absolute alcohol; finally twice with absolute ether, each time the minced gland being filtered through a fluted paper and replaced in the jar. The gland was then transferred to a dish and dried in a desiccator, all these processes being carried out at the same temperature, viz. -6° . Finally, still in the desiccator, it was brought up to the temperature of the laboratory, and then extracted for three days in a Soxhlet with absolute ether and dried in a vacuum desiccator. The dry residue was rubbed through a fine sieve to break up all lumps and remove connective tissue, etc.

The resulting white powder is hygroscopic, almost completely soluble in water or dilute saline giving a clear, faintly yellow, solution with a very high protein concentration, as shown by the heavy coagulum on boiling. It contains trypsin, amylase and lipase in high concentration and in a very active form. The presence in it of insulin has been demonstrated and also, as will be shown below, the factor responsible for the inhibition of lactic acid formation in muscle. Such a preparation has been kept in an ordinary stoppered bottle on the laboratory shelves for nine months with all its properties unimpaired, and there seems no reason why it should not keep indefinitely so long as moisture is excluded. This preparation is to be recommended as a method of making and storing trypsin and pancreatic amylase in a concentrated and stable form, and may prove useful in the case of other enzymes. It was hoped by using a perfectly fresh pancreas to obtain trypsin in the form of its

inactive precursor trypsinogen, but though a pancreas was obtained immediately on the death of the animal, and frozen at once, the resulting dry powder contained as much trypsin as the earlier specimen. In this preparation 150 g. of the fresh gland gave 25 g. of the dry extract before the sieving process, and 14 g. afterwards.

EXPERIMENTAL DETAILS.

Throughout this work beef was used and it was always prepared in a standard way. An assistant was present at the slaughter house when the beast was killed, and immediately on the death of the animal, a piece of the neck weighing about 2 lbs. was cut out and placed in a pan, surrounded by a freezing mixture, and was then brought straight to the laboratory. It remained untouched until the temperature had fallen to about 5° or 6°. The fat was then removed and a piece of muscle, as intact as possible, dissected out, minced, and at once cooled in ice. Portions of 4 g. were weighed out on a tared watch glass and transferred quantitatively to small (150 cc.) conical flasks containing the buffer solution, pancreatic extract, etc. The flasks were well shaken, closed by cotton wool plugs, and then placed in the thermostat, or allowed to stand at room temperature according to the conditions of the experiment. The buffer solution used was acid potassium phosphate, adjusted to the required hydrogen ion concentration by the addition of 0.2 *N* NaOH. The concentration of phosphate used was double that recommended by Clark and Lubs, as this gives a solution approximately isotonic with muscle and the optimum conditions for lactic acid formation [Meyerhof, 1921]. The system was made anaerobic, and therefore any loss of lactic acid by oxidation prevented, by the addition of potassium cyanide, 1 cc. of an *N*/100 solution being added to both control and experimental flasks. According to Meyerhof [1921] this also acts as an antiseptic, and renders unnecessary the addition of other preservatives, such as toluene, which have an inhibitory effect on the lactic acid production.

At the end of the period of incubation, whether in the water-bath or at room temperature, the whole series of flasks were placed in ice to prevent any further change.

Estimation of the Lactic Acid.

The contents of each flask were filtered through muslin into a small beaker. The flasks were washed out with about 40 cc. of alcohol which was added to the filtrate. The muscle residue was then ground three times with sand and alcohol and the whole transferred to a second small beaker holding about 50 cc. Both beakers were allowed to stand overnight. The heavy protein precipitate was then filtered off, washed three times with alcohol, and the alcohol carefully evaporated on the water-bath, using a small beaker (A) and avoiding boiling. The alcohol from the muscle residue was evaporated with similar precautions in another small beaker (B), the residue being re-ground three times and the washings added to the filtrate. All alcoholic

extracts were made faintly acid with sulphuric acid before evaporation. When the residue in A was nearly, but not quite, dry it was once more extracted three times with alcohol, filtered, and the filtrate added to beaker B. In this way a residue was obtained almost free from protein, phosphates, etc. The remainder of the procedure was as described by Meyerhof [1920] with slight modifications. In all cases the final solutions were made up to 50 cc. and kept at -2° until the actual distillation, for which 10 cc. were always used. The results are all expressed as percentages of lactic acid. In every case duplicate estimations were made¹.

PRELIMINARY EXPERIMENTS.

Exp. 2. A solution of the dried gland in water was made, filtered, and different quantities added to a series of flasks. 4 g. of minced beef were placed in each of seven flasks containing 10 cc. of phosphate solution at p_H 5.8. 0.1 g. glucose was added to each and also the pancreatic extract as indicated in the table. All specimens were placed at 37° for 30 minutes to allow for any possible time factor [Dakin and Dudley, 1913] and also to prevent destruction of insulin by a more alkaline reaction. At the end of that time 2.1 cc. of 0.2 *N* soda (sufficient to bring the p_H to 7.0) and 1 cc. of *N*/100 KCN were added to each flask and the series incubated at 37° for two hours. A determination of the initial lactic acid content of the beef was made on a separate sample.

Table I.

No.	Amount of dry gland equivalent to extract added	cc. of 0.0102 <i>N</i> iodine equivalent to 10 cc. of the final solution	% lactic acid	Effect
<i>Exp. 2.</i>				
1	Control (none)	10.95	.63	—
2	"	10.20	.59	—
3	10 mg.	5.65	.33	59 % inhibition
4	"	5.80	.34	57 % "
5	4 mg.	5.95	.34	57 % "
6	"	6.30	.36	53 % "
7	10 mg. heated to 85° for 10 minutes	7.20	.41	47 % "
8	Preformed lactic acid in sample of beef	2.45	.14	Increase due to incubation in control = 470 %
<i>Exp. 3.</i>				
1	Control	10.90	.62	—
2	"	10.65	.61	—
3	50 mg.	5.30	.31	50 % inhibition
4	"	5.30	.31	50 % "

Similar results were obtained in another experiment using a much stronger extract of the dry gland.

Exp. 3. All the details of the procedure the same as in *Exp. 2.*

There is clearly an inhibitory effect due to the extract, but it was shown

¹ In spite of repeated attempts we have had to abandon the shortened method of Meyerhof [1921] as too unreliable. No matter how highly purified a specimen of ammonium sulphate is used, the presence of so much of this salt in the final distillation mixture may cause a blank as high as 3 cc. of *N*/100 iodine. It has also been our experience that consistent distillation figures are difficult to obtain with this method, the ammonium sulphate not only giving rise to a blank but to a variable blank.

that this extract contained large quantities of trypsin, and therefore there is no evidence that the failure to develop as much lactic acid as in the controls was not due to digestion of the muscle by this enzyme. In order to show that the inhibition was not due to trypsin, it was necessary to prepare an extract containing the inhibitory factor, but known to be free from trypsin. The presence or absence of trypsin was demonstrated by the use of caseinogen solution as described in Cole's text-book.

The use of heat and alkali was avoided because their effect on the inhibitory factor was unknown, and it is difficult to be sure that the destruction of the enzyme is complete. It was not found possible to remove the trypsin by adsorption methods. Kaolin, alumina, charcoal and silicic acid were all tried without effect. Two methods were however found to be successful. In the first the trypsin was effectively neutralised by the addition of fresh serum. The serum was added to a watery extract, and the mixture tested for trypsin as before. The results of Exp. 6 are given in Table II.

Table II.

No.	Pancreatic extract	Serum cc.	Saline cc.	Trypsin	cc. 0.0105 <i>N</i> iodine equivalent to 10 cc. of final solution	% lactic acid	Effect
5	Control (none)	1	5	—	6.00	.35	—
7	"	0	6	—	5.60	.33	—
1	Watery extract equivalent to 25 mg. dry gland	1	0	—	4.60	.27	26 % inhibition
3	"	1 boiled	0	+	4.90	.29	21 % inhibition

In the second method the effect of extracting the dry gland preparation with alcohol of different strengths was tried. This was found to be the most successful and convenient method for removing the trypsin and was the one finally adopted for routine work. Full details of the preparation of such an extract are given below, but in Table III are given the results obtained by using extracts of the gland with alcoholic solutions of different strengths. All details of experimental procedure are as in Table I.

Table III.

No.	Nature of extract	Trypsin	cc. of 0.0104 <i>N</i> iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition
Exp. 3, No. 4	80 % alcoholic equivalent to 50 mg. dry gland	—	8.90	.52	+
	Control		10.65	.64	
Exp. 3, No. 5	80 % alcoholic equivalent to 25 mg. dry gland	—	10.55	.63	—
	Control		10.65	.64	
Exp. 4, No. 1	70 % alcoholic equivalent to 60 mg. dry gland	—	4.10	.24	+
	60 % alcoholic equivalent to 60 mg. dry gland		4.10	.24	
Exp. 4, No. 2	Control	+ ?	6.50	.38	+
	70 % alcoholic equivalent to 25 mg. dry gland		3.90	.23	
Exp. 6, No. 15 and 16	Control	—	6.00	.35	+

A 50 % alcoholic extract was found to contain trypsin and was therefore not used. These preliminary results show that there is an inhibitory factor other than trypsin present in the pancreas. It can be extracted by alcohol solutions up to 80 %, but a 60 % alcohol also extracts traces of trypsin and an 80 % alcoholic extract was found to be not so rich in the factor as one made with 70 % alcohol. The last is at the same time free from trypsin. In all later experiments, therefore, 70 % alcohol was used to extract the inhibitory factor from the dried gland and unless otherwise stated it is understood that the extract was prepared in the following way, although the actual amount of dry gland used varied.

Five grams of the dried gland were ground with 150 cc. of 70 % alcohol and allowed to stand with frequent shaking for 24 hours. The mixture was then filtered and the residue washed with about 25 cc. of 70 % alcohol. The filtrate and washings were evaporated *in vacuo* at 37° until the volume was reduced as much as possible. It is not possible to continue till all the alcohol has distilled off because of the excessive frothing on which even caprylic alcohol is without effect. The volume can generally be reduced to between 50 and 60 cc. in this way. The milky residue was then poured into centrifuge tubes and centrifuged for about ten minutes. By this procedure a small quantity of fatty material can be removed. The remainder was got rid of by shaking three times with an equal volume of ether (the residue after evaporation of the ether was shown to be exceedingly rich in phosphorus) which also removed the remaining alcohol. The ether was removed by careful warming, taking care that the temperature never rose above 40°, and by blowing an air current across the surface. The resulting watery solution was made up to 50 cc. It is usually quite clear, or only faintly opalescent, and colourless. The experiments to be described show that it contains the inhibitory factor in high concentration. As a rule, the extract was made up fresh for each experiment, but one extract kept at - 6° for about six weeks still retained its inhibitory action almost unimpaired.

EXPERIMENTS WITH TRYPSIN-FREE EXTRACT.

1. *With increased Lactic Acid Production.*

Laquer [1914] showed that the so-called "acid maximum" of frog's muscle was merely the result of the conditions in the dead muscle and was really reached by self-hindrance in the reaction. By suspending the chopped muscle in an alkaline solution this "acid maximum" could be raised considerably. Embden and his collaborators [1914] showed that another limiting factor was the amount of the hexosephosphate precursor available and Meyerhof [1921] found that by suspending chopped muscle in a phosphate buffer solution at p_H 9.0, the quantity of lactic acid formed could be increased up to about 1 %. A series of experiments were therefore carried out under the conditions likely to yield high concentrations of lactic acid in order to

discover what degree of inhibition could be produced. The technique of the experiments was the same as for those already described, except that instead of being incubated at 37° for two hours, the flasks were allowed to stand for 24 hours at room temperature. Also in these and later experiments no glucose was added to the muscle mixture. The phosphate solutions were adjusted to p_H 7.0 and 9.0 respectively by the addition of 0.2 *N* soda. In the latter case the additional soda was not added until 30 minutes had elapsed from the time of adding the extract. Potassium cyanide was added as before. The protocol of one Exp. (7) is given in Table IV.

Table IV.

No.	Inhibitory factor	Saline cc.	Phosphate	cc. of 0.0106 <i>N</i> iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
1 and 2	Control (none)	6	10 cc. at p_H 9.0	15.9	.95	—
3 and 4	"	6	" p_H 7.0	16.1	.97	—
5 and 6	1 cc. 70 % extract = 50 mg. dry gland	5	" p_H 9.0	10.8	.65	32
7 and 8	"	5	" p_H 7.0	10.6	.64	33

These figures show that even in the optimum conditions for the formation of lactic acid, the pancreatic factor exercises an inhibitory power and later experiments (Tables VI to X) show an even greater inhibition.

2. *Experiments to show that the inhibitory effect is not due to insulin.*

It has now been proved that there is in the pancreas a factor capable of inhibiting the production of lactic acid in chopped muscle and that this factor is not trypsin. It now remains to show that the inhibition is not due to insulin, which is also present in the pancreas and is also associated with carbohydrate metabolism. Both aqueous and 70 % alcoholic extracts of the dried pancreas might be expected to contain insulin, and that such is the case is shown by the following figures. Rabbits were used as experimental animals, and the blood sugars estimated by a modification of the Folin-Wu colorimetric method.

Table V.

Date	Rabbit	Time	Blood sugar %	Remarks
20. vii. 23	I	10-12	.14	Injected with aqueous extract equivalent to 1 g. of the gland preparation
		10-25	—	
		11-18	.07	
		12-55	.11	
26. vii. 23	II	11-50	.10	Injected subcutaneously with a similar dose of aqueous extract made isotonic with sodium chloride
		12-00	—	
		12-55	.08	
		2-00	.065	
		3-55	.04	Convulsed
		5-00	—	
		5-10	.03	
17. viii. 23	XI	5-15	—	Recovered by subcutaneous injection of glucose
		10-35	.133	Injected with alcoholic extract = 1 g. dry gland
		10-55	—	
		11-55	.067	
		2-10	.052	
		4-45	.073	

The 70 % alcoholic extract of the gland preparation has not yet been further fractionated, but it has been shown that insulin has no effect whatever on the lactic acid formation. The first experiments on this question were carried out by Miss D. M. Moyle working in this laboratory. Her results may be summarised as follows:

The experimental mixtures were left at p_H 7.1 for $3\frac{1}{2}$ hours at 37° .

(a)	{	Control	13.05 cc.	iodine	$\equiv$ 62 %	lactic acid
		+ 3 cc. insulin	13.20 cc.	"	$\equiv$ 63 %	"
(b)	{	Control	9.35 cc.	"	$\equiv$ 44 %	"
		+ 2 cc. insulin	9.90 cc.	"	$\equiv$ 47 %	"

(Two samples of crude insulin were mixed and 3.8 mg. of the mixture dissolved in 5 cc. of saline.)

These results were repeated and confirmed by us, and the effect of insulin compared with that produced by the 70 % extract. The results are given in Table VI. Two samples of insulin were used, each of which was known to be active. One was the purest commercial sample obtainable, and one therefore in which any possibility of contamination with the inhibitory factor was reduced to a minimum. The other was a specimen of crude insulin (for which we are indebted to Messrs Winter and Smith) which had undergone no purification. Two samples also of the 70 % extract were used. One was freshly prepared and the other had been kept in cold store at -6° for nearly two months. It seemed of interest to test this old extract (the same as that used for Exp. 7) for deterioration of the factor, because it was found that it had no longer any insulin-like effect on the blood sugar when injected into a rabbit. Exp. 7 had shown that a p_H of 7.0 gave as high values for the lactic acid content of muscle in the controls as p_H 9.0, so it was decided to work at the neutral point, since insulin is readily destroyed on the alkaline side. The insulin was added while the contents of the flasks were still acid at p_H 5.8, and soda was added after half-an-hour to make the p_H 7.0. Otherwise, all experimental details were as in Exp. 7. (*Note.* The lactic acid in this series was estimated by the shortened method of Meyerhof, referred to above, and the figures given are in each case the mean of two samples. The figures in brackets are the extreme values obtained in the distillations.)

Table VI. *Exp. 10.*

No.	Factor added	cc. of 0.0102 <i>N</i> iodine equivalent to 10 cc. of final solution	% lactic acid	Inhibition %
1 (a)	Control	14.60		
(b)	1 cc. saline	(14.40, 15.15)	.84	—
2 (a)	1 clinical unit pure commercial insulin	14.10		
(b)		(14.20, 13.65)	.81	—
3 (a)	5 mg. crude insulin	14.90		
(b)		(14.90)	.86	—
4 (a)	1 cc. old 70 % extract equivalent to 100 mg. dry gland	6.90		
(b)		(7.25, 6.35)	.40	52
5 (a)	1 cc. new 70 % extract equivalent to 125 mg. dry gland	5.30		
(b)		(5.70, 4.90)	.31	63

It is clear that the factor in the pancreas responsible for the inhibition of lactic acid formation in chopped muscle is not insulin, and it is also evident that even in crude samples of insulin, the inhibitory factor has been removed by the higher concentration of alcohol used in its preparation.

3. *Antiglyoxalase.*

There is also present in the pancreas another factor of which no mention has yet been made, and which is probably concerned with carbohydrate metabolism and with lactic acid production, and that is the antiglyoxalase discovered by Dakin and Dudley [1913]. These authors showed that there is present in practically all tissues, except the pancreas, an enzyme, glyoxalase, which is able to convert α -ketonic aldehydes into the corresponding optically active hydroxy-acids.

Thus lactic acid would be produced by glyoxalase from methylglyoxal.

They found that the pancreas and commercial pancreatin, on the contrary, contain an antagonistic substance, called by them antiglyoxalase, which inhibits the action of glyoxalase. If a ketonic aldehyde is added to a tissue preparation, together with a pancreatic extract, there is no formation of the corresponding acid. Thus, in so far as it inhibits the production of lactic acid, antiglyoxalase would appear to be analogous, if not identical, with the inhibitory factor described above. Although possible however, it has not yet been definitely established that methylglyoxal is one of the intermediary products in the breakdown of hexosephosphate to lactic acid in the muscle. To decide, if possible, whether methylglyoxal occurred as an intermediary in lactic acid production was (we are informed) the original intention of Winfield and Hopkins' experiments. Antiglyoxalase is described as a thermolabile body, being destroyed by heating for ten minutes at 85° . It is slowly destroyed by weak acids, but is much more resistant to alkali. Even after 24 hours' digestion at 37° with 1 % sodium carbonate, its inhibitory action on glyoxalase is unimpaired (Dakin and Dudley). In order to decide conclusively whether the inhibitory factor we have described is, or is not, identical with antiglyoxalase, it will be necessary to test its action on phenylglyoxal added to a tissue mixture. It is hoped to be able to do this shortly, but meanwhile its resistance to alkali was compared with that attributed to antiglyoxalase.

Extracts of the dried gland, both with water and 70 % alcohol, were made up to 1 % with solid anhydrous sodium carbonate, a drop or two of toluene added, and the mixtures placed in the incubator overnight at 37° —usually for about 20 hours. They were then neutralised with hydrochloric acid and added to minced beef suspended in phosphate in the usual way. The results are summarised in Table VII.

Table VII.

No.	Extract	Conditions	cc. iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition
Exp. V	Control	Heated at 37° for 2 hours at p_H 7.0	7.10	.42	—
1					
2	50 mg. commercial pancreatin	"	4.90	.29	Marked
3	50 mg. pancreatin digested with 1 % Na_2CO_3	"	6.30	.37	Slight
Exp. VI	Control	"	5.60	.33	—
9	1 cc. 70 % extract = 25mg. dry gland digested with 1 % Na_2CO_3	"	6.00	.35	None
14	Aqueous extract = 25 mg. dry gland digested with 1 % Na_2CO_3	"	5.60	.33	None
Exp. VII	Control	Room temp. for 24 hours at p_H 9.0	15.90	.95	—
9 and 10	1 cc. 70 % alcoholic extract = 30 mg. dry gland digested with 1 % Na_2CO_3	"	16.10	.97	None
Exp. XI	Control	Room temp. for 22 hours at p_H 7.0	12.50	.71	—
1					
2	1 cc. 70 % extract = 100 mg. dry gland untreated	"	5.30	.28	Marked
5	1 cc. 70 % extract digested with 1 % Na_2CO_3	"	10.85	.63	Slight

Though not entirely conclusive these results certainly suggest that in its inability to resist the action of alkali the pancreatic factor responsible for the inhibition of lactic acid formation in chopped muscle differs from anti-glyoxalase.

4. *Stability towards heat.*

Too few figures have been obtained yet to say definitely how resistant towards heat the inhibitory factor is. Dakin and Dudley found that anti-glyoxalase was completely destroyed by exposure to a temperature of 85° for ten minutes. Winfield and Hopkins state that the factor is thermostable and will withstand boiling. As already noted, in their preparations, the enzymes may have been protected by the proteins present, and this is also the case in the first experiment quoted below. An aqueous extract of the gland preparation gives a heavy coagulum on being heated, but this possible source of error is absent in the second experiment. The alcoholic extract remains unclouded at the end of ten minutes at 85°.

In each case there has been a considerable destruction of the factor, but this seems not to be complete. Further work must be done to find to what extent it is thermostable, but in respect to heat also it would appear to be more resistant than anti-glyoxalase.

Table VIII.

No.	Extract	Conditions	cc. iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
Exp. III	Control	Incubated for 2 hrs. at 37° at p_H 7.0	10.60	.61	—
3 and 4	Aqueous extract equivalent to 10 mg. dry gland	"	5.70	.33	46
7	Same heated to 85° for 10 mins.	"	7.20	.42	31
Exp. XI	Control	Room temp. for 22 hours at p_H 7.0	12.50	.72	—
2	1 cc. 70 % alcoholic extract = 100 mg. dry gland	"	5.30	.30	58
9	Same heated at 85° for 10 mins.	"	11.50	.66	8

Another interesting feature of the action of the inhibitory factor is illustrated in the results given in Table IX. Estimations were made on samples of beef to which the extract was added after the most part of the lactic acid had been formed. The object of such experiments was to ascertain whether the diminished yield of lactic acid produced by the factor was due to a hindrance in the formation of the lactic acid or to its removal, either by oxidation or by synthesis to a precursor, after its formation. It was found that when once the lactic acid had been liberated, under the conditions of our experiments the factor had no effect on it. This would suggest that its action is truly one of inhibition and its effect is not to catalyse the removal of the acid.

Table IX.

No.	Extract	Conditions (all at p_H 7.0)	cc. iodine equivalent to 10 cc. final solution	% lactic acid
Exp. X	Control	At 37° for 2 hrs. and then 22 hrs. at room temperature	15.30	.88
7	1 cc. 70 % extract equivalent to 125 mg. dry gland	At 37° for 2 hrs. then extract added and left for 22 hrs. at room temperature	12.80	.74
Exp. XI	Control	At 37° for 2 hrs.	12.15	.70
6	Control	At 37° for 2 hrs. then 1 cc. saline added and left at room tem- perature for 20 hrs.	14.40	.83
7	Control	At 37° for 2 hrs. then 1 cc. saline added and left at room tem- perature for 20 hrs.	14.40	.83
8	1 cc. 70 % extract equivalent to 100 mg. dry gland	At 37° for 2 hrs. then extract added and left at room tem- perature for 20 hrs.	13.10	.75

In Table X are summarised the results obtained in one experiment showing the effect of concentration, alkali, and heat on the same extract. All samples were kept for 22 hours at room temperature which varied from about 10° to 13° during the experiment, and all were at p_H 7.0. The details of technique were exactly as already described¹.

¹ In all tables, except no. 1, the percentage inhibitions given are really less than the actual inhibitions, because there is always some 'preformed' lactic acid, the amount of which is the true base line for the changes. This follows from the results given in Table IX.

Table X. *Exp. 11.*

No.	Extract	cc. 0.0102 <i>N</i> iodine equivalent to 10 cc. final solution	% lactic acid	Inhibition %
1	Control	12.50	.72	—
2	1 cc. 70 % alcoholic extract equivalent to 100 mg. dry gland	5.30	.30	58
3	0.5 cc. extract equivalent to 50 mg. dry gland	5.30	.30	58
4	0.25 cc. extract equivalent to 25 mg. dry gland	6.10	.35	52
5	1 cc. extract digested with 1 % Na_2CO_3	10.85	.62	14
9	1 cc. extract heated to 85° for 10 mins.	11.50	.66	8

There are one or two interesting properties of the factor which might be noted. It is never able to prevent entirely the formation of lactic acid. In every case, however much of the preparation was added, there was some formation of the acid. For example, assuming the initial value of the "pre-formed" lactic acid as 0.14 % (Table I) the lowest value we have obtained after addition of the factor and subsequent incubation was 0.23 % (Table III). Also an increase of 100 % in the amount of factor added, above a certain minimum, had no further effect in inhibiting the lactic acid production. Then again, many experiments show a suggestive proportionality between the total lactic acid formed and the degree of inhibition. In our opinion these results lend some support to the view of Meyerhof [1921] that there is more than one source contributing to the total lactic acid production in muscle. It may well be that the inhibitory factor is only capable of exerting its action on one of these sources, though with the data available, this can be nothing more than a suggestion.

DISCUSSION OF RESULTS.

The experiments described show conclusively that there is present in the pancreas a factor which can control the production of lactic acid in muscle by exercising a marked inhibitory action. All the data available at present refer, it should be understood, to its action on chopped muscle *in vitro*, and to that alone. The factor is not insulin. It would seem moreover to differ from the pancreatic substance which was originally described by Otto Cohnheim and was later shown by Levene and Meyer to induce, when mixed with muscle, disaccharide formation from glucose. The active substance studied by us is, to judge at least from the published statements, less soluble in strong alcohol and less thermostable than Cohnheim's substance. It is possible however that these differences may be due to variations in the method of extraction. There is some evidence, as we have pointed out, to suggest that our inhibitory factor is not identical with the antiglyoxalase of Dakin and Dudley.

There is as yet no evidence as to what the exact physiological functions of this new factor may be but certain speculations are perhaps permissible and are certainly interesting. An attractive suggestion is that it forms part

of a delicate regulating mechanism in which insulin is also included. Since it inhibits the formation of lactic acid there is at least a possibility that it may exercise a stabilising influence on the glycogen stores of the body; in the muscles if not in the liver. If this be the case then insulin and the inhibitory factor may be, in this particular connection, mutually antagonistic, for as Dudley and Marrian [1923] showed, when insulin is injected into a normal animal the glycogen almost disappears from the liver and muscles. It has been shown by Robison and Kay [1924] that in the living animal, insulin has the power of increasing the lactic acid precursor—lactacidogen—which in its turn would normally result in an increased lactic acid production. Is this effect balanced and controlled in the normal animal by the intervention of the inhibitory factor? In the experiments where insulin was added to the muscle *in vitro* no increased lactic acid formation over the control was observed. Under these conditions, however, there would be an insufficient supply of the precursor, for though phosphate was supplied, carbohydrate was not and the development of lactic acid would cease when the precursor was exhausted, whether by the normal enzymes of the muscle or jointly by these and insulin. The effect of insulin upon the velocity of lactic acid production was not studied.

It has always been an unexplained circumstance that the muscular metabolism of the diabetic shows no obvious abnormality. There have been attempts to estimate the lactic acid in diabetic muscle and though the results are somewhat contradictory the general view is that it differs little, if at all, from the normal. The energy supply, even in the diabetic, is immediately derived from the breakdown of carbohydrate. Is this because the breakdown of glycogen through hexosephosphate and lactic acid in the muscle follows a different course from the general carbohydrate metabolism of the body? Is the latter regulated by insulin which controls the concentration of glucose in most tissues, while the specialised events in the muscles, where the lactic acid is not only part of the "fuel" but also of the "machinery" are regulated by this new factor?

These suggestions as to the physiological functions of the inhibitory factor are of course purely tentative and highly speculative, but we consider that they warrant our continued research into its effect on the intact animal by administration alone and with insulin and it is hoped the results will form the subject of a later communication.

SUMMARY.

The presence in the pancreas of a factor which is capable of causing marked inhibition in the lactic acid production of chopped muscle, is shown.

This inhibitory factor is neither trypsin nor insulin and probably not antglyoxalase.

A method is described for making a stable preparation of the pancreas with all its enzymes intact and details are given for making an extract of this

preparation with 70 % alcohol which contains the inhibitory factor in high concentration.

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