LXV. THE RELATION BETWEEN AMYLASE AND LACTIC ACID FORMATION IN MUSCLE.

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(Received February 28th, 1931.)

THE work reported in this paper was begun with the intention of investigating further the nature of the inhibition of lactic acid formation in muscle which is brought about by pancreatic and other extracts. This phenomenon had been shown by Case and McCullagh [1928] to be caused by the amylase present in such extracts, and it was demonstrated that similar inhibition could be brought about by amylase from various other sources, both animal and plant. In an earlier paper the mode of action of the "pancreatic factor," then unidentified with amylase, had been described by McCullagh [1928] as a suppression of the esterification of phosphoric acid; in other words, as a prevention of the formation of hexosephosphates rather than a prevention of their breakdown to lactic acid. This hypothesis was adhered to in the subsequent communication.

Harrison and Mellanby [1930] agreed that the substance responsible for the inhibition is amylase, but they did not regard as admissible the above interpretation of the manner in which it acts. Actually it appears that their criticism was based, in large part, upon a misunderstanding with regard to the meaning which was intended to be borne by the explanation in question.

It is clear that a statement such as that "inhibition of lactic acid formation depends upon the suppression of esterification of carbohydrate and phosphoric acid" can be presumed to apply only to those cases where inhibition does in fact occur; that is to say, to cases where the carbohydrate involved is starch or glycogen. When activated glucose is the substrate, amylase causes no inhibition, as was shown both by Case and McCullagh and by Harrison and Mellanby. From these circumstances, taking into consideration the fact that starch and glycogen are natural substrates for amylase, while glucose and other hexoses are not, it seems a reasonable deduction that the inhibition observed is related to an interaction of some kind between polysaccharide and amylase, in virtue of which the lactic acid-forming process is checked at its first stage, namely that of esterification. In a sense, it may be said that Harrison and Mellanby are agreeing to this when they put forward the suggestion that "an explanation of the experimental observations can be based

on the well-known fact that amylase breaks down starch to maltose," which substance as Meyerhof has shown is practically unacted upon by the muscle enzyme system. This explanation, which as Harrison and Mellanby point out seems obvious enough at first sight, is in the present writer's opinion inadequate, in such a simple form, to account for the complete and instantaneous inhibition of lactic fermentation which is produced by very small amounts of added amylase. It is not conceivable that immediately after the addition to the system of amylase all the starch is at once transformed to maltose and in that way removed from the sphere of action of the esterifying and other enzymes. It seems much more likely that the explanation is to be sought in the formation of some amylase-starch compound or complex, whereby the starch is protected from and rendered inaccessible to the muscle enzymes. That something of this nature indeed takes place is suggested by the fact that even in the presence of considerably more amylase than is necessary to produce complete inhibition of esterification and lactic acid formation, the characteristic blue compound with iodine can be obtained, thus demonstrating the continued existence in the solution of unhydrolysed starch. The breakdown of the starch into maltose proceeds slowly and can be followed by the iodine method or a reduction method, while the lactic acid formation is completely checked *ab initio*.

The experimental work to be described was originally commenced in order to produce fresh justification for this point of view, but as will be seen, results were obtained which throw light from a different angle on the problem of the relation between amylase and lactic acid formation in muscle.

EXPERIMENTAL.

In order to test the theory of Harrison and Mellanby referred to above, namely that maltose formation is responsible for the inhibition, the following experiment was designed. If one were to incubate starch for a time with amylase in sufficient quantity to cause complete inhibition of lactic acid formation by a given muscle extract, and then, after this preliminary incubation, remove or destroy the amylase, it should be possible to show that there still remains starch which can now yield lactic acid when incubated with muscle extract.

Table I sets forth the results of one experiment carried out on these lines.

Table I.

					mg. lactic acid in 15 cc. sample		
					Before incubation	After incubation	Change
1.	Muscle extract + starch + buffer + water				10.9	27.8	16.9
2.	" " " + 5 cc. amylase				11.1	12.3	1.2
3.	" (starch + buffer + 5 cc. amylase)	A			11.2	28.8	17.6
4.	" " "	B			10.9	23.3	12.4
5.	" " "	C			10.9	18.2	7.3
6.	" " "	D			11.2	14.6	3.4
7.	" " "	E			11.3	13.9	2.6

In preparation A the starch, buffer and amylase were mixed and incubated together at 27° for 1 minute, then boiled for 5 minutes in order to destroy the amylase. B, C, D and E received similar treatment, the incubation times being 5, 10, 20 and 30 minutes respectively before the 5 minutes' boiling. All were cooled to room temperature before the muscle extract was added. The composition of the final mixture was in all cases:

25 cc. muscle extract,
10 cc. 2 % starch,
10 cc. phosphate buffer,
5 cc. water or amylase.

Incubation was carried out for half an hour at 27°. The technique of sampling and of estimation of lactic acid was exactly as described in former work [Case, 1929], as was the preparation of the muscle extract and the composition of the buffer. This applies to all the lactic acid experiments reported in this paper.

The amylase employed was a diluted preparation of saliva, 5 cc. of which had been shown by a preliminary test to give 100 % inhibition of lactic acid formation with an average muscle extract.

It follows from these results that for a period of at least half an hour after the addition of amylase, starch remains present in the system and can be utilised by the lactic acid-forming enzymes if the amylase is removed. It would seem then that the starch is in some way held by the amylase prior to any destruction, and held in such a way that it is inaccessible to the myozymase system. It was a conception of this kind that was foreshadowed in the concluding sentence of Case and McCullagh's [1928] paper, *viz.* "it may be that some molecular union between amylase and its substrate, prior to enzymic hydrolysis, can be demonstrated."

All that has been said above applies with equal force to the case of glycogen, which of course is the naturally-occurring substrate for the muscle enzymes. An experiment similar in every respect to that of which Table I is the protocol, except that starch was replaced by glycogen, yielded substantially identical results.

It is well known that the tissue of numerous organs, including muscle, contains amylase in varying quantities. With regard to muscle, it becomes of interest to inquire as to the nature of the rôle played by its amylase.

Ronzoni [1929] believes that "hydrolysis of starch is a necessary step in glycolysis of muscle extract." In support of this view, she claims to have produced an acceleration of esterification by adding amylase to muscle extracts which, owing to short periods of extraction, were deficient in this enzyme. The present writer has, however, never succeeded in obtaining confirmation of this. It is interesting to note in this connection that preparations of amylase from muscle itself, made by glycerol extraction or other means, resemble amylases from other sources in that their addition in sufficient quantity to

the myozymase system produces inhibition of lactic acid formation. This fact was realised by Ronzoni and is mentioned in her paper.

Now the muscle enzyme system as a whole is strikingly thermolabile. Heating for a few minutes at 37° is sufficient to destroy its power of forming lactic acid from carbohydrate, or of esterifying phosphate. Muscle-amylase itself has a much greater degree of stability. It should be possible therefore, by heating muscle extract for, say, a quarter of an hour at 37° , to leave the amylase activity intact while destroying the lactic acid-forming property of the extract. A comparison of the rates of starch or glycogen destruction in an extract so treated, and at the same time in an unheated portion of the same extract, should then give significant results. If in both cases carbohydrate disappeared at the same rate, the fact would be an indication that amylase action is the first step in the degradation of starch. In order to render such a conclusion admissible, it would be necessary to ensure that the starch concentration was small enough to be below the saturation value for the amount of enzyme present.

In Table II are embodied the results of a number of experiments made to test this point. In each instance a part of the muscle extract was heated for 15 minutes at 37° , the remainder being kept at 0° . After cooling the first portion to 0° again, incubation mixtures were set up consisting of 20 cc. of muscle extract, 10 cc. of phosphate buffer and 10 cc. of 0.4 % starch. The final concentration of starch was thus 0.1 %. The incubations were carried out at 27° , and at intervals 0.5 cc. samples were tested with iodine. In each case the time was noted at which the addition of iodine produced no coloration.

Table II.

		Time (in mins.) for achromic point with iodine				Time (in mins.) for achromic point with iodine	
		Normal extract	Heated extract			Normal extract	Heated extract
A	p_H			C	p_H		
	6.0	41	44		6.0	35	35
	7.0	37	38		7.0	36	39
	8.0	32	32		8.0	30	33
B	6.0	20	21	D	6.0	13	12
	7.0	20	18		7.0	13	14
	8.0	18	20		8.0	12	13

A, B, C and D represent different muscle extracts made at different times.

Variations of p_H between the above limits, although in each case altering to some extent the actual times taken for the attainment of the achromic point, are seen to make no difference to the relative values for heated and unheated extracts.

The important point emerging from these figures is that in every instance the rates of disappearance of starch are the same, in spite of the fact that in one case lactic acid is being produced in the ordinary way, while in the other case nothing is occurring beyond amylolytic hydrolysis of the starch. This

seems to furnish strong evidence in support of the conclusion that the breakdown of starch to lactic acid depends primarily upon the amylase present in the extract.

Table III demonstrates that lactic acid production is, in fact, eliminated in the heated extracts. The figures given are for incubation mixtures A to D of the previous table, the p_H being 7.0.

Table III.

	Lactic acid production in 1 hour (mg. in 15 cc. sample)	
	Normal extract	Heated extract
A	4.9	0.5
B	7.0	0.8
C	5.5	0.5
D	8.7	1.0

The figures here given for "heated extract" are not quantitatively reliable, on account of the fact that the amounts of lactic acid are too small to be estimated accurately. The significance of the table, however, is not thereby affected.

A somewhat similar series of experiments was next performed, with the difference that instead of starch, glycogen was employed, in a concentration of 0.1 %. As before, normal and heated extracts were incubated with buffer (p_H 7.0) and glycogen. Instead of following glycogen disappearance by an iodine method, 5 cc. samples were taken initially and at periods of 5, 10 and 30 minutes. The glycogen in these was estimated by the modification of Pflüger's [1909] method described by Barbour [1929]. The samples were delivered directly into 5 cc. of hot potassium hydroxide solution, thus preventing any further enzymic activity.

The results of these experiments are given in Table IV. As in Table II, E, F and G are separate muscle extracts.

Table IV.

Period of incubation in minutes	...	% glycogen in solutions							
		Normal extract				Heated extract			
		0	5	10	30	0	5	10	30
E		0.094	0.077	0.061	0.005	0.095	0.079	0.060	0.004
F		0.096	0.081	0.069	0.024	0.096	0.080	0.070	0.030
G		0.096	0.070	0.048	0.000	0.097	0.068	0.050	0.000

Here again we find a parallel disappearance of glycogen in the corresponding cases where the muscle enzyme has and has not been heated for 15 minutes at 37°; these results therefore corroborate those of Table II.

If the conclusion just drawn is correct, it is to be expected that any agency which suppresses the amylolytic activity of a muscle extract would suppress in corresponding degree the formation of lactic acid. Actually to remove the

amylase without affecting the other components of the system would be a difficult task; fortunately, however, another way out of the difficulty is open.

It has been known for some time that muscle-amylase, in common with liver-amylase, differs in some respects from the enzyme obtained from pancreas, saliva, malt and other sources. Barbour [1929] made a study of glycogenolysis by muscle-amylase, and found that the sole product is a trisaccharide, which seems not to be identical with any sugar hitherto described; though Lohmann [1926] had reported among the products of glycogenolysis in muscle extracts a trisaccharide which may have been the same as that described by Barbour. Lohmann at the time suggested that the substance might be identical with the amylotriase of Pringsheim, but Barbour's more complete investigation of its properties appears to render this unlikely. If glycogen is hydrolysed with pancreatic or salivary amylase the products consist mainly of isomaltose with a little glucose; no trisaccharide is formed.

It was found possible to confirm Barbour's work, and by starting with several grams of glycogen to obtain relatively large amounts of a substance answering to his description of the trisaccharide. In general, glycerol extracts of finely minced rabbit muscle were employed and were incubated for 24 hours or longer with phosphate buffer (p_H 6.5) and 5 % glycogen. The trisaccharide was isolated from the digest according to the directions given by Barbour.

Now Wohl and Glimm [1910] showed that in the case of malt-amylase, the addition of maltose, the chief product of hydrolysis, caused inhibition of the amylolytic activity when starch was the substrate; this fact was made use of by Case and McCullagh [1928]. In a similar way, addition to muscle-amylase of the trisaccharide referred to above should depress the activity of the amylase. That this is so was shown by Barbour, and the following figures obtained during the course of the present work afford confirmation.

Table V.

Incubation period (hours)	Glycogen content (%)			Hydrolysis (%)	
	0	5	20	5	20
Control	0.98	0.64	0.15	35	85
Trisaccharide	0.97	0.95	0.97	2	0
Glucose	0.97	0.66	0.20	32	79
Maltose	0.98	0.66	0.23	33	77
Sucrose	0.96	0.68	0.22	29	77

In this experiment each flask contained 5 cc. of muscle-amylase (glycerol preparation) and 5 cc. of 2 % glycogen (made up in phosphate buffer of p_H 7.0). The different sugars (0.5 g. of each) were dissolved in the glycogen solutions before mixing with the enzyme. The mixtures were incubated at 27° and the glycogen was estimated in 3 cc. samples by the method referred to earlier in the work.

It is clear that the trisaccharide exerts a marked specific inhibitory action upon muscle-glycogenase.

The next step was to try the effect of adding trisaccharide to incubation

mixtures of glycogen and myozymase. To this end tubes were set up as follows (S represents trisaccharide):

1.	10 cc. muscle extract + 5 cc. 2 % glycogen + 5 cc. buffer	
2.	„ „ „ „	+ 0.25 g. S
3.	„ „ „ „	+ 0.5 g. S
4.	„ „ „ „	+ 1.0 g. S
5.	„ „ „ „	+ 1.0 g. glucose
6.	„ „ „ „	+ 1.0 g. maltose
7.	„ „ „ „	+ 1.0 g. sucrose
8.	„ + 5 cc. water	+ 0.25 g. S
9.	„ „ „ „	+ 0.5 g. S

The production of lactic acid during one hour at 27° is shown in Table VI.

Table VI.

Tube No.	mg. lactic acid in 10 cc. sample		
	Before incubation	After incubation	Change
1	6.5	19.4	12.9
2	6.4	16.8	10.4
3	6.7	7.4	0.7
4	6.5	7.1	0.6
5	6.5	17.2	10.7
6	6.2	16.9	10.7
7	6.4	18.5	12.1
8	6.8	6.9	0.1
9	6.4	6.9	0.5

Here again it is seen that the addition of trisaccharide in a concentration of 2.5 % has completely suppressed the formation of lactic acid from glycogen, while the other sugars tried have had no such effect. Tubes 8 and 9 are intended to demonstrate that the trisaccharide itself is not acted upon by muscle enzymes.

This experiment was repeated using half quantities throughout, and with the addition of sodium fluoride in 0.02 *M* concentration. Instead of lactic acid, phosphates were determined before and after the incubation by the Briggs-Bell-Doisy colorimetric method. The results, as is apparent from the next table, bear out those of Table VI; that is to say, esterification has been inhibited in the systems containing the trisaccharide, but in no others.

Table VII.

Tube No.	mg. P as free phosphate in 3 cc. sample		
	Before incubation	After incubation	Change
1	1.02	0.40	- 0.62
2	1.04	0.52	- 0.52
3	1.03	1.00	- 0.03
4	1.03	1.02	- 0.01
5	1.04	0.55	- 0.49
6	1.02	0.57	- 0.45
7	1.01	0.54	- 0.47
8	1.05	1.02	- 0.03
9	1.05	1.03	- 0.02

DISCUSSION.

Considering jointly the significance of the experimental results embodied in Tables V, VI and VII, one is led to the conclusion that since lactic acid production in muscle is inhibited by the trisaccharide, and the trisaccharide inhibits the activity of muscle-glycogenase, of whose action upon glycogen it is the end-product, then the first stage in the breakdown of glycogen into lactic acid is one dependent upon amylolytic activity. This is the same conclusion that was arrived at from a consideration of the facts reported in Tables II, III and IV, and up to a point is in agreement with Ronzoni's view of the situation. But whereas Ronzoni postulates amylolytic hydrolysis as a necessary preliminary, the present writer is not of this opinion. As has been said, it seems fairly certain from Barbour's work that the only product of glycogenolysis by muscle-amylase is the trisaccharide; it appears that the intermediate production of dextrans does not occur. The trisaccharide itself is incapable of being further acted upon by the muscle enzymes, as is shown in Tables VI and VII. It may be said in parenthesis that Lohmann's trisaccharide was also non-fermentable.

The position may then be summarised by the statement that on the one hand the action of muscle-amylase is a necessary preliminary to the formation of lactic acid from glycogen, while on the other hand the product of the hydrolysis of glycogen by muscle-amylase is one which cannot be attacked by myozymase. The inference is therefore that this product, the trisaccharide, is not formed in the process of lactic acid production. Since it is the only product of glycogenolysis by muscle-amylase, it follows that in all probability hydrolysis does not occur at all. The muscle-amylase, if this is the case, is not functioning in the manner that normally would be expected of an amylase.

The suggestion put forward is that we have in muscle a system of enzymes working not only in a coordinated consecutive manner but in a conjoint and simultaneous way, in the sense that the amylase and another component of the system, presumably the esterifying enzyme, are so closely correlated and interdependent in their activity that they may, in fact, be considered to form a single colloid complex or aggregate. It is a generally accepted hypothesis that an enzyme molecule proper is attached to an inactive associated structure, probably of protein. It is necessary to imagine then that in this particular case both the amylase and the esterifying enzyme are attached to the same colloid structure and constitute to that extent a single entity. The first step in the transformation of glycogen to lactic acid seems to be then not merely a union of the carbohydrate with amylase, but a simultaneous union with both amylase and another enzyme component of myozymase—presumably involving two different sites on the carbohydrate molecule. In this connection it is tempting to allude to the extreme thermolability of the myozymase system as a whole. The very pronounced effect of even short exposure to quite low degrees of heat (*e.g.* 37°) is highly unusual, even in enzyme chemistry,

and it is difficult to regard this effect as consistent with the destruction of some enzyme component; it seems a more likely hypothesis that the exaggerated deleterious effect of temperature occurs as the result of the rupture of some delicate colloidal complex between one part of the system and another.

SUMMARY.

1. The nature of the inhibition of lactic acid formation in muscle caused by amylase is further investigated and the conclusion is drawn that an enzyme-substrate compound is formed prior to the hydrolysis of starch or glycogen by amylase.

2. Evidence is adduced in support of the hypothesis that the amylase of muscle is concerned in the process whereby lactic acid is produced from carbohydrate.

3. The concept of two enzymes functioning in a mutually dependent manner and not as individuals is introduced and is briefly discussed with reference to the case of muscle.

I am indebted to Sir F. G. Hopkins for his interest and encouragement, and to Prof. J. B. S. Haldane for much stimulating advice.

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