CXI. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

III. GLYCOLYSIS IN STERILE CELL-FREE EXTRACTS OF MUSCLE.

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Since the time of Claude Bernard, much investigation has been done on the subject of the "glycolytic enzyme" of blood and of muscle. The results of these investigations are very varied and even contradictory. One aspect of the subject, however, strikes one as worthy of further consideration. In the past, very few of the muscle extracts were bacteriologically sterile. It is certainly true that antiseptics were generally employed; but while on the one hand a negative result under these conditions proves nothing conclusive, since the antiseptic may be an inhibitor of the enzyme, on the other hand, a positive result, especially with an incubation period of more than three or four hours, makes one doubt the efficiency of the antiseptic. Whether a given antiseptic is an inhibitor of enzyme activity in the concentration required to prevent effectively the growth of microorganisms, or is an indifferent substance, cannot be demonstrated beyond doubt until the effect of its presence has been studied in an extract made sterile by some other method. If the problem of sterility is to be overlooked the period of incubation must not exceed three hours, and if in that period the loss of glucose is small, the results are not very convincing. The solution of this question lies in preparing extracts that are sterile, free from antiseptics and with a glycolytic activity of such magnitude that the loss of glucose is rapid. Such extracts have been prepared in this laboratory. The method consisted in filtering the muscle extract, previously frozen to a thick cream, through Berkefeld filter candles and then, at the end of the incubation, testing both for aerobic and anaerobic organisms by inoculation of a sample of the extract into Douglas broth incubated both aerobically and anaerobically.

This method of producing sterility was first applied to muscle extract by Brunton and Rhodes [1901]. They filtered an extract of sheep muscle through Pasteur-Chamberland filters: on keeping the filtrates in bottles plugged with cotton wool for several weeks, no bacterial growth was observed. The concentration of glucose fell from 1·5 to 0·75 % in 48 hours and this result was confirmed several times.

Since the experiments described in this paper were performed, Meyerhof [1926] also mentions that he was able to observe lactic acid formation from glycogen in extracts of muscle filtered through Berkefeld filters. His results were similar to those described here in that the first portion of the filtrate is inactive; but the experiments below were conducted with the intention of detecting destruction of glucose, not the breakdown of glycogen. It is in this sense that the term glycolysis is used here. In the investigator's experience it is more difficult to get an extract showing active glycolysis than one showing rapid lactic acid formation from glycogen. Glycolytic activity in this sense

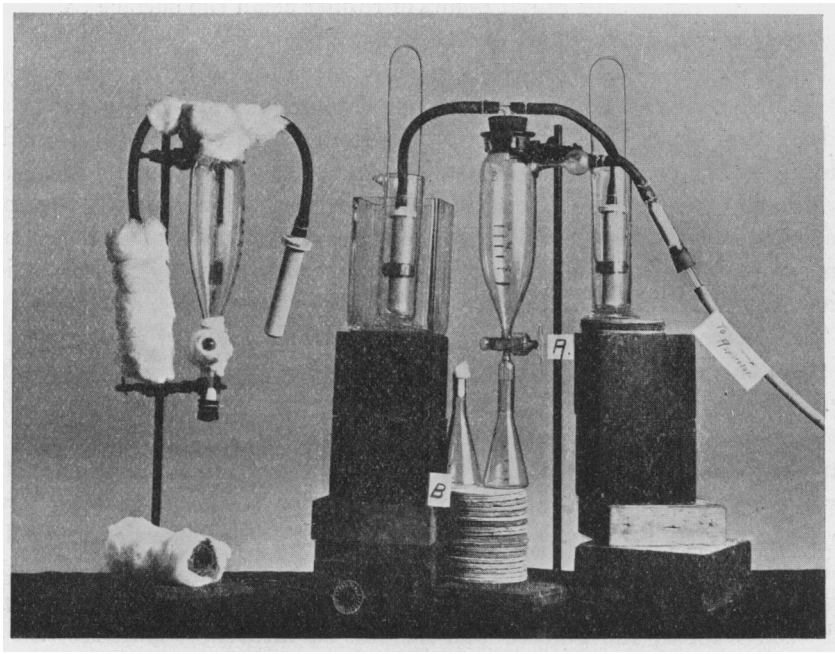

Fig. 1.

is a very uncertain phenomenon for some reason as yet obscure. Using certain precautions, however, it was possible in a great many cases, although not without many failures, to prepare sterile extracts of perfused rabbit and cat muscle, free from any intact cell structures, with a glycolytic activity of such a degree that the existence of the "glycolytic complex" in an active condition in cell-free extracts of muscle can no longer be considered doubtful.

Preparation and filtration of the extracts.

The animals were killed by a sharp blow on the head and immediately perfused with Ringer solution. The treatment thereafter was as is described in the first paper of this series, only in the cases described here the extracting

solution was one containing sodium bicarbonate and disodium hydrogen phosphate (hydrated) both to the extent of 0·5 %.

For the filtration the apparatus shown in Fig. 1 was employed. The two candles are joined by a T-piece fitted into a rubber stopper inserted into the top of the large collecting vessel known as a "Massen." The quickest filtration was obtained by reducing the pressure inside the "Massen" to about 100 mm. Hg and at the same time continually clearing the surface of the candles by the scrubbers as shown in the figure. These scrubbers consist of a ring of brass slightly wider than the diameter of the candle and lined on the inside with a strip of rubber pay-mat. By moving the scrubber up and down over the surface of the candle, the raised points of rubber clean the surface free of the slime that accumulates and chokes the filter.

For convenience the "Massen" was graduated to show every 50 cc. up to 300 cc. The collected filtrate was run out through the tap (*A*) into specially made conical flasks (*B*). The outlet tube from (*A*) is enclosed in a hood of wider bore; the conical flasks were therefore made with a long neck without any lip and of such a diameter that they could be pushed up inside the hood. The transference of the filtrate from the "Massen" to the conical flasks could thus be done without risk of infection, if the usual bacteriological precautions were taken.

The "Massen" and attached filters were sterilised by autoclaving at 120° for 20 minutes. The apparatus ready for sterilisation is shown on the left of Fig. 1. The tap (*A*) was tightly wrapped up in a pad of cotton wool, because when the suction is applied there often occurs at this tap a leak of air into the "Massen" in spite of the fact that the tap has been well greased by a mixture of white wax and lanolin. Since rubber shrinks on heating, the stopper with the T-piece tends to become loose and consequently was likewise covered up with a thick layer of cotton wool; it could thus be reinserted when the apparatus was cold without undue risk. When the required volume of the extract had been filtered, the glucose was introduced into the "Massen" via the tap (*A*). The tap bore was first cleared free of wax by a hot wire, the autoclaved glucose solution was introduced through the bore of the tap by means of a sterilised glass tube drawn out to a long thin point.

After shaking up, a few cc. were run out to wash out any glucose that might have adhered to the sides of the outlet tube. The extract was then measured out into the conical flasks, 25 cc. in each. One flask was taken for the initial values of glucose; the proteins were precipitated by tungstic acid and the glucose estimated by the method of Schaffer and Hartmann [1921]. The other samples were tested for sterility and analysed at the end of the incubation. For the detection of bacteria, two tubes of Douglas broth were each inoculated with about 1 cc. of the extract. One was incubated in the usual manner for aerobes. In the other the cotton wool plug was flamed and pushed in. The tube was fitted with a rubber stopper carrying a glass tube drawn out to a constriction ready for sealing: the air was sucked out and re-

placed by purified hydrogen; this alternate evacuating and refilling with hydrogen was done five times and lastly the tube was sealed when evacuated. This tube was incubated for the detection of anaerobes.

The difficulty of producing sterile filtrates was overcome after several failures but it was soon realised that all Berkefeld filters do not necessarily give sterile filtrates and unless one uses candles previously tested for their ability to hold back bacteria (in this case a peptone culture of *B. pyocyaneus* was used and the filtrate plated on agar), sterility is somewhat uncertain, and this entailed a great waste of effort. This difficulty was connected with the even greater one of producing extracts that were glycolytically active to a decided extent. Again after many failures the following details of technique had to be adopted.

The reaction of the extract must be on the alkaline side of p_H 7·0; on the acid side of this the proteins do not pass through the pores of the filter, the candles choke very early in the filtration and the filtrates are inactive. This was the experience of Mudd [1922].

The extracts must be well frozen to a good stiff cream before the filtration is started so that the extract enters the filters in a concentrated condition by the freezing out of the water. Even with these precautions the best results were invariably obtained by using new, dry, but autoclaved filters. There was always however the attendant risk of infection under these conditions as the candles had not been previously tested. It was not possible to use new candles on every occasion. The most effective method of clearing an old filter was to fill it with 0·2 *N* sulphuric acid and to autoclave it in this condition. This produces a rapidly filtering candle, and in many cases this treatment could be repeated four or five times without the structure being destroyed or the ability to hold back bacteria being lost. Such filters, washed free of acid by bicarbonate and sucked "dry" on the aspirator, lose weight to the extent of 30 g. if heated at 100° until of constant weight. When using filters in this condition, *i.e.* with the kieselguhr skeleton damp, it was found that the first 100 cc. of the filtrate could be rejected because there was little or no glycolytic activity in this fraction; but the next 100 to 150 cc. usually showed glycolytic activity. With new, dry filters the whole of the filtrate could be used. The process of drying by heating to about 100° rapidly leads to the crumbling of the filter and for that reason it was given up; but, if the early portions of the filtrate were rejected, these old filters could be used again and again.

Experimental results.

Although no lactic acid estimations were made in this series given below, later work has indicated that the end product is lactic acid, a fact now well established. Never, in any single instance (and 34 cases were investigated for this) was any evidence found of the formation of a more complex carbohydrate (disaccharide) as suggested by Levene and Meyer [1912]. The glucose concentrations are expressed as mg. per 100 cc. extract.

Table of results.

No. of exp.	Glucose Start	Glucose End	Glucose Loss	Time hrs.	Remarks
50 *B*	135	22	113	24	Tame rabbit
54 *C*	112	Trace	111	24	„
57 *B*	149	„	148	24	„
59 *B*	157	25	132	24	„
60 *C*	136	Trace	135	24	„
73 *B*	133	„	132	4	„
77 *B*	154	12	142	4	Cat
79	169	169	0	3	1st 50 cc. Tame rabbit
	182	173	9	3	2nd 50 „
	173	98	75	3	3rd 50 „ Damp filters
	187	55	132	3	4th 50 „
82	147	71	76	2	1st 50 cc. Tame rabbit
	146	19	127	2	2nd 50 „
	155	24	131	2	3rd 50 „ New dry filters
	146	88	58	2	4th 50 „
	152	129	23	2	5th 50 „
83	254	243	11	2	1st 50 cc. Tame rabbit
	285	243	42	2	2nd 50 „
	268	177	91	2	3rd 50 „
	258	197	61	2	4th 50 „ Damp filters
	251	120	131	2	5th 50 „
86	308	113	195	3	1st 100 cc. Tame rabbit
	332	103	229	3	2nd 100 „
87	512	319	132	3	1st 100 cc. Tame rabbit
89	351	21	330	8	Tame rabbit
91	641	402	239	3	„
93	574	407	167	3	Cat
95	332	152	180	2	„
96	336	119	217	3	Two tame rabbits
97	355	179	176	3	Cat
99	330	174	156	2	1st 50 cc. Two tame rabbits
	345	170	175	2	2nd 50 „
	327	122	205	2	3rd 50 „
	335	216	119	2	4th 50 „ New dry filters
	343	279	64	2	5th 50 „
100	345	129	216	2	Two tame rabbits
102	308	233	75	1	1st 75 cc. Cat
	305	152	153	2	2nd 75 „ New dry filters
	291	120	171	2	3rd 75 „
	291	38	253	2	„ „ plus 0·1 g. of $NaHCO_3$

Quoted above are 21 cases out of a total of 102 extracts filtered. The 81 cases not recorded include both those that were infected and those that showed little or no loss of glucose. The latter are interesting although not of direct value in a positive sense. Many of the early failures were due to the use of an unsuitable extracting solution. The results showed that a mixture of sodium or potassium bicarbonate with the chlorides or dibasic phosphates of those metals was quite suitable if the salts were used in concentrations not greater than about 1 %. A solution of 5 % Na_2HPO_4, $12H_2O$ gave an extract that coagulated during the filtration. The extraction of the muscle proteins was very extensive with this solution but that very fact rendered it highly unsuitable for filtration purposes.

Another source of failure, which was not appreciated until experiments had been done with unfiltered extracts, is that tame rabbits are not, for some peculiar reason, the best animals for the production of very glycolytic extracts.

During the course of the author's investigations on glycolysis, over 200 different extracts have been examined and the best animals for this type of investigation have been found to be either wild rabbits or cats. Even with these animals one occasionally encounters extracts of apparently normal individuals that show only a slight glycolytic activity, no matter how rigorously a standardised method of preparation is employed. In the case of tame rabbits, extracts prepared in the above manner show good glycolysis in only about 50 % of the cases. In this respect lactic acid formation from glucose must be distinguished from lactic acid formation from glycogen, the latter unlike the former takes place at a fairly uniform rate in all freshly prepared extracts. This is still under investigation.

Discussion.

The above results indicate that the glycolytic enzyme (or enzymes) can be demonstrated by the destruction of added glucose in filtrates from Berkefeld filters. No activator of any description, such as the pancreatic extract employed by Cohnheim [1903; 1904, 1, 2; 1907], was added. More important still, no intact cell structures were present, and the condition put forward by Fletcher [1911], that glycolysis can only take place if the muscle structure is preserved, is not indicated here at all. If the above type of extract be centrifuged in a Sharples supercentrifuge, a clear transparent solution is produced, similar to the filtrates from the Berkefeld filters, but of course not sterile. The removal, in this manner, of the muscle debris that passes through the mesh of the muslin used in the hand-press, has absolutely no effect on the glycolytic activity. The muscle enzymes are in solution in the same sense as the muscle proteins, although removed from the internal environment of the muscle fibres.

The question of sterility is not, in the author's opinion, an important one for the following reason. An examination of the literature of this subject gives one the impression that an incubation period of at least 24 hours is necessary before a decided loss of glucose can be detected; but an extensive examination of many extracts at intervals of 10 minutes over a period up to 2 hours, indicated that the greatest rate of destruction of glucose takes place in the first 10 minutes of the incubation. In extracts of cat muscle the loss of glucose in the first 10 minutes has been observed to be as high as 78 mg. per 100 cc. extract: with wild rabbit muscle it has been as high as 95 mg. This high rate lasts for about 40 minutes; consequently the period of incubation need not exceed, at the longest, 2 hours, and even 90 minutes is quite sufficient. In that time the effect of bacterial growth is not evidenced. The many cases of unfiltered extracts, prepared with no precautions against infection and showing no loss of glucose at the end of a 2 hours' incubation, indicate that it is the muscle enzymes and not bacteria that give rise to lactic acid in the early period of the incubation, and it is in that early period that the most important and interesting phase of lactic acid formation takes place.

SUMMARY.

Glycolysis has been observed in extracts of perfused muscle after filtration through Berkefeld filters. The results quoted are with those filtrates that were bacteriologically sterile. The original observation of Brunton and Rhodes on glycolysis in similar filtrates has been confirmed.

In concluding, the author wishes to express his gratitude to Prof. E. Waymouth Reid, F.R.S., who suggested and supervised this research; likewise his thanks to Mr W. Milne for taking charge of the sterilising and care of the apparatus, and also to Prof. W. J. Tulloch, for his advice on the bacteriological side of the problem.

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