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

II. ANTIGLYOXALASE AND GLYOXALASE.

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(Received August 3rd, 1925.)

1. THE LACTIC ACID INHIBITOR AND ANTIGLYOXALASE.

IN a recent paper on the pancreatic control of carbohydrate metabolism in muscle [Foster and Woodrow, 1924] there was described a factor found in the pancreas and capable of inhibiting the production of lactic acid by chopped muscle *in vitro*. It was pointed out that another substance found in the pancreas, namely the antiglyoxalase described by Dakin and Dudley [1913, 4], might have the same effect. According to these authors antiglyoxalase inhibits the action of glyoxalase, thereby preventing or diminishing the production of lactic acid from methylglyoxal and of mandelic acid from phenylglyoxal. If the lactic acid produced by muscle *in vitro* is derived from methylglyoxal by the action of glyoxalase, its formation would similarly be prevented by the addition of antiglyoxalase to the mixture. If, on the other hand, the production of lactic acid from carbohydrate in muscle is not through methylglyoxal, but some other intermediary product, glyoxalase will not form part of the system and therefore antiglyoxalase will not inhibit its production. It is also true that if a certain factor inhibits the production of lactic acid in muscle, but not the production of α -hydroxy-acids from α -ketonic aldehydes, the lactic acid is probably not derived from methylglyoxal but from some other intermediary compound.

The experiments described below were undertaken with the object of deciding whether the factor which inhibits lactic acid formation is identical or not with antiglyoxalase, and hence to get some evidence as to whether or not methylglyoxal is an intermediary product in the carbohydrate metabolism of muscle.

The presence of methylglyoxal has never been demonstrated in the tissues, and its properties render it extremely unlikely that it could persist in sufficient quantities for this to be possible; but there is every reason to believe that, in the liver at any rate, it can form a stage in the breakdown of glucose. This follows from the work of Dakin and Dudley [1913, 2] on the presence of glyoxalase

in the liver and more especially from their perfusion experiments [1914]. There is, however, not the same evidence for its presence in muscle. This will be discussed more in detail in Part III of this paper.

Though all the experimental work to be described has been done on muscle extracts, or chopped muscle, there is every reason to believe that the fundamental chemical reactions are the same as those involved in the production of lactic acid in the living, intact muscle [Meyerhof, 1920, 1921 and Hill, Long and Lupton, 1923].

In the paper referred to above [Foster and Woodrow, 1924] some evidence was brought forward to show that the lactic acid inhibitor and antiglyoxalase are not identical and this is confirmed by the work to be described in the present paper.

Experimental details.

The method described by Dakin and Dudley [1913, 3; 1914] for the detection of glyoxalase and antiglyoxalase has been followed exactly, and, unless otherwise stated the procedure has been the same in all experiments. The glyoxalase solution used was obtained by treating minced chicken muscle with five times its weight of water and, after allowing the mixture to stand for an hour, filtering it through muslin. 50 cc. of the glyoxalase solution were allowed to act on 0.2 g. phenylglyoxal dissolved in 10 cc. water. The mixture was incubated over night at 37° with an excess of precipitated chalk and a few drops of toluene in a conical flask. The resulting mandelic acid was extracted with ether and its rotation measured, exactly in the way described by the above authors. When the muscle extract was allowed to act on the phenylglyoxal alone, a relatively large residue of white crystals of mandelic acid was left after the evaporation of the ether. This was dissolved in 15 cc. of water, boiled with a trace of charcoal and filtered. The rotation of the solution was measured in a 2 dm. polarimeter tube and the figures given in the tables are the rotations observed with mercury green light. The solutions examined were always perfectly clear and colourless. When a solution was tested for antiglyoxalase it was added to the glyoxalase some time, usually an hour, before the phenylglyoxal and the mixture kept at 37°. The control mixtures were always similarly incubated with an equal volume of saline.

Foster and Woodrow [1924] showed that a 70 % alcoholic extract of a dried pancreas preparation (the alcohol being subsequently removed by vacuum distillation, etc.) contained the lactic acid inhibitor, and it was an extract prepared in this way which was tested for antiglyoxalase. The same preparation of dried pancreas was used throughout, but the extract was made fresh for each experiment. In some cases (Exps. 2 and 3) it was found that the extract did have some antiglyoxalase action, but by exercising great care in preparing it, in particular by using alcohol accurately diluted to 70 % and centrifuging till the solution was perfectly clear, an extract was obtained which had no inhibiting action on glyoxalase (Exp. 4, Nos. 1 and 4).

Exp. 1. Chicken muscle used as source of glyoxalase. Two pancreatic extracts tested A and B. (B had been prepared some weeks earlier and kept at -7° .)

Table I.

No.	20 % extract of chicken muscle cc.	Calcium carbonate suspension cc.	Inhibitory factor	Phenyl- glyoxal g.	Toluene drops	Rotation	Notes
1	50	10	None	0.2	5	-3.16°	Control
3	50	10	5 cc. A	0.2	5	-3.46°	Duplicate
4	50	10	5 cc. A	0.2	5	-3.16°	No inhibition
5	50	10	5 cc. B	0.2	5	-3.3°	No inhibition
7	50	10	5 cc. A heated to 85° for 10 mins.	0.2	5	-3.01°	No inhibition
9	50	10	5 cc. A boiled	0.2	5	-3.27°	No inhibition
			Incubated 1 hour				
			Incubated 20 hours				

This solution therefore did not contain antiglyoxalase. Some of the same preparation was found to inhibit the formation of lactic acid in pigeon muscle by about 50 %¹.

Exp. 2. All details as in Expt. 1, except that the chicken muscle extract had been stored some time at -7° and had apparently lost some of its glyoxalase as the controls gave low rotations.

Table II.

No.	Inhibitor	Rotation	Notes
1	None	-0.98°	Control
2	None	-1.01°	Control
3	5 cc. 70 % alcoholic extract of pancreas	-0.81°	Slight inhibition
4	" "	-0.90°	Slight inhibition

This preparation therefore did contain traces of antiglyoxalase.

Exp. 3. A 70 % alcoholic extract of pancreas was tested both for lactic acid inhibitor and for antiglyoxalase and chicken muscle was used in both cases.

A. Lactic acid estimations.

In each of four flasks were placed 10 cc. phosphate buffer solution p_H 7.5, 3 cc. saline or pancreatic extract and 1 cc. $N/10$ KCN and 4 g. minced muscle [see Foster and Woodrow, 1924]. Left at room temperature for 24 hours.

Table III.

No.	Inhibitor	% of lactic acid	Notes
1	3 cc. saline	(Lost)	Control
2	3 cc. saline	1.5	Control
3	3 cc. extract	0.911	40 % inhibition
4	3 cc. extract	0.787	47 % inhibition

There is therefore at least a 40 % inhibition of lactic acid production due to the extract.

¹ Unpublished experiment by Mrs D. M. Needham.

B. *Glyoxalase estimations.*

Table IV.

No.	Inhibitory factor	Rotation	Notes
1	6 cc. saline	-2.03°	Control
2	6 cc. saline	-2.12°	Control
3 }	3 cc. extract + 3 cc.	-1.82°	16 % inhibition
4 }	saline each	-1.64°	16 % inhibition
5 }	3 cc. insoluble residue	-1.64°	26 % inhibition
6 }	+ 3 cc. saline each	-1.42°	26 % inhibition
7	3 cc. extract + 3 cc. in-soluble residue	-1.55°	20 % inhibition
8	3 cc. extract + 3 cc. ether-soluble residue	-1.45°	29 % inhibition

Not only was the final purified 70 % alcoholic extract of the pancreas tested for antiglyoxalase, but also the various fractions removed in course of preparation. The figures show that the extract contained some antiglyoxalase, but so did the residues from the ether and centrifuging. The inhibition of the glyoxalase by 70 % alcoholic extract is only 16 % of the total (as against 40 % of the lactic acid), and as the residues gave higher figures it seemed probable that more careful preparation of the final solution would remove all the antiglyoxalase.

A similar but more conclusive result was obtained in Expt. 4 in which a water extract was made of the residue of the gland insoluble in 70 % alcohol. The alcoholic extract was made in the usual way. The residue was then dried in a current of warm air to remove all traces of alcohol, and ground with sand and distilled water and left overnight. Then the mixture was centrifuged till a clear supernatant liquid was obtained. This was made up to 1 % with sodium carbonate and incubated at 37° to remove trypsin, as recommended by Dakin and Dudley [1913, 4]. It was found that there was some trypsin still left, but it has been shown that antiglyoxalase is distinct from trypsin (Dakin and Dudley [1913, 2] and Exp. 7 below). 5 cc. of this extract were equivalent to 1 g. of the dried gland.

Exp. 4.

Table V.

No.	Extract	Treatment	Rotation	Notes
12 }	5 cc. saline	—	-3.21°	Control
11 }	5 cc. saline	—	-3.12°	Control
1 }	5 cc. alcoholic	—	-2.92°	Very slight
2 }	extract	—	-3.00°	inhibition
3 }	5 cc. alcoholic	Heated at 85°	-3.14°	No
4 }	extract	for 10 mins.	-3.04°	inhibition
5 }	5 cc. water ex-	—	-0.52°	Almost com-
6 }	tract	—	-0.51°	plete in-
				hibition
7 }	5 cc. water ex-	Heated at 85°	-2.16°	Some de-
8 }	tract	for 10 mins.	-2.13°	struction of
				antiglyoxalase
9 }	5 cc. water ex-	Boiled for 10	-1.72°	Some destruc-
10 }	tract	mins.	-1.64°	tion of anti-
				glyoxalase

These figures show that the extract made with 70 % alcohol was practically free from antiglyoxalase, whilst the watery extract was rich in this factor and could inhibit the glyoxalase. It would appear therefore that the lactic acid inhibitor is extracted by 70 % alcohol, whilst the antiglyoxalase is not.

SOME OBSERVATIONS ON ANTIGLYOXALASE.

In all the experiments with phenylglyoxal it was noticed that whenever there was any antiglyoxalase action, that is to say when no mandelic acid was produced after adding phenylglyoxal to a mixture of muscle extract and an extract of pancreas, there was a development of an orange coloration. The colour produced was always proportional to the degree of inhibition. If this was complete the colour produced during incubation of the mixture was a deep orange, and, with partial inhibition lighter colours were obtained. This phenomenon was noticed whatever pancreatic preparation was used, fresh fowl and ox pancreas, or a watery extract of the dried pancreas; and also when an alcoholic extract showed some inhibitory power, as in Exp. 2. The colour gradually increased during incubation.

The greater part of the colouring matter was precipitated during removal of the protein, by boiling after addition of ammonium sulphate, and the coagulum was always brightly coloured, but some colour persisted in the filtrate. Acidification with phosphoric acid produced no change but the ether solution and residue frequently turned red, and once or twice a blue colour was noticed at this stage. All traces of the colour disappeared on boiling with charcoal and the final solution was as clear and colourless as the controls. The control flasks in which mandelic acid was produced from the phenylglyoxal never showed any trace of this orange colour at any stage. Since this colour was invariably associated with a failure to convert the phenylglyoxal into mandelic acid it seemed worth while to investigate the connection between the two phenomena.

At first sight it seemed probable that the colour might be due to the excess of phenylglyoxal present when the glyoxalase was inhibited; but this can hardly be the case, as in the controls also there must be a large excess of unchanged phenylglyoxal present. A rotation of -3° only means a conversion of 30 % of the 0.2 g. phenylglyoxal added to each flask into mandelic acid. The unchanged excess is precipitated during the boiling with ammonium sulphate [Dakin and Dudley, 1913, 1].

Then it was found that on incubating a pancreatic extract with phenylglyoxal, in the absence of any muscle extract or any other tissue preparation, the mixture turned the same deep orange colour and an orange precipitate was formed. Evidently there was some factor in pancreatic extract which could bring about this change in the phenylglyoxal. Is the apparent "antiglyoxalase" action of pancreatic preparations due, not to their inhibitory action on the glyoxalase, but to the fact that they contain something capable of changing phenylglyoxal into a highly coloured body which cannot give rise to

mandelic acid? In other words, is their action not on the glyoxalase but on the phenylglyoxal? It is worth noting that in every experiment on anti-glyoxalase reported in their original paper Dakin and Dudley [1913, 4] used phenylglyoxal as substrate and not methylglyoxal.

In the experiments described below some evidence is brought forward to show that pancreatic extracts do in some way change the phenylglyoxal. It cannot, of course, be said that they have no action on glyoxalase, and therefore that "antiglyoxalase" does not exist, until the action of pancreatic extracts has also been tried on methylglyoxal. It may be mentioned in passing, however, that it is difficult to imagine the significance and function of an anti-glyoxalase since it is not found in the blood stream and the removal of the pancreas does not increase the glyoxalase action of the tissues [Dakin and Dudley, 1913, 4].

Experimental details.

If by previous incubation with pancreatic extract the phenylglyoxal has undergone some change, it will not give rise to mandelic acid when subsequently added to a glyoxalase preparation even if all the antiglyoxalase factor is removed. This was the principle underlying the following experiments. After incubation overnight the mixture of phenylglyoxal and a pancreatic extract, which was always orange, was boiled to destroy any antiglyoxalase and then added to a glyoxalase solution prepared from chicken muscle in the usual way. In order to demonstrate that any change in the phenylglyoxal was not due to the boiling, the solution (0.2 g. in 10 cc.) was boiled before being added to the control flasks. It was shown repeatedly that the phenylglyoxal was in no way affected by boiling or even autoclaving (Exp. 7). There were always controls with and without pancreatic extract as shown in the following table:

Exp. 5. Pancreatic extract 20 cc. = 1 g.

Table VI.

No.	Extract added	Treated	Rotation	Notes
1	20 cc. saline	None	-3.02°	Controls
2	20 cc. saline	None	-2.91°	Controls
3	20 cc. water extract	Raw	+0.01	Orange colour
4	20 cc. water extract	Raw	+0.07°	and inhibition
5	20 cc. water extract	Incubated with the phenylglyoxal 24 hours and then	-0.23°	Orange colour
6	20 cc. water extract	boiled 13 mins.	-0.25°	and inhibition
7	20 cc. water extract	Boiled 13 mins.	-0.16°	Orange colour
8	20 cc. water extract	Boiled 13 mins.	-0.39°	and inhibition

There is therefore no mandelic acid formed from the phenylglyoxal, which was first treated with the pancreatic extract; but this is no evidence that the effect was due to destruction of the phenylglyoxal since the addition of boiled extract to the mixture containing glyoxalase also prevents the formation of mandelic acid. This means that either the phenylglyoxal was unchanged by the previous action of the pancreatic extract, which seems unlikely in view

of the marked change in colour and the development of precipitate, or that the "anti" factor, whether it acts on the phenylglyoxal or glyoxalase, is thermostable. Dakin and Dudley [1913, 4] found their antiglyoxalase preparations destroyed by heating to 85° for 10 minutes and this was confirmed in the case of a very weak antiglyoxalase solution (see Exp. 4).

It is generally agreed that any factor of an enzyme character would be destroyed by autoclaving even if protected by other substances, such as protein, or by high concentration. Exp. 5 was therefore repeated and all the various solutions were autoclaved at 120° for 20 minutes. It was necessary to show, first of all, that the phenylglyoxal itself was unaffected by autoclaving, and this was done by comparing the effect of glyoxalase on autoclaved and unautoclaved phenylglyoxal. A mixture of rat muscle and liver was used as the source of glyoxalase.

Table VII.

No.	20 % extract of rat liver and muscle	Phenyl- glyoxal 2 %	Treatment	Rotations
1	50 cc.	10 cc.	Control	-2.67°
2	50 cc.	10 cc.	Autoclaved	-2.95°

The phenylglyoxal solution can therefore be autoclaved with impunity.

Exp. 7. Chicken muscle as source of glyoxalase.

Table VIII.

No.	Extract added	Treatment	Rotation	Notes
1	10 cc. saline	None	-2.42°	Control
2	10 cc. saline	None	-2.40°	Control
3)	10 cc. water	Incubated 24 hrs. with 0.2 g.	-0.09°	Inhibition. The mixture was
4)	extract	phenylglyoxal and then the whole autoclaved	-0.09°	bright orange before ad- dition of muscle extract
5)	10 cc. water	None	-0.10°	Inhibition. Orange colour
6)	extract	None	-0.05°	developed during incuba- tion
7)	10 cc. water	Autoclaved before use	-0.92°	Some development of orange
8)	extract	Autoclaved before use	-0.81°	colour and partial inhibi- tion

It is obvious that even autoclaving has not completely destroyed the inhibitory factor whatever it is. It is extremely improbable that anything of an "organised" nature would have survived such treatment. All trace of trypsin has gone, though it is difficult to destroy by boiling alone in such high concentration. It must be some thermostable substance present in the pancreatic extract which is producing the effect.

In order to make sure that the results observed were not due to differences of reaction, though this was very improbable in view of the excess of chalk used in every flask, the experiment was repeated exactly, except that every solution was neutralised to p_H 7.0 both before and after autoclaving. The result was the same as shown by Table VIII.

Exp. 8.

Table IX.

No.	Extract	Treatment	Rotation	Notes
1	10 cc. saline	—	-2.39°	Control
2	10 cc. water extract	Incubated overnight with phenylglyoxal and then autoclaved	-0.16	Inhibition
3	10 cc. water extract	Raw	-0.13°	Inhibition
4	10 cc. water extract	Autoclaved	-0.80°	Partial inhibition

Owing to the difficulty of removing the factor after its action on phenylglyoxal, these experiments do not definitely prove that its action is on the phenylglyoxal rather than the glyoxalase; but there are two facts which make this extremely probable. One is the development of the orange colour, which invariably accompanies inhibition, by the action of the pancreatic extract on the phenylglyoxal alone. The other is that the inhibition produced by the autoclaved extract on the whole mixture is only partial (as shown by Exp. 4, Nos. 7 and 8), while the inhibition produced by its action on the phenylglyoxal is complete.

IS METHYLGLYOXAL THE PRECURSOR OF LACTIC ACID IN MUSCLE?

In the paper already referred to [Foster and Woodrow, 1924] the suggestion is made that certain facts of muscular metabolism, in particular the liberation of lactic acid at special surfaces where it is required for a special purpose in the series of phenomena that result in muscular movement, would be more easily explained if there were any evidence to show that the muscle has a specialised carbohydrate metabolism of its own; that is to say, if the sequence of chemical changes, which take place in the production of lactic acid from the carbohydrate stores of the muscle, were not necessarily the same as those taking place in other organs, where the lactic acid is merely a stage on the way to the complete combustion of glucose to carbon dioxide and water, with the liberation of all its available energy. In the muscle lactic acid is needed in relatively large quantities at a given surface at a given moment, and is produced as the result of a special stimulus, normally through a nerve. If there were this alternative course in the breakdown of glucose the mechanism controlling or inhibiting these changes would be different from that in an organ not so specialised; the liver for example. As already tentatively suggested such a differentiation would help to explain some of the anomalies of the diabetic condition.

It must be admitted that up to the present there is very little evidence in favour of this hypothesis, but one significant fact has accidentally come to light in the course of the work described in the earlier part of this paper. Some months ago the author (in collaboration with Mr C. E. Woodrow) made repeated attempts to test the pancreatic extract for the presence of anti-glyoxalase in the way described above. Time after time the same difficulty occurred, and the experiments were useless because the controls were always negative. When a muscle extract prepared in the routine way was added to

phenylglyoxal no mandelic acid was obtained. This was repeated five or six times on different specimens of phenylglyoxal and no mandelic acid was ever produced. Later when another muscle extract (chicken) was used as the source of glyoxalase no further difficulty was experienced, and it was always possible to get an extract able to convert phenylglyoxal into mandelic acid. The reason for our lack of success in all the earlier experiments was that we had always used *rabbit* muscle. It is now clear, as the experiments described below prove, that *rabbit muscle* contains no glyoxalase.

It seemed impossible that the original discoverers of glyoxalase should not have found this exception to the general rule that, with the exception of the pancreas and certain specialised glands, all tissues contain glyoxalase, including the muscle of various animals, *e.g.* dog, fowl, cat, etc. An examination of their results [Dakin and Dudley, 1913, 1, 2, 3 and 4] shows that whenever the rabbit was the animal selected they used a mixture of muscle and *liver*. As the experiments described in detail below show, rabbit liver is very rich in glyoxalase, while from the muscle it is completely absent. This is a very significant fact.

Experimental results.

These experiments were all carried out in the routine way already described. In each case a 20 % extract of the muscle was used and the rotation is that obtained by the action of 50 cc. muscle extract on 0.2 g. phenylglyoxal. The final solution was always made up to 15 cc. and the reading taken in a 2 dm. polarimeter tube, mercury green being the source of light.

Table X.

Exp. no.	Source of glyoxalase	Rotation of mandelic acid	Notes
10	Rabbit muscle	-0.21°	No crystalline residue from the ether
	Rabbit muscle	-0.19°	
14	Rabbit liver	-2.50°	Typical crystals of mandelic acid
	Rabbit liver	-2.28°	
	Muscle from same animal	+0.44°	No crystalline residue
	Muscle from same animal	+0.39°	No crystalline residue
17	Rabbit liver	-2.17°	Mandelic acid crystals
	Rabbit liver	-2.26°	Mandelic acid crystals
	Muscle from same animal	+0.05°	No residue except traces of ammonium sulphate
	Muscle from same animal	+0.07°	

It is quite certain that rabbit muscle produces lactic acid in a perfectly normal way, in fact it is the muscle on which a great deal of experimental work has been done [Fletcher, 1911, 1913; Winfield and Hopkins, 1915]. The present author has frequently found that chopped rabbit muscle produces lactic acid in relatively large amount. The following figures will serve as examples. In each case the muscle was removed immediately after the death of the rabbit and rapidly minced. One fraction was at once ground in ice-cold alcohol and served as control, while the rest was left at room temperature for 24 hours in various buffered solutions and the lactic acid then estimated

in the routine way [Foster and Woodrow, 1924]. These figures are selected at random from a large number of experiments carried out for another purpose.

Table XI.

% of lactic acid in control	% of lactic acid after incubation	Notes
0.164 (1)	0.523 (1)	Incubated in a "Ringer" solution containing sodium bicarbonate (1) or borate (2)
0.164 (2)	0.664 (2)	
0.16	0.44	Incubated in a "Ringer" solution containing bicarbonate
0.13	0.47	
0.18	0.46	Incubated in a "Ringer" solution containing bicarbonate
0.19	0.46	
0.12	0.54	Incubated in a "Ringer" solution containing bicarbonate
0.14	0.53	
0.16	0.673	Incubated in a "Ringer" solution containing bicarbonate

It follows from these experiments that in rabbit muscle lactic acid is produced without the action of glyoxalase and, presumably therefore, it does not arise from methylglyoxal. On the other hand, the liver of the same animal is rich in glyoxalase and there is every reason to suppose, as Dakin and Dudley suggested [1913, 1914], that it plays an important part in the conversion of ketonic aldehydes into the corresponding α -hydroxy-acids, and that lactic acid is produced in this way in this organ from methylglyoxal. This is at present an isolated instance and the muscle of certain other animals, mammals as well as birds, has definitely been shown to contain glyoxalase, but it is possible that there are other animals like the rabbit which have no glyoxalase in their muscles. However, even one exception is important since it shows that methylglyoxal is not necessarily a precursor of lactic acid. It is significant that the amount of mandelic acid obtained by Dakin and Dudley [1913, 4] when muscle was used as the source of glyoxalase, was a good deal lower than that obtained from the same weight of liver. For example, for the dog the rotations observed were:

Liver	— 2.3° to — 2.58°
Muscle	— 0.45° to — 1.13°
Heart and tongue	— 0.45°

It is possible that where glyoxalase is found in muscle it serves to catalyse the breakdown of glucose into carbon dioxide and water, through methylglyoxal, in the general metabolism of the muscle, but plays no part in the special production of lactic acid as the result of a nerve stimulus, which results in contraction.

SUMMARY.

1. The factor found in the pancreas and able to inhibit the production of lactic acid by chopped muscle *in vitro* is not antiglyoxalase.
2. Some evidence is brought forward to show that the "antiglyoxalase" action is probably due to the action of some factor in the pancreas on phenylglyoxal rather than on the glyoxalase.

3. It is shown that rabbit muscle does not contain glyoxalase and the significance of this in the carbohydrate metabolism of muscle is discussed.

I wish to thank Dr Dudley for his interest and for the hospitality of his laboratory at one stage of this work, and Sir F. G. Hopkins for his continuous help and encouragement.

The author has been in receipt of a grant from the Food Investigation Board and the subject of this communication has formed part of a report to that Board.

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