LXXI. EXPERIMENTS ON RESPIRATION AND FERMENTATION.

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The researches of Batelli and Stern [1911] and of Meyerhof [1918 *et seq.*] on tissue respiration, and those of the latter on its relation to alcoholic fermentation, have led to the conception that the respiration both of surviving muscle and of "acetoned" yeast is controlled by a factor which also plays an essential part in the process of alcoholic fermentation. Harden and Young [1906] had shown that, in addition to potassium and phosphate ions, a soluble "co-enzyme" must be present to ensure the fermentation of glucose by yeast. Meyerhof stated that this co-enzyme was also essential as a "co-enzyme to respiration" and he named it the "respiration substance" [Meyerhof, 1919, 2]. In the case of the respiration of washed muscle or of washed "acetoned" yeast suspended in a boiled extract of tissue or yeast, it provided a general catalytic stimulus to the cell respiration as a whole, accelerating the oxidation of various oxidisable substances also present in the cell extract, by virtue of its property of acting as an oxygen carrier. It exerted a similar function during the course of alcoholic fermentation [Meyerhof, 1919, 2]. On the other hand he maintained [Meyerhof, 1920, 2] that when a muscle is chopped, but not washed, and suspended in a buffer solution or in Ringer, the oxygen consumption observed is due to the oxidation of the lactic acid formed during the chopping, to the practical exclusion of any other respiratory process.

In a previous paper [Holden, 1923] it was suggested that, in the case of washed frog muscle respiring in a yeast "boiled extract," the oxygen uptake should be attributed rather to the presence of oxidisable substances in the extract than to the action of any "co-enzyme to respiration" having a solely catalytic function. The necessity for assuming that a respiratory co-enzyme exists does not seem indeed to have been established.

The present paper describes the results of experiments in extension of those referred to above. The principle of the experiments is for the most part similar to that of those already described. "Boiled extract" of muscle or yeast was aerated in contact with washed muscle or with "acetoned yeast." The effects of the extract after aeration, on the oxygen uptake of washed muscle, or the fermenting power of washed "acetoned" yeast, were compared

with those of the original unaerated extract, samples of which were stored *in vacuo* at 0°.

The yeast extracts were more concentrated than those previously employed, being made by stirring baker's yeast in its own weight of boiling water and filtering off the insoluble residue. The muscle extracts were made by mincing the muscle rapidly, covering it with distilled water, heating rapidly to boiling with constant stirring, and filtering by suction. The extract was immediately adjusted to p_H 7·6, a portion being passed through a Berkefeld filter and stored *in vacuo* at 0° as before. When the aeration was performed at p_H 5·8 in contact with yeast, the stored sample was stored at that p_H.

The muscle used in the respiration experiments was finely chopped with scissors and washed as often as stated in the tables. Each washing was performed by shaking vigorously in a stoppered bottle with the volume of distilled water shown. The washing was continued for 20 minutes and the muscle residue was then filtered off on a small suction filter.

The "acetoned" yeast referred to was made as follows. 30 g. of baker's yeast was crumbled and placed in a glass dish in an eight-inch vacuum desiccator over strong sulphuric acid. Within 24 hours it dried to a brittle mass, which was finely ground and replaced in the desiccator for a further 24 hours. It was then sterilised by grinding in pure *anhydrous* acetone two or three times for five minutes each time, and air dried. The resulting product was a light, dry powder which while apparently quite free from living cells showed considerable fermentative and respiratory activities. After washing a few times with cold distilled water it lost these powers, but they were restored on the addition of boiled extracts. This method, unlike the various German commercial methods, does not involve the use of very high pressures for the purpose of dehydrating the yeast, but it seems to be quite suitable for the preparation of small amounts for laboratory use. It is important to use carefully purified acetone, as quite small amounts of water, when mixed with it, have a markedly deleterious action on the enzymes.

The "acetoned" yeast was washed by placing 0·1 g. in each of four centrifuge tubes and adding 35 cc. of distilled water to each. The tubes were closed, well shaken, and allowed to stand for ten minutes. They were then shaken again, again allowed to stand for ten minutes, centrifuged, and the supernatant liquid decanted. This was done six times in all, except when otherwise stated.

The extracts were aerated and sterilised as before. The oxygen uptake was determined by the use of Barcroft micro-respirometers as previously described. The experiments performed in order to determine the presence or absence of the "alcoholic co-enzyme" were done in Barcroft apparatus with conical bottles similar to those used for blood-gas analysis. They can be filled with an inert gas if desired, but after a few trials this was found to be unnecessary. They were placed in a water-bath and shaken as in the respiration experiments. Some preliminary experiments showed p_H 5·8 to be the optimum,

so all solutions were adjusted accordingly before use. In each experiment of this type four instruments were used side by side, all being shaken simultaneously in the same water-bath. In each was placed 0·1 g. of the washed "acetoned" yeast.

In addition the instruments contained respectively:

Instrument	Glucose g.	Suspension fluid
A	·3	3 cc. of "stored" extract with 1 % KH_2PO_4 added, adjusted to p_H 5·8
B	None	,, ,, ,, ,,
C	·3	3 cc. of "aerated" extract with 1 % KH_2PO_4 added; p_H 5·8
D	·3	3 cc. of 1 % KH_2PO_4 adjusted with NaOH to p_H 5·8

The results shown in the Tables I and II and in Figs. 1 and 2 are typical of a number of experiments.

Table I.

First respiration: The destruction of the "Respiration substance"							Second respiration: To test for the presence of the "Respiration substance"						
							Cmm. of O_2 absorbed per gram of muscle in one hour						
Extract	Vol. cc.	Muscle used	Weight grams	Times washed	Cc. of H_2O per gram of muscle for each washing	Time of aeration in hours	Phosphate solution	"Respired" extract	"Stored" extract	Muscle used	Times washed	Cc. of H_2O per gram of muscle for each washing	Temp. of respiration °C.
Rabbit muscle	50	Frog	5	6	50	4	34	94	266	Frog	8	50	21·5
Rat muscle	50	none	—	—	—	22	29	117	235	Rat	8	60	22·5
,,	29	Rat	2·9	6	50	5	29	95	225	,,	8	75	22·0
,,	43	,,	4·3	6	50	21	24	58	380	,,	8	75	22·4
Rabbit muscle	80	Rabbit	8	4	40	17	28	59	213	,,	8	75	22·2
,,	72	,,	7·2	4	40	18	39	76	511	,,	8	75	22·0

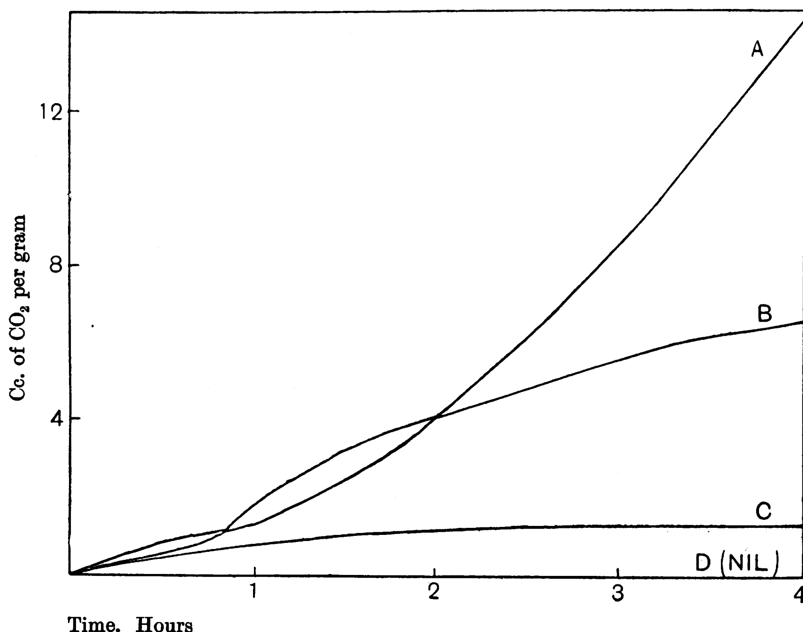

Fig. 1.

Table II. *The destruction of the alcoholic co-enzyme.*

No.	Extract	Time of aeration hours	Vol. of extract cc.	Substance added	Weight	Times washed	Cc. H_2O per g. each washing	Aeration p_H
1	Yeast	17	73	Rat muscle	7·3	4	40	7·6
2	Rabbit muscle	18	72	Rabbit muscle	7·2	4	40	7·6
3	Yeast	18	30	"Acetoned" yeast	0·6	—	—	5·8
4	Rabbit muscle	20	50	,,	1	—	—	5·8
5	Yeast	21	50	Frog muscle	5	6	50	7·6

The test for the presence of the co-enzyme. cc. of CO_2 per g. of yeast.

No.	Temp. °C.	Time in hours	A	B	C	D
1	22·0	4	14·56	6·65	1·34	0
2	22·0	4⅓	24·27	11·16	2·8	0·43
3	21·5	3½	28·3	2·62	3·55	0·06
4	24·0	4	7·32	4·86	0·97	0·27
5	21·1	4	12·1	—	1·25	0·14

The letters A–D *refer to the four instruments, whose contents were as follow:*

	Yeast g.	Glucose g.	Suspension fluid
A	0·1	0·3	3 cc. "stored" extract with 1 % KH_2PO_4 p_H 5·8
B	0·1	None	,, ,, ,,
C	0·1	0·3	3 cc. "aerated" extract with 1 % KH_2PO_4 p_H 5·8
D	0·1	0·3	3 cc. 1 % KH_2PO_4 adjusted to p_H 5·8

The diagrams of experiments 1 and 2 only are published. On these diagrams, Nos. 1 and 2, the letters correspond to the letters in the above table.

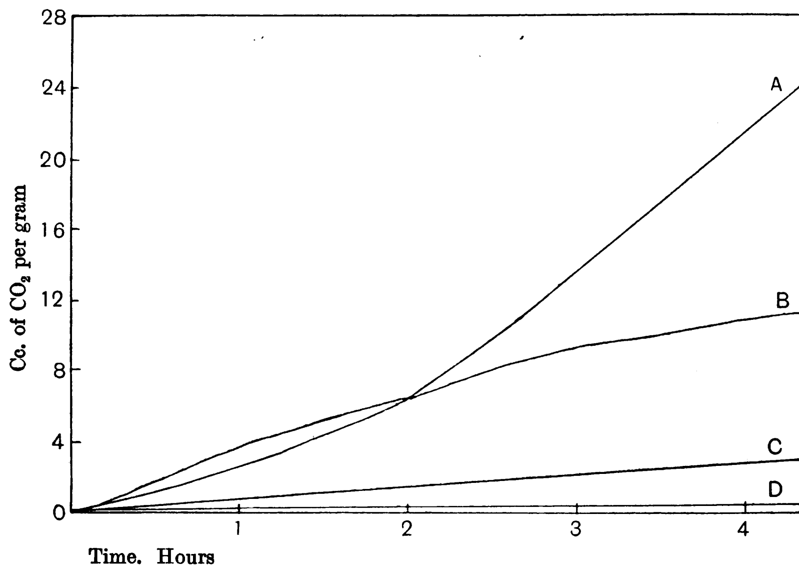

Fig. 2.

The "respiration substance" in muscle extracts, as in yeast extracts, is destroyed during respiration. The alcoholic co-enzyme in both yeast and muscle extracts is also destroyed by aeration in contact with washed muscle or with "acetoned" yeast. Yeasts from different sources differ in their power

of oxidising the alcoholic co-enzyme. Harden and Young [1907] showed that it is destroyed by yeast press-juice.

It may be mentioned that the fermentation co-enzyme in both yeast and muscle extracts is resistant to oxidation by air alone at ordinary temperatures, both at p_H 5·8 and 7·6. Little destruction can be observed after 48 hours of such treatment in some cases, and in no case was any marked destruction observed. The oxidation is dependent upon the presence of the washed tissue or the acetone-treated yeast cell.

Experiments were performed in order to determine whether either glutathione [Hopkins, 1921] or pancreatic insulin could function as the alcoholic co-enzyme in the presence of potassium phosphate. In both cases the results were negative. Glutathione showed a small stimulating effect on the oxygen uptake of the washed yeast. The insulin was wholly without effect. Nor could either oxidised or reduced glutathione restore the lost co-enzymic function to an extract which had been aerated with washed muscle. For a supply of active insulin I am indebted to Messrs Winter and Smith.

In view of Meyerhof's [1920, 2] opinion that the respiration of chopped, unwashed muscle is due to the oxidation of lactic acid formed during chopping, it was considered advisable to attempt to estimate the part played by lactic acid in the respiration of washed muscle in an extract. 0·5 g. of washed muscle was allowed to respire in 3 cc. of yeast extract and the oxygen uptake was measured. The lactic acid was estimated in some more of the same extract by Meyerhof's method [1920, 1].

The velocity of oxygen uptake was very much greater than could be obtained from pure solutions of lactic acid of similar concentrations, and in one experiment was greater than could be accounted for by the complete oxidation of all the lactic acid present. The results are given in Table III.

Table III. *Comparison between the lactic acid content of a cell extract and its power of stimulating the respiration of washed muscle.*

No.	Extract	Muscle	Times washed	O_2 per gram in phosphate	O_2 per gram 6 cc. extract	Temp. °C.	Time of respiration min.	R.Q.	Lactic acid mg. per cc.	Difference in cmm. between O_2 in extract and in phosphate at N.T.P.	Cmm. O_2 required to oxidise lactic acid in 6 cc. of extract
1	Yeast	Rat	4	29	777	22·7	60	—	·18	691	810
2	Yeast	Rat	4	61	1016	22·0	120	·92	·086	884	384
3	Yeast	Rat	4	55	1002	22·5	100	—	·23	875	1032
4	Rabbit muscle	Rat	4	38	368	22·5	80	·90	2·45	305	10992

Again when washed muscle or washed "acetoned" yeast was permitted to respire in a yeast or a muscle extract to which extra lactic acid had been added, the velocity of oxygen uptake was no greater than the sum of the velocities of oxygen uptake for the extract and for the lactic acid solution employed separately. Hence it would seem that there is in such extracts no substance capable of acting as a co-enzyme to the oxidation of lactic acid by washed muscle or yeast.

The results are set forth in Table IV and in Fig. 3.

Table IV. *The oxygen uptake of washed muscle and yeast respiring in cell extracts to which lactic acid had been added.*

			Respiration conditions						O_2 absorbed per gram in cmm.				
									X	Y	Z	W	
No.	Extract	Substance	Weight g.	Times washed	Cc. of H_2O per gram each washing	pH	Time min.	Temp. °C.	In 1% KH_2PO_4	In extract	In 0·5% lactic acid in 1% KH_2PO_4	In extract containing 0·5% lactic acid added	Calculated Y+Z−X
1	Yeast	Rat muscle	0·5	4	75	7·6	80	21·7	70	494	442	830	866
2	Yeast+ 1%KH_2PO_4	"Acet." yeast	0·1	4	350	5·8	90	22·3	550	1790	2990	4290	4230
3	Rabbit muscle +1%KH_2PO_4	"Acet." yeast	0·1	6	350	5·8	80	22·3	270	1640	2440	3260	3810

The values calculated in the last column are based on the assumption that the lactic acid is oxidised independently of the substances in the extracts. Fig. 3 shows the curves obtained in experiment No. 1, Table IV. The curves are lettered to correspond with the letters at the top of the columns.

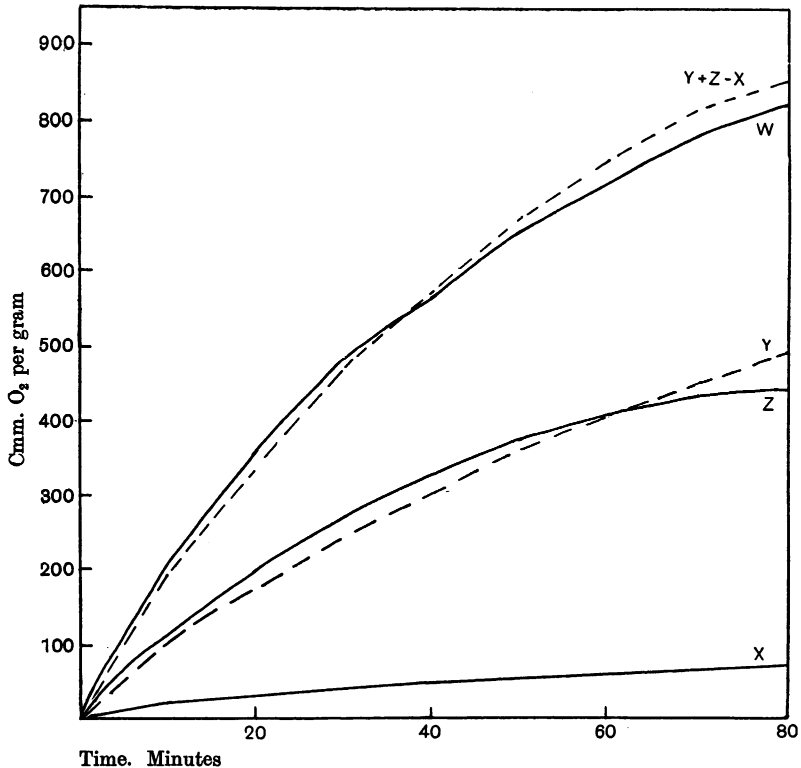

Fig. 3.

Discussion of Results.

The existing views on the respiration of such systems as chopped muscle and "acetoned" yeast may be stated as follows. When muscle is chopped, and suspended in buffer solution, the oxygen uptake is due solely to the

oxidation of the lactic acid present [Meyerhof, 1920, 2]. When washed chopped muscle or washed "acetoned" yeast is placed in tissue or yeast extract, the resulting respiration is due to the action of a "co-enzyme to respiration" in the extract catalysing the oxidation of oxidisable substances present, and hence providing a general stimulus to the cell respiration as a whole. This "co-enzyme to respiration" is identical with the alcoholic co-enzyme and is an oxygen carrier [Meyerhof, 1919, 2].

It seems to be tacitly assumed that the resting respiration of muscle is due almost entirely to the oxidation of the small quantity of lactic acid continually being formed and destroyed.

Certain facts may be noted. Muscle is known to possess several oxidative systems, each capable of dealing specifically with one type of substance, for instance the lactic acid, the succinic acid, and the glutathione systems. It has been shown that washed muscle is capable of oxidising various substances other than lactic acid without the aid of any oxygen carrier or other co-enzyme, which is soluble in water and therefore removable by washing; *e.g.* the sodium salts of lactic, succinic and citric acids are thus oxidised.

The glutathione system is an example of a true "co-enzyme to respiration" [Hopkins and Dixon, 1922]. Here the oxidisable substances are in the muscle residue. Under the conditions of the experiments described in this paper the oxygen consumption of this last system is small; moreover [Holden, 1923] it is possible to stimulate the respiration of washed muscle by yeast extracts from which glutathione has been removed by treatment with lead acetate.

Meyerhof [1919, 2] has himself shown that part at least of the "respiration substance" is precipitated by alcohol. Since lactic acid is not so precipitated, the fraction insoluble in alcohol must, from the standpoint taken in this paper, contain other oxidisable substances.

It will be observed that the claim based upon the work here described is that the stimulating effect of a tissue or yeast extract on the respiration of washed muscle may be attributed to the simultaneous oxidation of various substances present. The individual concentration of any one of these may be small, but collectively they may account for a considerable oxygen uptake. The existence of a co-enzyme is not disproved, but as yet it has not been shown to be necessary.

The experiments, in which extracts are used to which lactic acid has been added, show that there is no evidence of a soluble co-enzyme acting in the oxidation of this substance. Nor is the effect of an extract on the respiration of washed muscle due in any considerable degree to its lactic acid content.

One or more of the oxidisable substances in tissue and yeast extracts can function as the co-enzyme to alcoholic fermentation. It cannot be an oxygen carrier since it is capable of undergoing irreversible oxidation by washed muscle. It may be a hydrogen carrier of which the molecule is not dehydrogenated, but destructively oxidised during respiration. The fact that gluta-

thione, which can function as a hydrogen carrier, and which is present in yeast, cannot act as the alcoholic co-enzyme, cannot be considered as decisive evidence in view of the specificity of enzyme action.

The nature, the mode of action, and the significance in animal metabolism, of the alcoholic co-enzyme must still be regarded as obscure.

Summary.

The "respiration substance" in an extract of mammalian muscle made with boiling water is destroyed by aeration in contact with washed rabbit, rat, or frog muscle.

Under similar conditions the alcoholic co-enzyme in yeast or muscle extracts is also destroyed, though it is not readily oxidised by air alone.

Neither glutathione nor pancreatic insulin can act as the alcoholic coenzyme to washed "acetoned" yeast, in the presence of potassium phosphate, and at a suitable hydrogen ion concentration for the natural co-enzyme to act.

The "respiration substance" would seem to be in reality a collection of irreversibly oxidisable substances, though not, in the main, lactic acid.

A new method of making "acetoned" yeast is described.

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