LVII. THE EFFECT OF PYRUVATES, ALDEHYDES AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE BY YEAST JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE.

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Oppenheimer [1915] observed that the fermentation of glucose by maceration extract was greatly stimulated by the addition of a pyruvate or pyruvic acid and that acetaldehyde had a similar but less pronounced effect. The estimations were made by weighing at comparatively long intervals but it is obvious in the case of acetaldehyde, that the stimulation chiefly occurs at the commencement of the fermentation. The results with pyruvates are not always so clear and are apparently complicated by some unconsidered factor since in some cases the total CO_2 evolved exceeds that obtainable from the glucose and pyruvate in the normal course of fermentation (e.g. Br_1, Table III, p. 240 in which 316 milligrams of CO_2 are obtained from 0·4 g. of glucose + 0·04 g. pyruvic acid which would together only yield in the normal course 220 mgms. or including the observed autofermentation 230 mgms., and Br_2 in the same table where 295 mgms. are obtained against 212 normally obtainable).

Neuberg somewhat later [1915] observed a similar stimulating action of pyruvates and other α-ketoacids on the fermentation of glucose, mannose, fructose and saccharose and remarked that the activation was most pronounced at the commencement of the fermentation. Experiments continued for 19–20 hours (p. 82) showed little difference in the total fermentation in the presence and absence of pyruvate.

Neuberg subsequently examined the effect of a large number of aldehydes [1918] on alcoholic fermentation and found that they were all vigorous activators. He pointed out that the effect was most marked with glucose and mannose, less so with fructose and cane sugar and suggested that this fact might be related to the observation of Harden and Young [1909] that fructose under certain circumstances can stimulate the fermentation of glucose. The stimulation, like that produced by pyruvate, was most marked at the commencement of fermentation.

Meyerhof [1918] has made an interesting contribution to this subject in his study of the kinetics of cell-free fermentation. Lebedev [1912] observed that when maceration extract was mixed with a fermentable sugar a period of *induction* occurred during which no CO_2 was formed and no change in rotation occurred, and this has frequently been confirmed. Meyerhof now finds that this induction is not observed when the extract contains even a trace (0·2 millimolecular concentration) of a hexosephosphate and hence has never been recorded for juice prepared by Buchner's process, in which recognisable amounts of hexosephosphate are present. The period is moreover shorter in presence of cane sugar than of fructose or glucose and is diminished when these two sugars are warmed for 4–6 hours at 80° with a neutral phosphate mixture. The induction period is also greatly lessened by grinding the dried yeast with glass powder before maceration.

Following on the induction period, the velocity of fermentation more or less gradually attains a maximum corresponding to the concentration of phosphate present. This is termed by Meyerhof the "Gäranstieg" and has been studied in some detail. The effects of the addition of excess of phosphate described by Harden and Young [1908], viz. a diminished maximum and a more gradual attainment of the maximum[1], are found by Meyerhof to be also produced by the addition of salts such as sodium chloride or nitrate and hence the phenomenon is partly to be explained as a general salt effect. The rate of attainment of the maximum becomes greater as the hexosephosphate concentration increases, but this characteristic phenomenon cannot entirely be abolished by the addition of hexosephosphate. Meyerhof discusses the cause of the phenomenon and shows by an ingenious application of the effect of arsenates that it is probably not due to the production of a specially labile form of sugar from the hexosephosphate. The attainment of the maximum was also found to be more rapid the greater the concentration of the coenzyme, added in the form of boiled extract of yeast or muscle.

The facts that the activating effect both of α-ketoacids and of aldehydes was chiefly manifested at the commencement of the reaction, that the experiments both of Oppenheimer and Neuberg were made with maceration extract which contains a large amount of mineral phosphate and that the effect was less marked with fructose than with glucose led us to enquire whether this action was a general stimulation of the fermentation process or a more specific acceleration of the reaction in presence of free mineral phosphate. The results show that when aldehyde is added to fermenting mixtures of yeast juice or

[1] Meyerhof [1918, p. 196] erroneously attributes to Harden and Young in explanation of this phenomenon the suggestion that the *sugar* forms with high and low concentrations of phosphate different esters of different stability, one of which, as the phosphate is used up, passes into the other. What they actually suggested was [Harden and Young 1908] that the phosphate is capable of forming two or more different unstable associations with the *fermenting complex* (by which was meant the complex of enzymes concerned, not the sugar). The alternative suggestion has also been made [see Harden 1914] "that the addition of increasing amounts of phosphate causes a progressive but reversible change in the mode of dispersion of the colloidal enzyme."

zymin (acetone yeast) with glucose no marked acceleration in the normal rate of fermentation occurs. If a suitable amount of phosphate be then added, sufficient to cause only a gradual rise of rate to the maximum in the control experiment with glucose, the effect of the presence of the aldehyde is greatly to diminish the time required for the attainment of the maximum, so that the volume of gas evolved in the period immediately following the addition of the phosphate is greatly increased. At the same time a considerably higher maximum is attained. On the completion of the esterification of the phosphate, the rate again diminishes both in the presence and absence of aldehyde and the total evolution is not greatly different in the two cases. Similar phenomena are produced by the addition of pyruvates. The effect varies with the concentration of aldehyde and is common, but in unequal measure, to the four aldehydes tested (formic, acetic, propionic and butyric). So far only glucose has been employed as the fermentable sugar, but experiments are in progress with fructose and cane sugar.

This striking effect of aldehydes, which are known to be readily reducible by yeast, strongly suggested that the cause of the delay in attainment of the maximum after the addition of phosphate was lack of an acceptor for hydrogen. In order to test this idea, methylene blue, which is also readily reducible by yeast, was substituted for the aldehyde, with the result that it was found to produce a very similar effect. When increasing amounts of methylene blue are added a point is soon reached at which the maximum, although it is more rapidly attained, is considerably lowered. This is probably due to the inhibitory effect of the dye on the enzyme complex. Even with the most favourable concentration of methylene blue however the rise of rate was not so rapid as with the aldehydes, and the maximum was unchanged.

According to the pyruvic acid theory, which may now be taken as established, the final stage of the alcoholic fermentation of sugar is the reduction of acetaldehyde (produced by the decomposition of pyruvic acid), a reaction which proceeds so rapidly that only an extremely small concentration of the aldehyde is present during normal fermentation.

$$CH_3 . CO . COOH \longrightarrow CH_3 . CHO + CO_2 \xrightarrow[+2H]{} CH_3 . CH_2OH + CO_2.$$

Further, the production of the pyruvic acid from sugar appears only to be possible when some acceptor for hydrogen is available, this being normally supplied by the acetaldehyde produced in a later stage of the reaction.

On this view it would seem to follow that no rise in the rate of fermentation can occur without the provision of an additional quantity of a hydrogen acceptor. Some such acceptor is probably more or less rapidly formed and reduced during the period of delay, which follows on the addition of phosphate, this process being accompanied by a corresponding increase in the formation of pyruvic acid, until sufficient of this is being produced to provide the amount of acetaldehyde necessary for the maximum effect. When, however, the easily reducible aldehydes or methylene blue are added, these act as

acceptors and a much more rapid or even instantaneous attainment of the maximum becomes possible, as was actually observed in our experiments.

Whether this acceptor is the same substance as yields glycerol in the sulphite fermentation of Neuberg and Reinfurth, and is supposed by Neuberg to be methylglyoxal, is uncertain. It may be pointed out however that the precursor of glycerol assumed by Neuberg and Kerb [1913] must be much less rapidly reduced than acetaldehyde in the course of normal alcoholic fermentation since the ratio of glycerol to alcohol under these circumstances is only small. No experiments have yet been made to decide whether an enhanced glycerol production occurs during the period of delay.

It seems probable that the delay following the addition of phosphate when fructose is employed as the fermentable sugar is also due in part to lack of an acceptor. The facts that fructose yields a much higher maximum rate with phosphate and that the optimum concentration of phosphate is much higher than for glucose can accordingly be interpreted to mean either that fructose yields a hydrogen acceptor much more readily than glucose or that the acceptor formed is much more rapidly reducible. This question is at present under investigation. If this conclusion be granted, a simple explanation is afforded of the remarkable "induction" observed by Harden and Young [1909] when fructose was added to a mixture of yeast juice and glucose or mannose to which a considerable excess of phosphate had been added. Under these circumstances the rate of attainment of the maximum fermentation was greatly accelerated even when the phosphate concentration was kept constant and moreover the volume of CO_2 evolved under these circumstances was much greater than could be obtained from the fructose added. In the light of the foregoing remarks it now appears that the function of the fructose under these conditions is probably to provide a hydrogen acceptor and this, once formed, enables the fermentation of the glucose to proceed rapidly, as explained above, even in the presence of a concentration of phosphate, which in the absence of an acceptor causes a prolonged delay.

It is further probable that the hydrolysis of the hexosephosphate, both that originally present and that slowly formed in the fermenting mixture, results in the formation of fructose, which in its turn yields a hydrogen acceptor and thus assists in the increase of the rate of fermentation. Meyerhof's observations on the marked effect of hexosephosphate on the rate of attainment of the maximum would thus receive a simple explanation.

Owing to the method of experiment employed by us, the full effect of the addition of hexosephosphate could not be observed as the fermenting mixtures always contained this substance formed from the phosphate originally present. The addition of a further quantity of hexosephosphate produced very little effect.

Whether the lack of acceptor combined with Meyerhof's "salt-effect" of the excess of phosphate provides a complete explanation of the delay in

attainment of the maximum after the addition of phosphate or whether time is required for some other change, such as transformation of the sugar into a fermentable form, as maintained by Euler, remains as a subject for further investigation.

Experimental.

The yeast juice and zymin employed were both prepared from a brewery top-yeast. The acetaldehyde used in Exp. 2 was a preparation obtained from Kahlbaum. For the other experiments it was prepared from paraldehyde by distillation with dilute sulphuric acid. The formaldehyde was a dilution of formalin and the concentration was estimated by Ripper's method. The propionic aldehyde was prepared by heating a mixture of calcium propionate and formate and the butyric aldehyde by the oxidation of *n*-butyl alcohol with potassium dichromate and sulphuric acid. The methylene blue was Grübler's "Methylenblau med pur." The pyruvate solution was made by dissolving 1 g. of pyruvic acid in water, neutralising with *N* KHO and making to 100 cc.

Effect of the addition of pyruvate and acetaldehyde to yeast juice in presence and absence of free phosphate.

Exp. 1. 25 cc. Yeast-juice + 1 g. glucose. No toluene. T = 25°.

	1% Pyruvate cc.	1% Acetaldehyde cc.	H_2O cc.		0·3*M* Na_2HPO_4 cc.	H_2O cc.
1	5	0	0	and subsequently	5	0
2	5	0	0		0	5
3	0	5	0		5	0
4	0	5	0		0	5
5	0	0	5		5	0
6	0	0	5		0	5

Measurements were commenced at 2.20. The additions of phosphate and water were made at 3.20.

	Pyruvate		Aldehyde		Water	
	1	2	3	4	5	6
cc. CO_2 evolved before addition of phosphate in 55′	23·6	23·8	20·9	21·2	22·3	23·2
cc. CO_2 evolved after addition of phosphate in successive periods of 5′						
	Na_2HPO_4	H_2O	Na_2HPO_4	H_2O	Na_2HPO_4	H_2O
1	17·8	1·9	29·8	2	5·2	1
2	24·0	2	12·8	2	7·8	3
3, 4	6·7	4·6	7·4	3·7	28·1	4·2
5, 6	4·7	3·4	1·2	3·4	7·3	3·7
Total in 30′	53·2	11·9	51·2	11·1	48·4	11·9

It will be seen that the addition of pyruvate or aldehyde did not appreciably alter the normal rate of fermentation, as in the 55′ preceding the addition of phosphate all six flasks gave approximately the same amounts of gas. Further,

the three flasks 2, 4 and 6 to which H_2O was subsequently added continued to ferment at equal rates, giving almost exactly equal amounts of gas in 30′ (11·9, 11·1 and 11·9 cc.).

On the addition of phosphate, flask 6, containing no aldehyde or pyruvate, showed the normal behaviour, the rate gradually rising to a maximum which was attained in 10–15′ and amounted to 15 per 5′ (calculated by plotting the figures given in the table). In presence of aldehyde the rate rose to its maximum of 29·8 in the first 5′, whilst in presence of pyruvate an intermediate result was obtained the evolution being 17·8 in the first 5′ and 24 in the second.

Exp. 2. 25 cc. Yeast-juice + 1 g. glucose. No Toluene. T = 25°.

	1% Pyruvate cc.	Water cc.		0·3*M* Na_2HPO_4 cc.	H_2O cc.
1	5	0	and subsequently	5	0
2	5	0		0	5
3	0	5		5	0
4	0	5		0	5

Measurements were commenced at 3.10 and the additions of phosphate and water were made at 3.40.

	Pyruvate		Water	
	1	2	3	4

cc. CO_2 evolved before addition of phosphate between 3.10 and 3.30.

6·9	7·1	7·2	7

cc. CO_2 evolved after addition of phosphate in successive periods of 5′.

	Phosphate	Water	Phosphate	Water
1	3·4	0·3	0·3	0·5
2	5·6	0·5	1	0·7
3	4·4	0·4	0·8	0·4
4, 5	10·2	0·8	1·6	0·8
Total in 25′	23·6	2	3·7	2·4
Total in 140′	49	9·7	31·2	9·2
Total in 17 hrs.	88·6	49·5	76·2	46·1

This is an example in which a quantity of phosphate largely in excess of the optimum was added. In the absence of added phosphate (Nos. 2 and 4) the fermentation was substantially the same with and without pyruvate. After addition of phosphate in the presence of pyruvate the rate rose much more rapidly than in its absence and in the latter case, probably owing to the continued action of excess of phosphate on the enzyme complex, the total was considerably less.

Effect of varying concentrations of acetaldehyde on the fermentation of glucose by zymin in presence and absence of phosphate.

Exp. 3. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0·2 cc. Toluene. Acetaldehyde 1% solution (by weight). T = 25·8°.

	1% Acetaldehyde cc.	
1	0	and subsequently 10 cc. 0·15*M* Na_2HPO_4 to each.
2	1·2	
3	6	
4	12	

Incubated for 1 hour and the phosphate then added.

	1	2	3	4
		Acetaldehyde		
	0 cc.	1·2 cc.	6 cc.	12 cc.
cc. CO_2 evolved before addition of phosphate in 45′.	34·4	33·6	20·7	3·4
cc. CO_2 evolved after addition in successive periods of 5′.				
1	2·7	3·4	12·5	5·5
2	3·6	4·6	12·8	8·4
3	5·3	5	12·3	9·7
4	6·2	7·2	11·7	9·4
5	7·7	7·7	9·8	9·0
6	9·1	9·6	9·3	7·9
7	9·0	9·3	7·2	7·5
8	8·0	7·5	3·6	7·4
Total in 40′	51·6	54·4	79·1	64·8
Total in 110′	113·6	118·8	119·6	103·1

The characteristic effect is here well marked. In the control (No. 1) the maximum of 9·1 cc. is slowly reached in 30–35′ and the rate then as gradually diminishes. 1·2 cc. of 1% aldehyde [1 in 1667] produce practically no effect, 6 cc. on the other hand [1 in 334] produce a very marked effect, the maximum is much higher (12·8 cc.) and is very rapidly attained (5–10′). The total evolved in 110′ is however only about 5% greater than that of the control. A point of considerable interest is that both 6 cc. and 12 cc. of 1% aldehyde diminish the normal rate of fermentation although they both produce a considerable acceleration in the rate at which the maximum is attained after the addition of phosphate. This is especially marked in the case of the 12 cc. of aldehyde solution, which diminished the normal rate of fermentation to 1/10 of that of the control. This effect is probably due to a specific inhibition of the hexosephosphatase, thus diminishing the liberation of phosphate on which the normal rate depends.

Effect of formic, acetic, propionic and butyric aldehydes on the fermentation of glucose by yeast-juice in presence of phosphate.

Exp. 4. 25 cc. Yeast-juice + 1 g. Glucose + 0·2 cc. Toluene. T = 25°.

1	5 cc. Water	and then 10 cc. 0·3*M* K_2HPO_4.
2	„ 0·78 % Formaldehyde	
3	„ 1 % Acetaldehyde	
4	„ 1·3 % Propionic Aldehyde	
5	„ 1·6 % Butyric Aldehyde	

Incubated for 50′ before addition of phosphate.

	1	2	3	4	5
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO_2 evolved in 30′ before addition of phosphate					
	7·3	4·1	7·0	7·7	7·2
cc. CO_2 evolved after addition of phosphate in successive periods of 5′.					
1, 2	15·5	23·6	57·1	58·1	53·7
3	12·5	22·4	16·4	16·6	16·1
4	15·3	20·7	2·7	2·7	2·8
5	18·4	7·0	1·6	1·5	1·7
6	11·9	1·4	1·2	1·6	1·4
7	2·5	1·5	1·8	1·6	1·4
	76·1	76·6	80·8	82·1	77·1

Exp. 5. As above, but 15 cc. of 0·3*M* K_2HPO_4 added. Incubated 25′.

	6	7	8	9	10
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO_2 evolved after addition of phosphate in successive periods of 5′.					
1, 2 (10′)	10·3	22·9	56·8 (28·4, 28·4)	55·0 (26, 29)	48·1 (23, 25·1)
3	5·5	22·3	27·6	27·2	30·1
4	6·6	23·9	20·5	19·9	22·4
5	8·0	21·1	4·8	7·9	3·8
6	10·3	15·7	1·6	2·1	1·8
7	11·3	2·4	1·3	1·6	1·5
8	15·3	1·4	1·4	1·4	1·2
9	17·8				
10	13·9				
11, 12	4·5				

With this sample of juice very high maxima were attained, but as the first readings were made 10′ after the addition of phosphate the exact maximum could not be directly observed. They were obtained by plotting the total fermentations and reading the evolutions per 5′ from the curves, with the following approximate results.

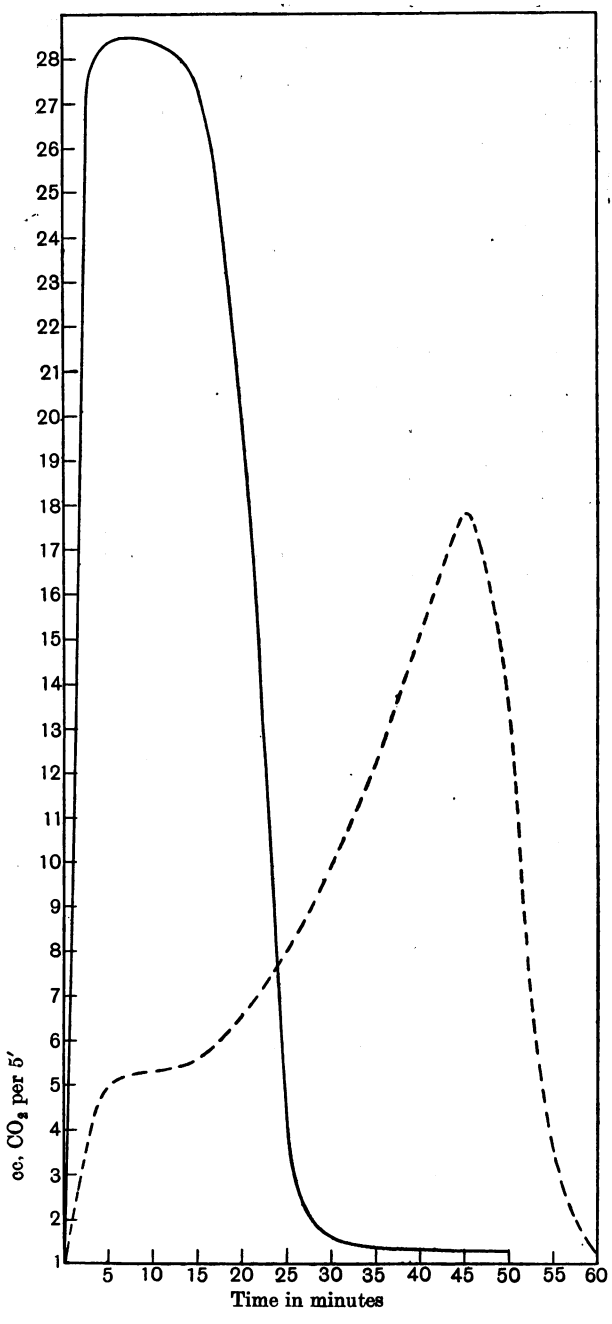
Exp. 4.

	1	2	3	4	5
Maximum	18·4	22·4	34	35	27
Minutes after addition when attained ...	20–25	30–15	0–5	0–5	0–5

Exp. 5.

	6	7	8	9	10
Maximum	17·8	23·9	25·4	29	30·1
Minutes after addition when attained ...	40–45	15–20	0–5	5–10	30–15

The effect here is very marked and moreover in several cases the maximum is reached in the first 5′ of fermentation. The results with the acetaldehyde in No. 8, Exp. 5, are plotted in Fig. 1.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1
cc. CO_2 per 5′
5 10 15 20 25 30 35 40 45 50 55 60
Time in minutes

Fig. 1.

Effect of (a) Acetaldehyde, (b) Na Hexosephosphate on the fermentation of glucose by zymin in presence of phosphate.

Exp. 6. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0·2 cc. Toluene. T = 25·8°.

1% Acetaldehyde

1	0	1	10 cc. 0·3*M* K_2HPO_4
2	1·2 cc.	2	
3	6 cc.	3	
4	0	4	10 cc. H_2O + 10 cc. 0·3*M* K_2HPO_4
5	0	5	Na Hexosephosphate from 0·2 g. Basalt + 0·52 g. K_2HPO_4 in 20 cc. (equivalent to 10 cc. 0·02*M* Hexosephosphate and 10 cc. 0·3*M* K_2HPO_4).

(1, 2, 3, 4, 5 subsequently)

Incubated for 65′ before the additions were made.

	Water 1	1·2 cc. Acetaldehyde 2	6 cc. Acetaldehyde 3	4	5
cc. CO_2 evolved before additions in 50′	39·2	33·8	28	38·9	38·4

Additions made at 1·05.

cc. CO_2 evolved after addition in successive periods of 5′.

	Phosphate	Phosphate	Phosphate	Phosphate and water	Phosphate + Hexose-phosphate
1	2·7	3·2	13·6	0·3	3·5
2	2·9	2·7	15·6	1·8	3·8
3	4·3	4·1	15·4	3·0	4·4
4	5·0	5·4	15·6	4·3	6·1
5	7·3	7·7	13·4	5·0	15·6
6	9·1	9·5	12·1	8·1	
7	10·5	10·7	11·1	9·2	8·8
8	11·4	12·3	10·0	10·3	8·8
9	11·0	10·4	8·0	10·3	8·6
10	10·9	10·8	6·4	9·2	8·0
11	9·6	9·1	3·6	8·9	14·6
12	8·4	8·1	3·4	8·6	
Total in 60′	93·1	94	128·2	79·0	82·4
Total in 120′	157	169·2	169·3	152·3	144·9

(*a*) The result with acetaldehyde confirms that obtained in Exp. 3, but the total in 2 hrs. is slightly larger in presence of acetaldehyde than in its absence.

(*b*) The addition of hexosephosphate has only a small effect in accelerating the attainment of the maximum. The total in 2 hours is slightly less than that in the control.

Effect of varying concentrations of methylene blue on the fermentation of glucose by zymin in the presence of phosphate.

Exp. 7. 4 g. Zymin + 2 g. Glucose in 20 cc. 0·2 cc. Toluene. T = 25·5°.

1	Methylene Blue 0	and subsequently 10 cc. 0·3*M* K_2HPO_4 to each.
2	„ 0·1 g.	
3	„ 0·2 g.	
4	„ 0·3 g.	

The results of 1 and 2 are shown in the curves (Fig. 2) in which *A* and *B* represent the evolutions per 5′ in absence and presence of methylene blue respectively.

In presence of 0·2 g. and 0·3 g. of methylene blue the course of the reaction was almost the same as with 0·1 g. but the maximum attained was in each case slightly lower (0·1 g., 13·2 cc.; 0·2 g., 12·5; 0·3 g., 12·7). The methylene blue in (2) became colourless in about 1 hr. and in (3) in about 2 hours.

The totals evolved in 2hr. 15 min. in the 4 flasks were almost identical, as will be seen from the following statement.

No.	M.B. g.	Max. attained (cc. in 5′)	Total in 2 hrs. 15 min. cc.
1	0·0	13·2	181·2
2	0·1	13·2	184·5
3	0·2	12·5	176·8
4	0·3	12·7	178·9

When a still larger concentration of methylene blue is employed, the maximum is considerably lower than in its absence but is more quickly attained. The dye apparently partially inhibits the enzyme complex.

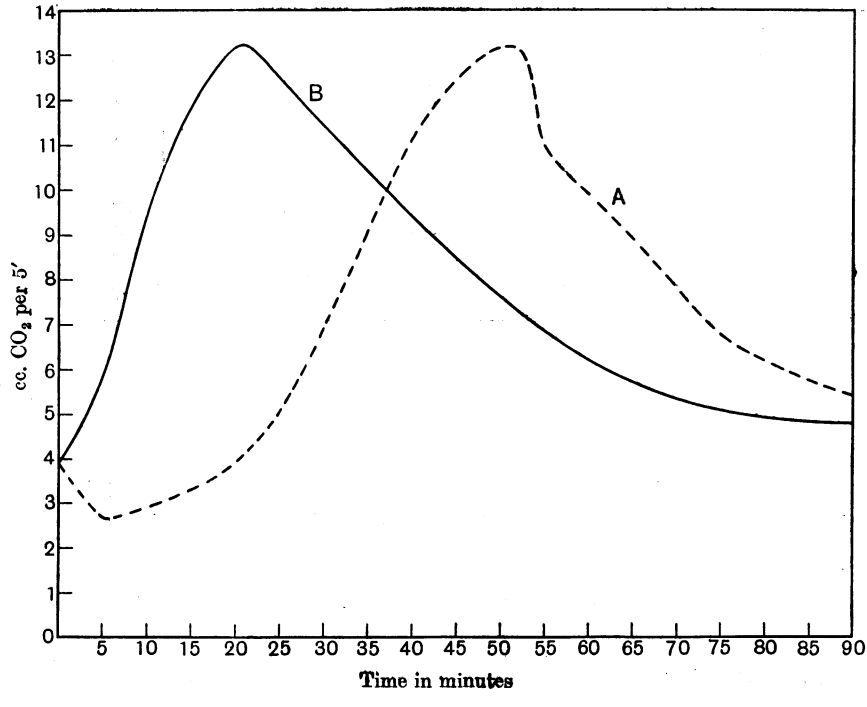

Fig. 2.

Exp. 8. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0·2 cc. Toluene. T = 26°.

1 Methylene Blue 0
2 ,, 0·5 g.
3 ,, 0
4 ,, 0·5 g.
} and subsequently { 6 cc. 0·3*M* K_2HPO_4 / 10 cc. 0·3*M* K_2HPO_4

Result. After the addition of phosphate.

	Phosphate added 0·3*M*	M.B. g.	Max. attained	Time required to attain maximum	Total evolved in 1 hr. 35 min.
1	6	0	11	30–35′	107·0
2	6	0·5	8·8	0–5′	97·4 in 1 hr. 55 min.
3	10	0	12·2	30–35′	147·1
4	10	0·5	10·6	5–10′	149·7

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