LXXX. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

III. THE LAG BETWEEN PHOSPHATE ESTERIFICATION AND CARBON DIOXIDE EVOLUTION.

IV. OXIDATION-REDUCTION POTENTIALS OF YEAST PREPARATIONS.

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(Received May 5th, 1930.)

III. The lag between phosphate esterification and carbon dioxide evolution.

EULER AND JOHANSSON [1913] were the first to suggest that in the reaction of phosphate with yeast preparations fermenting sugar the phosphate was esterified some time before the equivalent carbon dioxide was liberated. These workers considered that a preliminary activation of sugar, followed by formation of hexosediphosphate and a triosemonophosphate, was responsible for the lag in evolution of gas. In addition to the lag between the reactions of phosphate esterification and carbon dioxide formation there is a delay in the esterification after the phosphate is added. When yeast preparations (particularly maceration extract and preparations containing much water) are added to sugar there is a considerable induction time before any fermentation occurs; the free phosphate present in the preparation then reacts during the period called the "Gäranstieg" by Meyerhof [1918]. This induction period and the "Gäranstieg" caused by the free phosphate inherent in preparations are not discussed in the present work.

In a recent paper on the reaction of phosphate with dried yeast fermenting fructose [Boyland, 1929] a lag similar to that of Euler and Johansson was described. This lag between the processes of esterification and carbon dioxide formation was not due to physical causes such as supersaturation, and it was suggested that it was due to the relatively slow decomposition of products formed after, or simultaneously with, phosphate esterification. The nature of this lag is investigated in the following experiments.

Harden and Young [1908] showed that in the reaction of phosphate with yeast preparations the time necessary for the attainment of the maximum rate of carbon dioxide evolution was much greater with glucose than with fructose,

and that this time could be reduced by the addition of relatively small quantities of fructose [Harden and Young, 1909]. Harden and Henley [1920, 1921] found that this time was much reduced by the addition of hydrogen acceptors such as methylene blue, pyruvates or aldehydes. Acetaldehyde was the most active agent in this way, being about fifty times as efficacious as fructose. Under almost all conditions the reaction of one molecule of phosphate in fermentation yields one molecule of carbon dioxide, whether monophosphoric or diphosphoric ester is formed. Combining the recent ideas of Harden [1930] with those of Neuberg and Kerb [1913], the sequence of fermentation in presence of a trace of aldehyde might be represented as follows:

Of these reactions the only one likely to be affected by hydrogen acceptors is the oxidation of methylglyoxal to pyruvic acid (equation 3), and as hydrogen acceptors have such a marked effect on carbon dioxide evolution this oxidation is probably the slowest or controlling stage in the beginning of the reaction of phosphate. It should therefore be responsible for the lag in the appearance of carbon dioxide after phosphate esterification and for this reason the effect of glucose, fructose and hydrogen acceptors on the lag was examined.

EXPERIMENTAL.

The preparation used in most of these experiments was the "zymase" free from phosphatase, made by the acetone precipitation of yeast juice, which has previously been described [Boyland, 1930]. It was possible to obtain reliable preparations free from hexosephosphatase by grinding the yeast juice precipitate with half its weight of kieselguhr together with the sugar and water necessary for the fermentation. The whole was then centrifuged and the clear centrifugate used for the fermentation. Harden and Macfarlane [1930] have indicated that hexosephosphatase is probably adsorbed by kieselguhr.

The methods used were largely the same as those of previous experiments, the analytical procedure being identical. When hydrogen acceptors were added, the addition was made 15 minutes before the addition of phosphate. The concentrations of the added substances are calculated on the final volume of the fermenting solution after the addition of phosphate.

EFFECT OF HYDROGEN ACCEPTORS ON THE REACTION.

The experiments of Table I, in which the lag is expressed as the difference between the phosphate esterified and the carbon dioxide evolved at any particular time, show that hydrogen acceptors cause a marked increase in the rate of reaction of phosphate, without diminishing the amount of lag as measured by molecular concentrations. Hydrogen acceptors sometimes increase the lag in amount, though not the period during which it persists; it may be possible

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Table I. *The lag between phosphate esterification and carbon dioxide evolution in fermentation with the acetone precipitate of yeast juice.*

In each case 1 cc. 2M K_2HPO_4 added to 2 g. yeast preparation in 20 cc. sugar solution.

		Results in total millimols.							
Time after phosphate addition (mins.)		5	10	20	30	40	50	60	70
Exp. 180									
Glucose alone	P esterified	0.08	0.20	—	0.83	—	1.65	2.00	2.00
	CO ₂ evolved	—	0.09	—	0.63	—	1.55	2.10	2.10
	Lag	0.08	0.11	—	0.20	—	0.10	—	—
Exp. 203									
Fructose alone	P esterified	0.10	0.25	—	0.84	—	1.90	2.00	—
	CO ₂ evolved	—	0.12	—	0.70	—	1.82	2.06	—
	Lag	0.10	0.13	—	0.14	—	0.08	—	—
Exp. 204									
Fructose with methylene blue (0.05 %)	P esterified	0.12	0.28	—	1.15	—	1.99	1.99	—
	CO ₂ evolved	—	0.10	—	0.90	—	1.94	2.03	—
	Lag	0.12	0.18	—	0.25	—	0.05	—	—
Exp. 199									
Fructose with acetaldehyde (0.15 %)	P esterified	0.30	0.80	1.60	1.96	—	—	—	—
	CO ₂ evolved	0.16	0.64	1.56	1.97	—	—	—	—
	Lag	0.14	0.16	0.10	—	—	—	—	—

Table II. *The effect of varying concentrations of acetaldehyde on the lag.*

10 cc. 0.6 M K_2HPO_4 added to 4 g. dried yeast in 30 cc. 10 % fructose.

Concentrations expressed as molecules per litre.

Concentration acetaldehyde	Change after 20 minutes' reaction		
	P esterified	CO ₂ evolved	Difference or lag
Nil	0.017	—	0.017
0.015	0.022	0.009	0.013
0.030	0.036	0.025	0.011
0.045	0.037	0.024	0.013
0.060	0.043	0.024	0.019
0.075	0.042	0.018	0.024

that in the presence of certain concentrations of acetaldehyde, pyruvic acid is formed more quickly than it can be decomposed by the carboxylase, and under such conditions the decarboxylation would become the master reaction. The lag occurs in the reaction with all the yeast preparations examined (acetone precipitate of yeast juice, yeast juice, zymin and dried yeast). It varies in extent with different preparations and must depend upon the balance of enzymes present. It is greater with larger additions of phosphate and it cannot be much reduced by any concentration of acetaldehyde. Table II shows the lag with dried yeast fermentations with varying concentrations of acetaldehyde. The results are also set out in Fig. 1 and the optimum concentration of acetaldehyde is fairly well marked.

As this lag is not removed by hydrogen acceptors—which increase both the rate of esterification and of carbon dioxide evolution—and is fairly constant in amount for any one preparation, it is probable that esterification is normally arrested by accumulation of methylglyoxal. The removal of methylglyoxal (by oxidation at the expense of hydrogen acceptors) allows more sugar to be esterified.

TRIOSE IN THE REACTION.

If the lag in fermentation is due to the relatively slow oxidation of methylglyoxal, then that compound should accumulate in fermentation when the lag is at its greatest. The triose present during the reaction was therefore estimated by the distillation method described by Neuberg, Färber, Levite and Schwenck [1917]. The filtrate from the trichloroacetic acid precipitation was distilled with 10 % sulphuric acid, while water was dropped in to keep the concentration of sulphuric acid constant. All trioses were thus converted into methylglyoxal, which was precipitated from the distillate by heating with a solution of *p*-nitrophenylhydrazine. When cold the precipitate of methylglyoxal *bis*-nitrophenylhydrazone was filtered off in a Gooch crucible, washed with cold water, dried *in vacuo* and weighed. The presence of acetaldehyde does not affect the estimation because the *p*-nitrophenylhydrazone of acetaldehyde is soluble in water in the concentrations in which it would occur in the distillate from fermentations.

Fig. 1.

That there is an increase in the amount of triose present when the lag in fermentation is at its maximum is shown by the results obtained in Table III. The agreement between the amount of lag and the amount of triose formed is not very exact but the changes are of the same order.

Table III. *The triose present during the period of lag.*

Conditions as in Table I. Results expressed in total millimoles.

Time after addition of phosphate (mins.)		0	5	10	15	20	30	50
Exp. 180								
Fermenting glucose	Lag	—	0.08	0.11	0.25	—	0.20	0.10
	Triose present	0.16	0.23	0.36	0.34	—	0.25	0.21
	Additional triose formed	—	0.07	0.20	0.18	—	0.09	0.05
Exp. 199								
Fermenting fructose with acetaldehyde	Lag	—	0.14	0.16	0.10	0.04	—	—
	Triose present	0.09	0.19	0.17	0.11	(0.25?)	0.10	—
	Additional triose formed	—	0.10	0.08	0.02	(0.16?)	0.01	—

Although the method of distillation with dilute sulphuric acid yields methylglyoxal in the distillate, methylglyoxal itself does not appear to be present in the trichloroacetic acid filtrates from fermentations. Methylglyoxal is precipitated by dinitrophenylhydrazine in cold acid solution, but the filtrates from fermentations never give the methylglyoxal derivative with this reagent in the cold. If dinitrophenylhydrazine is added immediately after removal of the proteins, a precipitate of acetaldehyde dinitrophenylhydrazone (M.P. 151–152° after recrystallisation from pyridine and ether) is obtained. But, if the trichloroacetic acid filtrate is allowed to stand for more than an hour before the addition of the hydrazine, no precipitate is obtained. Thus even acetaldehyde changes in the solution and it is possible that methylglyoxal, which is still more reactive, changes almost immediately to some compound, possibly a polymer, which is reconverted into methylglyoxal on distillation with acid.

Kluyver and Struyk [1927] suggest that a triosephosphoric ester is formed in fermentation. Such a compound might not be precipitated by hydrazines, yet yield methylglyoxal on distillation with acid. There is, however, no definite support for the existence of such a triosephosphoric ester.

IV. Oxidation-reduction potentials of yeast preparations.

If acetaldehyde acts as an oxidising agent or hydrogen acceptor in fermentation, it must have a definite apparent oxidising potential. It cannot have a true oxidation-reduction potential, because it is reduced irreversibly. The reduction is not a simple electronic change and the reduction product, alcohol, is a feeble reducing agent in aqueous solution. The apparent potential of acetaldehyde was determined by adding it to solutions of leuco-dyes of known oxidation-reduction potential in buffered phosphate solution at p_H 6.0. In this way acetaldehyde was able to oxidise reduced indigodisulphonate, methylene blue and thionine, so that its oxidation potential at p_H 6.0 must be higher than 0.10 volt. Acetaldehyde will not oxidise potassium ferricyanide or the leuco-compounds of 1-naphtholindophenol-2-sulphonic acid and 1-naphtholindo-2:6-dibromophenol-2-sulphonic acid, so that its oxidation potential is probably below 0.15 volt. These determinations were carried out at p_H 6.0 because this was found to be about the p_H of fermenting yeast preparations containing the usual amount of phosphate and saturated with carbon dioxide.

The p_H of fermentation was determined by use of the glass electrode. The reaction of phosphate with yeast juice fermenting fructose was carried out in the cup of a glass electrode similar to that described by Bayliss, Kerridge and Verney [1926]. The readings over 40 minutes during the reaction varied from p_H 6.2 to 6.3. This result is in agreement with the results obtained by Biilman and Katagiri [1927] for phosphate solutions containing glucose and alcohol and saturated with carbon dioxide. These workers used the quinhydrone electrode, which cannot be applied to yeast solutions on account of their oxidation-reduction potential.

Although glucose is more strongly reducing than fructose at p_H 7.5–9.0 [Wurmser, 1927], in solutions as acid as p_H 6.0 it is doubtful if glucose and fructose have any oxidising or reducing power. In buffered phosphate solution at p_H 6.0 neither glucose nor fructose could reduce methylene blue or oxidise leuco-naphtholindophenoldisulphonate in 12 hours at 30°. It is therefore improbable that the different behaviour of glucose and fructose in fermentation with free phosphate is due to difference in oxidising or reducing properties. The difference in behaviour possibly lies in a difference in the ease of esterification of the two sugars.

Cannan, Cohen and Clark [Clark and collaborators, 1928, p. 306] had found that the oxidation-reduction potential of suspensions of washed yeast cells at p_H 5.75 fell from 0.10 to – 0.05 volt in 5 hours. Having determined that the p_H of yeast preparations remained steady during the reaction of phosphate, it became possible to determine the oxidation-reduction potential by adding dyes, and noting the time necessary for decoloration. The acetone precipitate of yeast juice, being free from hexosephosphatase, was very useful for this determination of the oxidation-reduction potential of fermentation with free phosphate, because no fermentation or reduction of dyes occurred until free phosphate was added. The time taken for the reduction of various dyes added in minimal concentrations (0.01 %), so as to have as little poisoning effect as possible, are shown in Table IV. The figures for the approximate E_0' (the E_h at which the dyes are 50 % reduced) at p_H 6.0 are taken from papers by Clark and his collaborators [1928]. The results show that the oxidation-reduction potential falls from 0.10 to – 0.03 while the greater part of the reaction with phosphate occurs.

Table IV. *The time taken for the reduction of dyes added to the acetone precipitate of yeast juice fermenting glucose in the presence of K_2HPO_4 .*

Dye added (0.01 %)	E_0' at p_H 6.0	E_h for 98 % reduction	Time for reduction (mins.)
1-Naphtholindo-2:6-dibromophenol-2-sulphonate	0.19	0.14	2
1-Naphtholindophenol-2-sulphonate	0.18	0.13	5
Thionine	0.10	0.05	10
Methylene blue	0.05	0.00	15
Indigotetrasulphonate	0.00	– 0.05	30
Indigotrisulphonate	– 0.03	– 0.08	Partially reduced
Indigodisulphonate	– 0.07	– 0.12	Not reduced

Experiments were next carried out with higher concentrations of the dyes so that they exerted some poisoning effect on the system. In this way the rate of fermentation in the presence of free phosphate was measured at different oxidation-reduction potentials. Results of such experiments are shown in Table V. It will be seen that dyes more strongly oxidising than thionine or more strongly reducing than indigodisulphonate do not accelerate the fermentation, but in addition to methylene blue both thionine and indigotetrasulphonate act as hydrogen acceptors. The failure of indophenol dyes to increase fermentation may be due to their quinone structure, so that they

might combine with the protein of the enzyme, or to the phenolic structure of their reduced forms so that they might inhibit fermentation in the same way as phenol. Both quinone and quinol hinder fermentation. The dyes, however, do not seem to combine in any way, as on addition of potassium ferricyanide at the end of fermentation the colour of the dye is always restored. Potassium ferricyanide, a different type of compound and a stronger oxidant than any of the dyes used, is reduced in fermentation but the fermentation is not increased.

Table V. *The effect of different hydrogen acceptors and reducing agents on fermentation.*

1 cc. 2*M* K₂HPO₄ added to 2 g. acetone precipitate of yeast juice in 20 cc. 10 % glucose solution.

Substance added						<i>E_h</i> during reaction (approximate)	Millimolecules CO ₂ evolved in 20 minutes
None	...	...	...	...	...	0.00?	0.16
0.05 % indigodisulphonate	...	...	...	...	...	– 0.03	0.10
0.05 % indigotrisulphonate	...	...	...	...	...	– 0.02	0.10
0.05 % indigotetrasulphonate	...	...	...	...	...	– 0.01	0.52
0.05 % methylene blue	...	...	...	...	...	0.01	0.47
0.05 % thionine	...	...	...	...	...	0.03	0.40
0.05 % naphtholindo-2: 6-dibromophenol-2-sulphonate	...	...	...	...	...	0.14	0.18
0.05 % naphtholindophenol-2-sulphonate	...	...	...	...	...	0.13	0.10
0.05 % acetaldehyde	...	...	...	...	...	—	0.86
Leuco-dyes							
0.05 % leuco-indigodisulphonate	...	...	...	...	...	– 0.10	0.08
0.05 % leuco-methylene blue	...	...	...	...	...	0.00	0.42
0.05 % leuco-naphtholindophenol-2-sulphonate	...	...	...	...	...	0.12	0.12

The effect of adding leuco-dyes throws some light on the mode of action of dyestuffs in fermentation. Leuco-indigodisulphonate almost stops fermentation, but leuco-methylene blue accelerates fermentation to nearly the same extent as the oxidised dye and leuco-naphtholindophenolsulphonate has no effect on the fermentation. As reduction does not therefore have any great effect on the action of dyes, it is probable that they act as hydrogen carriers rather than as hydrogen acceptors. Thus it might be expected that methylene blue would be reduced by methylglyoxal and oxidised again by acetaldehyde (the last reaction can be shown to take place in the test-tube without enzyme). If this is the case, only those dyes which can be oxidised by acetaldehyde and reduced by methylglyoxal will be active in fermentation and the apparent reducing potential of the active methylglyoxal of fermentation should be about – 0.02 volt at *p_H* 6.0.

The action of acetaldehyde in accelerating the reaction of phosphate is probably different from that of dyes. The reduction of aldehyde differs from that of the dyes which accelerate fermentation in not being reversible, so that acetaldehyde acts purely as hydrogen donator, not as hydrogen carrier. It is probably more effective in fermentation because of some specificity of the oxidase for acetaldehyde.

SUMMARY.

1. The lag between phosphate esterification and carbon dioxide evolution, in fermentation by yeast preparations, is not removed by the addition of those hydrogen acceptors which reduce the time necessary for the attainment of the maximum phosphate rate of carbon dioxide production.

2. The lag is accompanied by an increase in the amount of triose present; it is possible that accumulation of methylglyoxal prevents further esterification of phosphate.

3. During the reaction of phosphate in fermentation the p_H of the solution, saturated with carbon dioxide, remains constant between 6.2 and 6.3.

4. Those dyes which accelerate fermentation by acting as hydrogen acceptors or carriers have an E_0' between 0.00 and 0.10 volt at p_H 6.0; it is probable that they must be reducible by methylglyoxal and oxidised again by acetaldehyde.

My best thanks are due to Prof. A. Harden for much helpful criticism, and to Dr Winifred Pleass, who carried out the p_H determinations with the glass electrode.

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