XXXI. THE EQUATION OF ALCOHOLIC FERMENTATION. II.

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EVIDENCE has been accumulating for some time that with certain yeast preparations a very large proportion of the phosphorus esterified during the fermentation of a mixture of sugar and phosphate is found at the close of the fermentation in the form of a monophosphoric ester. In his early experiments with yeast juice Robison [1922] obtained as much as 15–30 % of the total esterified P in the form of monophosphate, equivalent to a molecular ratio of diphosphate/monophosphate of 2·8 to 1·6, but in later experiments, Robison and Morgan [1928], as much as 47–50 % (molecular ratio 0·5) has been observed. These results were obtained by repeated additions of phosphate to the fermenting mixture. In our previous paper two low ratios (0·61 and 0·72) were noted in this laboratory. The general experience drawn from a large number of experiments is that with dried top yeast from a brewery high ratios are almost invariably obtained, whereas with yeast juice the results are much more variable and low ratios are frequent.

The matter is further complicated by the fact that the monophosphate formed by dried yeast (brewer's top yeast) consists very largely of trehalose-monophosphate [Robison and Morgan, 1928] whereas this substance has not yet been detected among the products from yeast juice. Kluyver and Struik [1928], working at Delft with maceration extract prepared from dried yeast, have also obtained considerable proportions of monophosphate and consider that the ratio in which di- and mono-phosphate are finally present depends on the dilution of the extract, more monophosphate (up to 80 % of the total esterified) being obtained with the more dilute juice.

As pointed out in the previous paper the production of monophosphate necessitates some modification of the equation originally proposed by Harden and Young in which only the formation of diphosphate is taken into account. In that paper it was shown that, notwithstanding the considerable variation in the proportions of di- and mono-phosphate produced, the ratio of CO_2/P esterified was approximately constant at about 0·9. It was suggested that this indicated that about 10 % of the phosphate was esterified without production of carbon dioxide. As regards the ratio of $\text{CO}_2/\text{hexosediphosphate}$ it was pointed out that small deviations from the theoretical value of 2 might be accounted for on the ground of partial hydrolysis of the diphosphate with

formation of monophosphate. No experimental evidence in favour of this supposition is available and, as was then remarked, this explanation is inapplicable when a large proportion of monophosphate is formed.

In order to obtain more information on all these points a further series of experiments has been made in which (a) dried yeast of various origins, (b) maceration extracts made from two of the samples of dried yeast used in (a), and (c) yeast juice were employed.

EXPERIMENTAL.

The methods adopted were described in the previous paper [1927]. The estimation of the proportions of mono- and di-phosphoric esters produced has received further consideration. It has been found by Robison that the precipitate of barium salts produced by adding baryta and barium acetate to the trichloroacetic acid filtrate from the fermented liquid may not contain the whole of the barium hexosediphosphate present. A certain amount may remain in solution along with the monophosphate. This is precipitated by the addition of one-ninth of a volume of alcohol, so that greater accuracy is obtained by estimating the organic phosphorus in the alcoholic filtrate and calculating this as monophosphate. Even with this modification the estimation is not susceptible of great accuracy, as a small amount of the monophosphate is sometimes precipitated under these conditions.

A second possible source of error lies in the fact that in the presence of free phosphate, hexosediphosphate is almost completely removed from solution by the treatment with baryta and barium acetate, so that no further precipitate is produced by the addition of alcohol. Since the baryta treatment at the commencement of the experiment is carried out in presence of free phosphate, it is essential to precipitate with 10 % alcohol at the end of the experiment, at which period no free phosphate is present, in order to get comparable results. In the work recorded in the previous paper the alcohol method was not employed, so that the amounts of monophosphate there recorded are probably all somewhat too high and those of diphosphate somewhat too low.

The samples taken before and after esterification are treated in the same way and the numbers quoted below represent the differences between the amounts present at the beginning and end of the esterification period.

Correction for normal rate of fermentation. It was pointed out in the previous paper that the steady rate of fermentation at the end of the experiment is usually higher than at the commencement, owing to the increased concentration of esterified phosphate. Boyland [1929] has found that with dried yeast the steady rate increases with the total esterified phosphorus up to a certain point and then remains constant. It follows from this that the correction to be applied should vary during the process of esterification. In the experiments described by Boyland, in which periodic estimations of esterified

Table I.

Exp. No.	Material used	Sugar	P		Correc- tion for		CO ₂ cc. at N.T.P.		Dura- tion. No. of 5 min. periods	mg. P as		CO ₂ / com- bined P	CO ₂ / diphos- phate	CO ₂ / mono- phos- phate	Diphos- phate/ mono- phos- phate
			cc. 0.6 M K ₂ HPO ₄ added	mg. P	com- bined CO ₂ cc.	Correc- tion for hydro- lysis cc.	Cor- rected vol. cc.	Hexose diphos- phate		Hexose mono- phosphate					
(a) <i>Dried yeast:</i>															
80	D.Y. 14. 6. 27	Fructose	10	180	21.5	63.1	135.4	14	159.8	15.1	1.07	2.35	12.4	5.29	
82	"	"	15	272	35.3	69.5	189.9	15	233.9	25.6	1.01	2.25	10.3	4.56	
84 a	"	Glucose	8	144	15.4	68.5	101.3	16	—	—	0.97*	—	—	—	
b	"	Gl. + acetaldehyde	8	144	15.4	47	109.4	10	—	—	1.05†	—	—	—	
85 a	"	Glucose	8	144	17.7	65.3	100.9	17	135.8	10.8	0.95	2.06	12.9	6.3	
b	"	Gl. + acetaldehyde	8	144	17.7	52	112.8	14	134.3	12.3	1.06	2.33	12.7	5.45	
93	D.Y. 1. 12. 27	Fructose	10	180	27	108.5	129.8	15	166.5	20.5	0.96	2.16	8.76	4.06	
94	"	"	10	180	23.5	95	126.1	16	161.6	21.4	0.95	2.16	8.15	3.77	
98	D.Y., Y. III	"	10	164.7	23.6	82.4	131.5	15	129.2	36.3	1.09	2.82	5.01	1.78	
(b) <i>Maceration extracts:</i>															
92	Maceration ext. D.Y. 1. 12. 27	Fructose	10	180	24	69.2	126	16	107.5	72.5	0.97	3.24	2.41	0.74	
97	"	"	10	164.7	25.5	65.2	115.3	15	22	140	0.98	11.1	1.49	0.08	
99	Maceration ext. D.Y., Y. III	F. + acetaldehyde	10	164.7	23.9	84.1	105.3	18	163.2	5.2	0.86	1.79	28.1	15.6	
(c) <i>Yeast juice:</i>															
100	Yeast juice	Fructose	10	163.3	27.2	42.2	106	12	151	12.6	0.90	1.94	11.6	5.9	
101	"	"	10	163.3	26.5	45.2	105.9	13	118.4	43.9	0.90	2.48	3.33	1.35	
102	"	"	10	160.5	22.1	16.1	109.5	11	124	39.5	0.93	2.44	3.84	1.57	

† Total esterified P = 143.8 mg.

* Total esterified P = 144.5 mg.

phosphate were made, it has been possible to apply a suitable correction at each point. The result of this is that his total correction is less than that calculated from the final rate only and his residual volume of gas correspondingly greater. The difference may amount to as much as 10 %, but varies very greatly with the conditions of the experiment, and is much less for yeast juice and zymin than for dried yeast. In the table of results, as in the previous one, the final rate only has been used, as we have no intermediate observations on which to base any modification. Our final volumes of CO_2 and ratios of CO_2/P esterified would therefore be expected to be somewhat lower than those obtained by Boyland.

RESULTS.

Experiments have been carried out with dried yeast, two samples of which had been prepared at different times from mild ale yeast and one from a baker's yeast of Dutch origin: two experiments were made with maceration extract prepared from one of the mild ale dried yeasts and one with maceration extract from the Dutch baker's yeast; finally three experiments were made with yeast juice from mild ale yeasts. The results are given in Table I.

DISCUSSION.

Ratio of CO_2 to total P esterified.

(a) *Dried yeast.* The average value for this ratio is 1.013 when the final rate of fermentation is taken as the basis for the correction for the hydrolysis rate. The application of the more accurate correction, in which allowance is made for the increase in the hydrolysis rate due to increase in the concentration of hexosephosphate during the fermentation, would bring this to about 1.06. It is noteworthy that, whereas in Exps. 84 *a* and 85 *a*, in which glucose was employed, the evolution of CO_2 was comparatively slow and protracted, the repetition of the experiments with the addition of acetaldehyde (Exps. 84 *b* and 85 *b*) gave a much more rapid evolution of gas and also a higher ratio of CO_2 to esterified phosphate. This is almost certainly due to the smaller and more accurate correction for hydrolysis required.

On the whole then we may conclude that in the case of the dried yeasts so far studied the ratios of CO_2 to esterified phosphorus are slightly higher than unity and markedly higher than those hitherto observed with yeast juice or zymin. The origin of this small amount of extra CO_2 is at present unexplained [see Boyland, 1929].

(b) *Maceration extracts.* These were prepared by Lebedev's method [1912] from two samples of the dried yeast used in the foregoing experiments. The extracts in Exps. 92 and 97 were prepared from the dried yeast (English mild ale yeast) used in Exps. 93 and 94, but that used in No. 97 was prepared about 2 months after that used in No. 92. These two extracts yield ratios almost identical with those given by the dried yeast from which they were prepared, viz. 0.97 and 0.98, which would be raised to about 1.01 and 1.02

by applying the revised correction for hydrolysis. The extract employed in Exp. 99 which was made from the Dutch baker's yeast used in Exp. 98, on the other hand, has yielded a much lower ratio (0.86, 0.9 corrected) than the corresponding dried yeast (1.09, 1.11 corrected). Further experiments are necessary before any definite conclusion can be drawn as to the behaviour of maceration extract.

Kluyver and Struik [1928] have devoted much attention to this aspect of the subject and regard the ratio as indeterminate. Their experimental ratios, obtained with maceration extract, had the values 0.60, 0.71, 0.72, 0.89, 0.67, 0.71 and 0.82, most of which are below the limits we have observed. These figures are, however, not strictly comparable with ours, as the correction for the evolution of CO_2 due to change of acidity in passing from phosphate to hexosephosphate was calculated and not ascertained experimentally. They are obtained from experiments with maceration extracts using in some cases comparatively small amounts of phosphate and rather slow evolution of gas, so that the correction for hydrolysis rate is relatively larger and correspondingly less accurate than in our experiments.

It must also be remembered that the yeast employed by Kluyver and Struik was different from those we used and the difference between our results may be due to this. Further work is certainly necessary to decide this point.

(c) *Yeast juice*. The three experiments with yeast juice (from English mild ale yeast) (Nos. 100, 101 and 102) all gave low ratios, viz. 0.90 (0.91 corrected), 0.90 (0.92 corrected) and 0.93 (0.93 corrected), in substantial accordance with the three results quoted in our previous paper (0.87, 0.95 and 0.83).

Ratio of hexosediphosphate to hexosemonophosphate.

With dried yeast the amount of combined phosphorus in the form of hexosediphosphate is invariably considerably greater than that in the form of hexosemonophosphate, varying in our present experiments from 92.6 to 88.3 % of the total phosphorus esterified with the English mild ale yeasts, while the Dutch baker's yeast gave the lower result of 78.1 %. On the other hand the maceration extract prepared from two different dried yeasts gave very different results. Of the two extracts prepared from English mild ale yeast one, No. 92, gave 59.7 % of the total esterified phosphorus as diphosphate, and the second, No. 97, only 13.9 %. The sample prepared from a Dutch baker's yeast on the other hand (No. 99) yielded 96.9 % as diphosphate, scarcely any monophosphate being formed.

The three samples of yeast juice also gave variable results, two of them (Nos. 101 and 102) yielding 73 and 75.9 % of the total phosphorus esterified as diphosphate, whilst the third (No. 100) yielded 92.2 %.

It must further be remembered that there is an important qualitative difference between the monophosphate which is formed by dried yeast, which

is largely trehalosemonophosphate, and that formed by yeast juice and zymine, which appears to be the hexosemonophosphate studied by Robison [1922]. The nature of the monophosphate formed by maceration extract has not yet been carefully examined from this point of view.

Kluyver and Struik have also made a series of determinations of the relative amounts of mono- and di-phosphate produced by maceration extracts under various conditions of dilution and phosphate concentration. They [1928] believe that the proportion of monophosphate to diphosphate is a function of the dilution of the juice, the more dilute juice producing the greater proportion of monophosphate. Preliminary experiments made in this laboratory are not in agreement with this conclusion, which will be dealt with in a later communication.

The equation of alcoholic fermentation.

The fact which is now well established that in some instances a very large proportion of the esterified phosphorus is found in the form of hexosemonophosphate, whilst at the same time the molecular ratio of CO_2 to total esterified phosphorus is approximately unity, renders it impossible in these cases to express the result by means of Harden and Young's equation. The ratio CO_2 /diphosphate in such cases is frequently very high and it is difficult to account for such large deviations from the theoretical ratio by any secondary changes such as were suggested in our previous paper.

As far as our experiments go the most definite result is that the molecular ratio of carbon dioxide to the total phosphorus esterified falls within the limits of 0.9 to 1.1, the tendency being for a low ratio with yeast juice, maceration extract and zymine, and for a high ratio with dried yeast. In other words the evolution of one molecular volume of CO_2 appears always to be accompanied by the introduction of a phosphoric group into a sugar molecule, either a mono- or a di-phosphoric ester being formed. The possibility of the occurrence of a certain amount of esterification unaccompanied by evolution of CO_2 is also not excluded, but according to our results this must be comparatively unimportant. We are yet far from understanding the actual course of events resulting in the rupture of a sugar molecule, nor can one yet say what determines the proportion of mono- and di-phosphate produced. These questions require and are receiving further investigation.

An important point which emerges from these results is that it is necessary before venturing on any generalisation to examine preparations of the most varied origins under as many modifications of conditions as possible.

SUMMARY.

1. In fermentations of glucose or fructose in presence of phosphate carried out with dried yeast, the ratio of extra carbon dioxide evolved to phosphorus esterified tends to be slightly higher than unity.
2. With maceration extract and yeast juice this ratio tends to be somewhat lower than unity, as previously found with zymine.
3. The ratio of hexosediphosphate to hexosemonophosphate formed during the fermentation is usually high for dried yeast, but is very variable for maceration extract and yeast juice, the product in extreme cases consisting almost entirely of either the diphosphate or monophosphate.
4. The most constant ratio which we have observed is that of extra CO_2 to total P esterified, which only varies about $\pm 10\%$, although the proportions of diphosphate and monophosphate may vary from 96 % of diphosphate to 86 % of monophosphate.
5. The equation of Harden and Young cannot be applied to those cases in which a large proportion of hexosemonophosphate is produced, whilst the ratio $\text{CO}_2/\text{total P}$ esterified remains at about 0.9.

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