CLXII. THE EQUATION OF ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN and FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute, London.

(*Received September 7th, 1927.*)

The fact that the hexosediphosphate which is produced by the action of yeast preparations on a mixture of a hexose and a phosphate is accompanied by a monophosphate renders it necessary to reconsider the equation originally proposed by Harden and Young [1906] to represent the reaction:

$$2C_6H_{12}O_6 + 2Na_2HPO_4 = C_6H_{10}O_4(PO_4Na_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O.$$

This equation was originally established on the basis of the ratio of carbon dioxide evolved to the phosphate which went into organic combination as experimentally observed and of the composition of the hexosephosphate produced. It was further confirmed by experiments in which the ratio was determined of the sugar added in presence of excess of phosphate to the carbon dioxide produced.

It is however obvious that if some of the phosphate and some of the sugar form a compound of a different composition (a hexosemonophosphate) experiments of the kind just indicated are not sufficient to afford a rigorous proof of the equation.

It was therefore decided to make a more detailed study of the reaction and to endeavour to determine the exact proportions of the hexosemonophosphate and hexosediphosphate produced, as well as the amount of carbon dioxide evolved.

Determination of the hexosemonophosphate and hexosediphosphate produced in the reaction.

The following method has been developed from a suggestion for which we are indebted to Dr R. Robison.

The fermentations are stopped by adding enough 25 % trichloroacetic acid to raise the concentration to 3·5 %. The liquid is filtered and the amount of free and total phosphorus in the filtrate is estimated by Neumann's method. To the rest of the filtrate is added excess of barium acetate and enough hot baryta to render the liquid just pink to phenolphthalein. Enough CO_2 is then added just to remove the pink colour. The liquid is allowed to stand in the cold room for 18 hours and filtered. The free and total phosphorus are then estimated in the filtrate.

It is assumed that in this treatment the large concentration of Ba ions present decreases the solubility of the barium hexosediphosphate to such an extent that this salt is almost completely precipitated along with the free phosphate present, whilst the readily soluble barium hexosemonophosphate is left in solution.

The amount of the latter is calculated from the combined phosphorus found in the liquid. The amount of diphosphate is estimated from the total combined phosphorus less that remaining in solution after precipitation.

Attention must be drawn to two possible errors, which affect the results in opposite directions. (1) If too much baryta is added, some of the monophosphate may be precipitated in the form of a basic salt. (2) The presence of a considerable concentration of monophosphate may enhance the solubility of barium hexosediphosphate. The extent to which these occur cannot readily be gauged.

In the earlier experiments the total free and combined phosphorus was precipitated by adding 50 % alcohol in conjunction with the barium acetate and baryta. The precipitate so formed was washed with 60 % alcohol to remove sugar, and its content of free and total phosphorus and copper-reducing power determined. From these data by means of a simultaneous equation the amounts of hexosemonophosphate and hexosediphosphate were then calculated. The results were unreliable owing to the presence of barium compounds of glucose or fructose in the washed precipitate.

Attempts were also made to precipitate the hexosediphosphate alone from the fermented liquid by the addition of brucine, but the results obtained did not agree among themselves, probably owing to the slow precipitation of free phosphate by brucine.

Determination of the CO_2 produced in the reaction.

The total volume of CO_2 produced was determined in the usual manner.

Part of this is due to the conversion of phosphoric into the stronger hexosephosphoric acids, the potassium salts of which are hydrolysed to a smaller extent in solution than the phosphates and therefore take up less CO_2 in the form of bicarbonate.

To estimate this part of the gas parallel experiments are made in each of which the same amount of phosphate is added.

To one, excess of trichloroacetic acid is added immediately after the addition of phosphate and the evolved gas measured. This gives the CO_2 combined in the form of bicarbonate at the commencement of the observation.

To the second, the same quantity of trichloroacetic acid is added after all the free phosphate has been combined and the evolved gas measured. This gives the residual bicarbonate. The difference between the volumes of gas in the two cases is taken to represent gas evolved owing to the difference in properties of the hexosephosphates and phosphates.

To reduce this correction to a minimum a mixture of four volumes of

0·6 *M* KH_2PO_4 and one volume of 0·6 *M* K_2HPO_4 was used whenever possible. This mixture can be successfully employed with yeast-juice[1]. In parallel experiments with the same sample of juice the addition of 10 cc. of 0·6 *M* K_2HPO_4 or 10 cc. of the 0·6 *M* phosphate mixture produces the same evolution of CO_2 within the limits of experimental error, when all corrections have been made. The phosphate mixture was found unsuitable for use with zymin or dried yeast, probably on account of its acidity (p_H 6·4).

Another deduction remains to be made from the total volume of gas produced. It has been assumed in past experiments, that, throughout the whole period, during which the added phosphate is undergoing esterification, a steady rate of evolution of CO_2 is maintained by the constant rate of supply of free phosphate by the action of the hexosephosphatase of the yeast preparation on the hexosediphosphate present in the liquid, as shown by the equation

$$C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = C_6H_{12}O_6 + 2Na_2HPO_4.$$

This amount cannot be ascribed to the phosphorus added and represents the amount of gas which would have been evolved in the absence of added phosphate.

Some uncertainty attaches to this calculation. The possibility exists that, in the very varied range of concentrations both of phosphate and hexosephosphate which occur in an experiment, the rate of action of the hexosephosphatase may be modified. This is obviously the case when a high concentration of phosphate is employed, since the rate of fermentation is at first considerably depressed and only regains its original value after a more or less prolonged period of induction. In Exps. 66 and 68 (Table I), for example, in which 15 and 20 cc. of 0·6 *M* K_2HPO_4 were used, on the addition of the phosphate the rates fell from 4·8 to 2·4 and 1·8 cc. per 5 minutes and did not regain their original value for 50 and 100 minutes respectively.

Hence, in determining the equivalent between hexosephosphate formed and carbon dioxide evolved only such quantities of phosphate can be used as cause no preliminary depression in the rate of fermentation.

Again, at the close of the experiment, when the concentration of hexosephosphate has become much larger than its original value, the steady rate of fermentation is frequently somewhat greater than before the addition of phosphate.

The complete conversion of the quantities of phosphate which have been used in these experiments requires from 1·5 to 3 hours. The normal rate of fermentation for 25 cc. yeast-juice is about 2–3 cc. per 5 minutes; for 4 g. zymin, 4·5 to 5 cc. and the volume equivalent to the phosphate added is 67·2 cc. for 5 cc. of 0·6 *M* phosphate.

---

[1] The statement made by Harden [1923] that the correction is negligible "when a mixture of five molecular proportions of K_2HPO_4 with one of KH_2PO_4 is used" should read "when a mixture of five molecular proportions of KH_2PO_4 and one of K_2HPO_4 is used."

Hence an error of 0·5 cc. in the estimated "normal" rate per 5 minutes in an experiment lasting 1·5 hours involves an error of 9 cc. or 13 %.

The addition of phosphate does not produce so great a relative increase in the rate of fermentation given by zymin as it does in the case of yeast-juice. As the result of this the correction in the case of zymin (see table) is very much higher—and correspondingly less accurate—than in that of yeast-juice.

EXPERIMENTAL METHOD AND RESULTS.

Experiments were carried out both with yeast-juice and zymin (yeast dehydrated with acetone). 25 cc. of yeast-juice were employed in each case + 3 g. fructose or glucose + 0·3 cc. toluene; in the case of zymin 4 g. were used + 3 g. fructose or glucose + 30 cc. H_2O + 0·3 cc. toluene.

In each case three separate quantities of the mixture were incubated at 30° until the rate of evolution of CO_2 became constant. The desired amount of phosphate solution previously saturated with CO_2 at 30° was then added to two of them, which were then treated as already described.

The third was employed to obtain a determination of the hexosemonophosphate and hexosediphosphate in the fermenting mixture at the commencement of the experiment. For this purpose trichloroacetic acid was added in order to stop the fermentation and then the same amount of phosphate as was added to the other two. In the filtrate from this hexosemonophosphate and hexosediphosphate were determined. As a check the same process was sometimes carried out with the yeast-juice or zymin without the addition of phosphate.

In calculating the results the amounts of monophosphate and diphosphate found at the commencement of the experiment were subtracted from those found at the end, the differences being regarded as the amounts of the two esters formed during the experiment. The results are contained in Table I.

Table I.

			P		CO_2, cc. at N.T.P.										
Exp. no.	Material used	Sugar	Mixture used 0·6 *M* phosphate	mg. added	Total evolved	Correction for combined CO_2	Correction for hydrolysis	Corrected vol.	Duration no. of 5 min. periods	Mg. P as hexosediphosphate	Mg. P as hexosemonophosphate	CO_2/Combined P	CO_2/Diphosphate	CO_2/Monophosphate	Diphosphate/Monophosphate
46	Yeast-juice	Fructose	10 cc. acid mixture	181·6	155·4	7·6	31·5	116·3	14	133·2	53·4	0·87	2·43	3·03	1·25
50	,,	,,	,,	181·6	148·7	5·3	23·5	119·9	14	96·7	79·6	0·95	3·45	2·10	0·61
58	,,	,,	,,	181·6	148·4	7·5	29·3	111·6	16	144·2	41·1	0·83	2·14	3·75	1·75
54	Zymin 1	,,	10 cc. K_2HPO_4	179·7	215·2	21·0	90·8	103·4	17	128·8	39·8	0·85	2·23	3·62	1·62
56	,,	,,	,,	179·7	234·3	21·9	113·2	99·2	18	123·2	43·1	0·83	2·24	3·21	1·43
60	Zymin 2	,,	5 cc. K_2HPO_4	89·85	92·6	10·4	53·9	28·3	23	50·6	35·0	0·46	1·55	1·12	0·72
62	Zymin 5	,,	,,	89·15	142·2	14·4	69·7	58·1	15	83·2	8·0	0·88	1·93	10·08	5·18
64	,,	,,	10 cc. K_2HPO_4	178·3	208·9	21·0	106·7	81·3	23	134·0	38·6	0·65	1·68	2·91	1·73
66	,,	,,	15 cc. K_2HPO_4	267·4	233·1	32·2	uncertain		29	215	44·9	not determined			2·39
68	,,	,,	20 cc. K_2HPO_4	356·6	305·6	42·6	,,		44	304·2	59·6	,,			2·55
72	,,	Glucose	5 cc. K_2HPO_4	89·15	151	12·1	75·6	63·3	17	64·4	26·8	0·96	2·72	3·27	1·20
74	,,	,,	,,	89·15	146·4	11·8	74·4	60·2	17	73·0	18·2	0·91	2·28	4·57	2·00
75	,,	Fructose	,,	89·15	136·1	11·8	68·0	56·3	15	81·1	10·1	0·85	1·92	7·69	4·00
76	,,	Glucose	7·5 cc. K_2HPO_4	133·7	184·4	16·1	93·6	74·7	23	115·4	19·2	0·90	1·79	5·37	3·00
77	,,	,,	5 cc. K_2HPO_4	90·75	137·5	12·6	65·2	59·7	15	67·9	22·7	0·91	2·43	3·64	1·50

DISCUSSION.

Ratio of CO_2 to total P esterified.

The fact that the ratio of CO_2/P is invariably below unity seems to indicate quite clearly that some esterification has taken place without evolution of CO_2 in accordance with the requirements of the original equation. As the estimation of the total amount of combined P is susceptible of a high degree of accuracy this conclusion may be regarded as well established. This is in agreement with Meyerhof's view [Meyerhof and Lohmann, 1927] that in alcoholic fermentation a monophosphoric ester is first formed without evolution of CO_2, if it be supposed that some of this still exists in the solution (either in its original supposedly active form or in a stabilised form) when the sample is taken for analysis.

Excluding the two exceptionally low results of Exps. 60 and 64, which are discussed later (p. 1221), the average ratio for the remaining eleven experiments is 0·89, the variations being from 0·83–0·96. If we assume that Harden and Young's equation correctly represents the change which results in evolution of CO_2, this indicates that an average of 11 % of the total phosphorus which has entered into combination has been esterified without evolution of carbon dioxide.

Since moreover, as will be seen later (p. 1222), there is no evidence from these experiments that diphosphate is formed without evolution of CO_2, it is probable that this direct esterification results in the formation of a monophosphate. This process then accounts for a considerable proportion, and in some cases for the whole, of the monophosphate present at the end of the experiment.

Ratio of CO_2 to hexosediphosphate.

Experiments have been made with three different preparations of yeast-juice and with three of zymin. Both glucose and fructose have been used. It will be seen that the results vary considerably, the values for the molecular ratio CO_2/hexosediphosphate extending over a comparatively wide range (1·55–3·45). How far this is due to imperfections in the analytical methods employed and how far to variations in the conditions it is difficult to decide. Duplicate analyses of the same experimental liquid give satisfactorily concordant results. On the other hand a series of three experiments (Nos. 72, 74 and 77) with the same sample of zymin at fortnightly intervals, under what were intended to be precisely similar conditions, gave decidedly different values both for the ratio CO_2/hexosediphosphate (2·72, 2·28 and 2·43) and for diphosphoric ester/monophosphoric ester (1·20, 2·0, 1·5). The barometric pressure was slightly different on these three occasions (751, 757·9 and 753·8 mm.), but whether the resulting small differences in the amount of CO_2 retained by the solution and the p_H of the liquids would affect the result is not known.

The lowest result obtained for this ratio, 1·55, was given by a sample of zymin (Exp. 60) which had been preserved for two years and fermented sugar at a much lower rate than the freshly prepared samples. The duration of the experiment was thus considerably prolonged and the corrections to be applied to the gas volume (see p. 1218) rendered correspondingly uncertain. The same is true of Exps. 64 and 76 in which an excessive amount of phosphate was added and the duration of the experiment was unduly prolonged. Excluding these three experiments, in only one experiment out of the twelve remaining did the monophosphate exceed the diphosphate. This gave the highest value obtained, 3·45, for the ratio CO_2/diphosphate. The remaining experiments, in all of which the diphosphate exceeded the monophosphate, gave ratios varying from 1·92–2·72 (nine experiments), the average being 2·38, whilst the average of the series of ten experiments is 2·26. This average agrees approximately with the value 2 which is required by Harden and Young's equation, but the individual variations are somewhat higher than would be expected, even when regard is taken of the uncertainty of the analytical methods and of the correction applied for hydrolysis to the total volume of CO_2 evolved.

The fact that this ratio, CO_2/diphosphate, is almost without exception above the value, 2, required by the equation might, however, be simply explained by assuming that the CO_2 was produced in accordance with the equation, but that some of the hexosediphosphate was subsequently partially hydrolysed, yielding a monophosphate and inorganic phosphate, the latter of which again entered into reaction.

The monophosphate actually present in the liquid analysed would then be derived from two distinct reactions, (*a*) direct esterification (see p. 1220), (*b*) partial hydrolysis of hexosediphosphate. This idea is quite in accordance with the fact that the monophosphate isolated from yeast-juice is probably a mixture [see Robison, 1922; Meyerhof and Lohmann, 1927]. On the other hand no definite evidence of the production of a monophosphate by hydrolysis of diphosphate by yeast enzymes is at present available, although this readily occurs on hydrolysis by acids [Neuberg, 1918], and Robison [1923] has obtained evidence that it probably also occurs when the diphosphate is hydrolysed by the phosphatases of cartilage and muscle. Quite recently Neuberg and Leibowitz [1927] have actually prepared the monophosphate from the diphosphate by the action of taka-diastase. Further work on this question is in progress.

It must however be remembered that if this be the origin of part of the monophosphate, a corresponding amount of inorganic phosphate must be liberated, which again enters into the reaction, since there is very little free phosphate present at the stage when the samples are taken. The carbonic acid liberated by these secondary reactions:

$$C_6H_{10}O_4(PO_4H_2)_2 + H_2O = C_6H_{11}O_5(PO_4H_2) + H_3PO_4$$

$$2C_6H_{12}O_6 + 2H_3PO_4 = C_6H_{10}O_4(PO_4H_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O$$

is comprised in that entered in the table under the heading "Correction for hydrolysis" and should, if the idea under discussion is correct, be not less than equivalent to the part of the monophosphate formed by hydrolysis. This is the case in every instance except that of Exp. 50, the exceptionally high ratio of CO_2/diphosphate and the large production of monophosphate in which remain unexplained.

Further light on the subject could probably be obtained by making a series of estimations of the relative amounts of monophosphate and diphosphate present at various stages of the fermentation, and efforts are being made to do this. Experiments of this kind as regards the total esterification have been made by Euler and Johansson [1913] and by Meyerhof and Lohmann [1927], but the distribution of phosphorus between the mono- and di-esters has not yet been ascertained.

The present experiments afford no evidence of the formation of diphosphate either directly or indirectly by esterification of monophosphate without evolution of CO_2 [cf. Meyerhof and Lohmann, 1927].

Ratio of diphosphate to monophosphate.

The ratio diphosphate/monophosphate is highly variable, the values obtained ranging from 0·61 to 5·18.

No definite relation of this value to any of the conditions of the experiment—concentration of phosphate (Exps. 62, 64, 66, 68), nature of sugar (Exps. 72, 74, 76, 77), nature of yeast preparation—can be made out. As already mentioned, in only two cases out of fifteen was the production of monophosphate greater than that of diphosphate, on many occasions (*e.g.* Exps. 62, 75, 76) it was very much less. The average value was 2·06. The factor controlling the relation of these two products has so far escaped detection.

Ratio of carbon dioxide to monophosphate.

The ratio of CO_2/monophosphate, like that of diphosphate/monophosphate is very variable—ranging irregularly from 1·12–10·08, with an average of 4·16. The variations are such as almost to preclude the possibility of the existence of a definite relation between the amounts of CO_2 evolved and monophosphate produced.

SUMMARY.

1. The reaction which occurs when a mixture of a hexose and inorganic phosphate is fermented by yeast-juice or zymin (yeast treated with acetone) has been re-examined and the amounts of carbon dioxide, hexosemonophosphate and hexosediphosphate produced have been determined.

2. The ratio CO_2/total P esterified is on the average 0·9, indicating that, if Harden and Young's equation be taken as correct, about 10 % of the phosphorus is esterified without evolution of CO_2. The product of this esterification is probably a monophosphate.

3. The ratio CO_2/diphosphate is on the average 2·38 but varies considerably in individual cases. The fact that this ratio is almost invariably somewhat greater than the value 2 required by Harden and Young's equation suggests that the diphosphate is originally produced in accordance with the equation, but that a part of it is subsequently partially hydrolysed, with formation of a monophosphate.

4. The ratios CO_2/monophosphate and monophosphate/diphosphate are highly variable and show no definite relations.

REFERENCES.

Euler and Johansson (1913). *Z. physiol. Chem.* **85**, 192.
Harden (1923). Alcoholic Fermentation (London), p. 43.
Harden and Young (1906). *Proc. Roy. Soc. Lond.* **77**, 405.
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
Neuberg (1918). *Biochem. Z.* **88**, 432.
Neuberg and Leibowitz (1927). *Biochem. Z.* **178**, 481.
Robison (1922). *Biochem. J.* **16**, 809.
—— (1923). *Biochem. J.* **17**, 286.