61. ON THE METHOD OF FORMATION OF PYRUVIC ACID BY BARLEY

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PYRUVIC acid is consumed in barley respiration [James & Norval, 1938] and can also be made to accumulate by poisoning the living plant with acetaldehyde or aromatic sulphonic acids [James & James, 1940]. Organic phosphates are present [Arney, 1939] which appear to be connected with respiration in a phosphorylating cycle [James & Arney, 1939]. It is, therefore, a matter of interest to discover whether the sugars consumed in barley respiration pass through such a cycle in their conversion into pyruvic acid.

Material

Leaves of young barley plants (var. Plumage Archer) were cut and frozen overnight at about -12° . They were allowed to thaw at room temperature and their sap was squeezed out through muslin by hand. This sap was usually allowed to stand in the refrigerator (at about 2°) for 24 hr. before use. Any sediment which formed was filtered off, a clear yellow liquid resulting. In a few experiments, as will be described, further purification was attempted. None of these saps by themselves yielded appreciable amounts of pyruvic acid, even after incubation (see p. 589) under the standardized experimental conditions, and they showed very little carboxylase activity. They were, therefore, very convenient for investigating the formation of pyruvic acid from the substances which it was desired to examine. Intact tissues could not be used since they would not have taken up the substrates sufficiently quickly.

Methods

Extraction. After a period of incubation at 30° in the presence of thymol as antiseptic, usually for 48 hr., any pyruvic acid present was separated from the sap. 10 ml. barium acetate were pipetted into the digest and the reaction adjusted to pH 8 by running in $M/5$ baryta. The barium precipitate was centrifuged off and another drop of barium acetate added, to make sure that precipitation was complete. 25 % trichloroacetic acid was then added, to give a final concentration of 5 %. The mixture was allowed to stand for 15 min. and then brought to the boil. The protein flocculum was filtered off and the clear filtrate transferred to a large distillation flask. Distillation was carried out under reduced pressure with a fine stream of air passing through the solution. Water and other volatile substances came over at $50-55^{\circ}$ and about 50 mm. pressure.

Identification. Pyruvic acid was identified in the distillate by the isolation of its 2:4-dinitrophenylhydrazone by the method of Peters & Thomson [1934]. The hydrazones obtained were compared with an authentic sample from twice redistilled pyruvic acid, giving M.P. 216° , a canary yellow colour and diamond-

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shaped platelets, single and clustered. This hydrazone gave a deep red coloration with alcoholic KOH, which was not discharged by adding water.

The separation of the pure substance from barley preparations presents numerous difficulties, and it was commonly found that the final product showed orange-tinted zones on crystallizing out. To avoid further laborious purifications, well-formed crystals were selected mechanically from the pure yellow zones for determination of the melting-point. Even when the quantity available was fairly considerable, it was found convenient to use the hot-box already described [James & James, 1940], selecting a small number of characteristic crystals for the purpose. These were either allowed to crystallize out on a coverslip placed in the crystallizing dish, or were scraped up and placed upon it with a clean scalpel.

Enzyme block. Loss of pyruvic acid might be expected, principally from the activity of carboxylase, [James & Norval, 1938]. Barley saps as used in the present experiments released CO₂ from added pyruvic acid. The release was slow and was shown, in another series of experiments, to be much less than when solid leaf material was present.

Low concentrations of 1-naphthol-2-sulphonic acid were used to restrict this carboxylase activity. Complete inhibition was not attempted, since the necessary concentration of the naphtholsulphonic acid caused heavy precipitations in the sap. High percentage yields of pyruvic acid could not, therefore, be expected, and were not obtained. Losses due to dephosphorylation of substrates by phosphatases are also known to occur [James & Heard, 1940].

Experiments

Sap only. (a) Exp. 48. A sample sap prepared as above was precipitated with N/5 barium acetate (1 part acetate to 20 parts sap). The precipitate was centrifuged off and the excess barium removed with a few drops of saturated ammonium sulphate. 5 ml. clear filtrate were added to 1 ml. 0.2 % 1-naphthol-2-sulphonic acid; and made up to 10 ml. with glass-distilled water. Crushed thymol (3 parts per 1000) was shaken with the digest which was put in a conical flask plugged with cotton-wool and incubated at 30° for 48 hr. No alkali-soluble 2:4-dinitrophenylhydrazone could be isolated.

(b) Exp. 70A. A sample of sap from young and vigorously growing seedlings was prepared in the usual way and was used immediately, without either barium precipitation or standing at low temperature for 24 hr. 40 ml. were mixed with 5 ml. 1 % sulphonic acid and 5 ml. glass-distilled water. Crushed thymol (3 per 1000) was added and incubation carried out as before. On extraction by the usual method, a small quantity of a 2:4-dinitrophenylhydrazone, soluble in alkali, was obtained. It was red in colour and crystallized poorly, but a narrow zone of spindle-shaped crystals was found which gave a rather indefinite M.P. 205–208°. These results suggest that small amounts of hydrazone-forming substances had been isolated, probably including traces of pyruvic acid. Since it was already known that under suitable conditions barley could be induced to accumulate pyruvic acid, no attempt was made to obtain definite yields in these digests. The absence of pyruvic acid from freshly extracted saps has already been shown [James & Norval, 1938; James & James, 1940].

Sucrose. Exp. 50. Incubation under the usual conditions was carried out with 24 ml. sap, 3 ml. 20 % sucrose (B.D.H. Analar) and 0.3 ml. 0.2 % sulphonic acid. Crushed thymol was added. The sap used in this experiment was precipitated with barium, as described under (a) above. This treatment might be

expected to reduce the concentration of phosphate, free and combined, in the sap. No trace of alkali-soluble hydrazones could be isolated.

Glucose and fructose. Special experiments with additions of glucose and fructose were not carried out, since these sugars were known to be present in fresh sap in fair quantity. Their presence did not, in itself, lead to the formation of appreciable quantities of pyruvic acid.

- *Adenylic acid. Exp. 68B.* This experiment was carried out with sap from young green seedlings. The sap was filtered and allowed to stand at 2° for 3 days before use. Its sugar content might be expected to be low. 40 ml. sap were incubated for 48 hr. at 30° with 5 mg. adenylic acid and 0.36 ml. 3 % 1-naphthol-2-sulphonic acid, in the presence of thymol (3 per 1000). It was cleared and distilled and the 2:4-dinitrophenylhydrazones formed in the usual way. The yield was small, with a narrow zone of canary-yellow crystals, probably the pyruvic acid derivative, round the edge. The bulk of the product was orange-coloured and oily. The result was markedly similar to that obtained with fresh sap only (see (b) p. 589).

Sucrose + adenylic acid. Exp. 68A. This experiment was carried out simultaneously with the adenylic acid experiment, with an equal aliquot of the same sap. All conditions were identical, except for the addition of 1 g. sucrose to this second digest. A much increased yield of alkali-soluble hydrazone was obtained which crystallized out wholly, though the last crystals to form were somewhat orange in colour. There were numerous bright yellow crystals of the spindle to diamond shape characteristic of the pyruvic acid hydrazone. They gave a deep red colour with alcoholic KOH, which was not discharged on adding water. M.P. 218°, corrected.

Glucose + adenylic acid. Exp. 69. A further supply of sap was prepared exactly as before and used after 24 hr. standing at low temperature. It was possible to use a rather larger digest: 70 ml. sap + 5 mg. adenylic acid + 1.75 g. glucose (B.D.H. analar) + 0.63 ml. 3 % 1-naphthol-2-sulphonic acid + thymol (3 per 1000). The result was similar to that of the previous experiment. A good yield was obtained of characteristic canary-yellow crystals, slightly contaminated with orange, which gave M.P. 216.5°, corrected, and the usual colour reaction with KOH.

Hexosediphosphate. A commercial sample of barium hexosediphosphate containing inorganic phosphate as impurity was used. The ratio of unhydrolysable P to P hydrolysed in 3 hr. was 0.64 as compared with 0.66 calculated from Lohmann's analyses of purified hexosediphosphate. We are indebted to Mr C. R. C. Heard for these figures. Immediately before use, aliquots of the barium salt were dissolved in dilute HCl and the barium centrifuged off after precipitation by a little saturated ammonium sulphate. The solution was neutralized (purple to litmus) with a few drops of strong KOH and made up to volume.

Exp. 47. A preliminary experiment was carried out using 20 ml. of clarified sap (as in (a) p. 589) + 4 ml. of a hexosediphosphate solution equivalent to 0.4 g. barium salt + 4 ml. 0.2 % 1-naphthol-2-sulphonic acid + crushed thymol (3 per 1000). The digest was made up to 40 ml. with glass-distilled water. Incubation was carried out under the usual conditions and hydrazones were prepared. There was a small yield of bright yellow crystals, some developing typical diamond forms. They gave a permanent red colour with Lu's [1939] reagent (6 vol. 10 % Na_2CO_3 + 4 vol. N NaOH). There was not sufficient for the determination of a melting-point by the usual laboratory method, and the hot-box for micro-determinations had not been prepared when this experiment was

carried out. A melting-point was, therefore, not obtained; but there seems little doubt that a small quantity of pyruvic acid had been isolated. Since the clarified sap used in this experiment gave an entire blank when incubated alone (p. 589), it is reasonable to assume that the pyruvic acid originated from the added hexosediphosphate. Confirmation was afforded by an experiment on a rather larger scale.

Exp. 54. 40 ml. sap + 10 ml. hexosediphosphate solution ($\equiv 0.1$ g. Ba hexosediphosphate) + 6 ml. 0.2 1-naphthol-2-sulphonic acid + thymol were incubated for 48 hr. at 30° . Isolation of alkali-soluble hydrazones was carried out as usual, yielding a considerable deposit of crystals, mostly canary-yellow, though a little reddish in places. Alcoholic KOH gave a permanent deep red colour which was not discharged on the addition of water. The abundant deposit was largely 'run together' but good diamond-shaped platelets were formed in the sparser zones. These gave M.P. 216° , corrected.

Phosphoglycerate. A pure preparation of the crystalline acid barium salt was given us by Prof. R. A. Peters, to whom we wish to express our gratitude. It was brought into solution in the same way as the hexosediphosphate, immediately before use.

Exp. 67. This experiment was carried out using an aliquot of the same lot of sap as was used for the adenylic acid and adenylic acid + sucrose experiments. 4 ml. phosphoglycerate solution ($\equiv 30$ mg. BaH_2 phosphoglycerate) and 0.72 ml. 3 % 1-naphthol-2-sulphonic acid were made up to 1000 ml. with sap. After the usual incubation, the preparation and isolation of alkali-soluble hydrazones were carried out, yielding a deposit of yellow crystals. There were crystal clusters and single crystals of typical pyruvate form, giving the usual permanent red colour with KOH; M.P. 216° , corrected.

Lactic acid. Lactic acid is not oxidized by barley saps alone, but on addition of ascorbic acid rapid oxidation takes place [James & Cragg, 1940]. The product of this oxidation was identified as pyruvic acid by means of the above technique, slightly modified.

Exp. 38. 200 ml. sap, clarified as in (a), were incubated with 40 ml. $N/5$ lactic acid + 40 ml. $N/10$ ascorbic acid (B.D.H.) + 4 ml. sulphonic acid + 116 ml. phosphate buffer, pH 7.0 ($= 400$ ml. in all). Incubation lasted 17 hr. at 30° . The digest was then killed with 5 % trichloroacetic acid. After it had stood overnight in a refrigerator, 25 % trichloroacetic acid was added to a final concentration of 5 %. After further standing, the digest was brought to the boil and distilled. A fraction coming over at $50-55^\circ$ and 20 mm. was taken and treated with an amount of 2:4-dinitrophenylhydrazine, which proved to be inadequate. When the hydrazone was finally separated it was oily, and there was a strong smell of free pyruvic acid. Further hydrazine was added and a plentiful precipitate of yellow crystals was finally obtained. They had typical pyruvate forms, gave deep red coloration with alcoholic potash and had M.P. 216° , corrected.

Inhibition of pyruvate formation by sodium fluoride

Exps. 47 and 54 above show that barley saps convert hexosediphosphate into pyruvic acid. Phosphoglycerate and lactate are also converted into pyruvate and either might, in theory, be an intermediate in the conversion of hexosediphosphate into pyruvate. The reaction phosphoglycerate $\rightarrow$ pyruvate, or, more exactly, its partial reaction 2-phosphoglycerate $\rightleftharpoons$ phosphopyruvate is markedly sensitive to poisoning by sodium fluoride. Inhibition of the hexosediphosphate $\rightarrow$ pyruvate reaction by sodium fluoride would therefore suggest conversion via phosphoglycerate, whereas tolerance of this poison would rule it out.

Exp. 70. A fresh preparation of sap was divided into 3 aliquots of 40 ml. each and the usual amounts of sulphonic acid and thymol were added.

A was made up to 50 ml. with glass-distilled water;

B with a solution of hexosediphosphate ($\equiv 40$ mg. Ba hexosediphosphate);

C with equivalent hexosediphosphate and NaF to a final concentration of $M/40$.

The three digests were incubated side by side for 48 hr. at 30° and alkali-soluble hydrazones isolated in the usual way. The results may be summarized as follows:

Table 1

	Digest	Distillation	Products
A	Sap + water	Complete below 50°	Partly crystalline, reddish. Narrow zone spindle-shaped crystals
B	Sap + hexosediphosphate	Temp. rose above 50°	Much larger and yellower deposit than A
C	Sap + hexosediphosphate + $M/40$ NaF	Complete below 50°	Quantity and colour very similar to A

The experiment suggested a marked inhibition due to sodium fluoride, but was necessarily inconclusive because the amount of product could only be determined by inspection, and because it was found that amounts of pyruvic acid added to sap could not be quantitatively recovered by the distillation. We therefore sought for means of estimating pyruvic acid formation more exactly. Lu's [1939] method, utilizing the colour of the hydrazone in strongly alkaline solution, was found to be useless because of the presence of varying amounts of other substances in the sap which gave intense colours and which could not be separated without serious loss of pyruvate. Straub's [1936] *o*-hydroxybenzylidene-pyruvate method suffered from the same disadvantage when applied to barley sap, but occasionally a sample of sap was obtained in which the interfering substances were present in relatively small amounts. Graded additions of pyruvic acid could then be added and a calibration graph constructed for that particular sap. This proved extremely laborious, since many samples of sap had to be examined before a suitable one was found. The first two suitable saps obtained gave results which confirmed the suggestion of the qualitative experiment above.

Exps. 56 and 63. The sap was prepared in the usual way, and the digests were set up as described below (see Table 2): Digest A was fixed and estimated immediately. Digests B and C were incubated for 46 hr. in the presence of thymol; they were then cleared with barium and trichloroacetic acid by the same method as was used in the isolation experiments, and each digest was made up to 10 ml. Aliquots of 4 ml. were taken from these and 4 ml. of salicylic aldehyde in alcohol and 2 ml. of 166 % KOH were added. The red colour of *o*-hydroxybenzylidene pyruvate was then developed by warming at 37° for 10 min., cooling and centrifuging exactly as described by Straub. The colorimetry was carried out with a Spekker absorptiometer, using a No. 6 (blue) screen. The results are given as arbitrary readings from the instrument, since the colour of the controls is not due to pyruvic acid. The assumption that the colour produced during incubation is in the main due to pyruvic acid is based on the results of the isolation experiments. The instrument numbers rise with increasing concentration of the coloured compound, though not necessarily in strict proportion.

Table 2

	Sap ml.	Hexosediphosphate ml.	1-Naphthol-2-sulphonic acid ml.	NaF ml.	Water ml.	Readings	
						Exp. 56	Exp. 63
A	2	1	—	—	1.0	322	298
			0.5	—	0.5	—	—
B	2	1	0.5	—	0.5	444	429
C	2	1	0.5	0.5	—	297	399

Final concentrations: NaF = $M/40$, 1-naphthol-2-sulphonic acid = 0.1 %. In Exp. 56 the hexosediphosphate added was equivalent to 3 mg. pyruvic acid and, making the necessary assumptions, the pyruvic acid estimated after incubation of digest B was about 1.2 mg.

In both the experiments the presence of NaF (digest C) has led to a smaller production of 'pyruvic acid' than occurred in its absence (digest B). In Exp. 63, inhibition was only partial at the concentration used, but in Exp. 56, at the same concentration, it was complete.

DISCUSSION

It is a commonly held belief that the mechanism of glycolysis in the higher plants is similar to that of alcoholic fermentation in yeast. The experiments described above show that barley saps are able to promote many of the reactions now believed to occur in fermentation. They do not, strictly speaking, show that these reactions occur normally in the living barley plant, but the corresponding evidence is rather scanty, even for yeast. We have, however, shown by other methods (papers quoted in the introduction) that some at least of these reactions are likely to be taking place in living barley tissues. Our experiments were all carried out aerobically and a final formation of alcohol was, therefore, not to be expected. The exact effect of oxygen is reserved for future consideration, but it is a matter of interest that these glycolytic reactions would take place in its presence.

The reactions we have actually shown to be catalysed by barley sap are indicated by the heavy side arrows in the following diagram. The formation of pyruvate from lactate differs from the other reactions in consuming oxygen [James & Cragg, 1940], whereas they only tolerate it.

The following remarks apply to the glycolytic reactions in barley systems.

Sucrose → pyruvate. In saps with low phosphate concentrations, this reaction fails to occur [Exp. 50], but it may be brought about in saps with a normal content of inorganic phosphate by the addition of adenylic acid [Exp. 68A]. Adenylic acid has not yet been isolated from barley, but the presence of a labile phosphate carrier is suggested by the fact that the organic esters contain a small readily hydrolysable fraction [Arney, 1939]. This small amount might be rendered inoperative in the extracted saps by dilution.

Glucose → pyruvate. This reaction presents the same general characteristics as the sucrose → pyruvate reaction, i.e. it will only occur if means are provided

for phosphorylation [Exp. 69]. It is therefore a reasonable assumption that both sugars are converted into phosphate esters before their actual splitting. Barley saps always contain a relatively large amount of free phosphate unless special steps are taken for its removal. We do not at present know whether this is continuously drawn upon for phosphorylation, or whether a closed cycle, excluding free phosphate, is established.

Hexosediphosphate $\rightarrow$ *pyruvate*. It is probable that a plexus of sugar-phosphate esters occurs in barley as in other plants and organisms. Hexosediphosphate is likely to be one of the esters and probably the most abundant. This is indicated by analysis of the phosphate fractions by the usual methods [James & Heard, 1940]. Added hexosediphosphate is decomposed [James & Heard, 1940] with formation of pyruvate by barley saps (Exps. 54, 56, 63, 70) without addition of adenylic acid or any other agent, even when free inorganic phosphate has been precipitated beforehand (Exp. 47). Inhibition of this reaction by $M/40$ NaF (Exps. 56, 63, 70) suggests that its course lies by way of phosphoglycerate and phosphopyruvate. Another probable stage, that involving triosephosphate, is not considered here, since the evidence collected depends on other methods.

Phosphoglycerate $\rightarrow$ *pyruvate*. Phosphoglycerate has not yet been isolated from barley. Saps breaking down hexosediphosphate in the presence of NaF accumulate a phosphate ester highly resistant to acid hydrolysis which is likely to be phosphoglycerate [James & Heard, 1940]. Added phosphoglycerate in the absence of NaF gave pyruvate (Exp. 67).

SUMMARY

1. Cell-free barley saps were prepared by a simple method. On incubation for 48 hr. at 30° in the presence of thymol and 1-naphthol-2-sulphonic acid, they gave rise to little or no pyruvic acid.
2. Addition of sucrose did not cause pyruvic acid formation. Glucose and fructose would be present in the sap itself, and therefore were also incapable of giving pyruvic acid.
3. Addition of adenylic acid to starved sap (i.e. sap low in sugar-content) gave traces of what was probably pyruvic acid.
4. Addition of sucrose + adenylic acid, or glucose + adenylic acid, led to the formation of readily identifiable quantities of pyruvic acid.
5. Hexosediphosphate and phosphoglycerate were also converted into pyruvic acid. Pyruvate formation from hexosediphosphate was markedly inhibited by $M/40$ NaF.
6. Lactic acid was oxidized to pyruvic acid in the presence of ascorbic acid and atmospheric oxygen.

These results, 1-5, indicate a conversion of sugars into pyruvic acid through a phosphorylating cycle similar, at least in outline, to the reactions of yeast.

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