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# Glycolysis by an Extract from Pea Seeds

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It appears that no complete well-characterized glycolytic system in extracts from plant tissues, capable of transforming hexoses and hexose monophosphates into pyruvic acid or carbon dioxide and ethanol, has been described.

The conversion of carbohydrate into ethanol (Beevers & Gibbs, 1954; James & Ritchie, 1955) and lactate (Barker & el Saifi, 1952) in intact plant tissues provided evidence for a functional glycolytic system. This has been supported by the identification of individual glycolytic enzymes and certain intermediates in extracts from plants (Axelrod & Beevers, 1956). More integrated systems were found by Tanko (1936) and Hanes (1940), who prepared pea-seed extracts capable of converting starch into hexose monophosphates and fructose 1:6-diphosphate (FDP). James, James & Bunting (1941) showed that the production of pyruvic acid by crude barley-leaf saps was increased by the addition of sugars, FDP and 3-phosphoglyceric acid (3-PGA). Stumpf (1950) obtained a pea-seed extract which formed carbon dioxide from FDP in the presence of arsenate by a series of reactions analogous to those of yeast and muscle glycolytic systems. However, phosphoglyceric kinase was absent and the pyruvic kinase yielded adenosine diphosphate (ADP) rather than adenosine triphosphate (ATP).

During investigations on the synthesis of sucrose from glucose 1-phosphate (G 1-P) and fructose by pea-seed extracts, evidence was obtained for the operation of a glycolytic system (Turner, 1954, 1957; Turner & Mapson, 1958). The crude pea extract was partially purified by centrifuging at 20 000 *g*, treatment of the supernatant with ammonium sulphate to 80 % saturation, suspension of the precipitated protein in water and dialysis against phosphate buffer. With hexose and hexose phosphates as substrates and with a yeast nucleotide fraction and $Mg^{2+}$ ions as cofactors, the partially purified pea extract esterified inorganic phosphate and formed FDP and carbon dioxide. For sucrose synthesis the yeast nucleotide fraction could be replaced by a mixture of diphosphopyridine nucleotide (DPN), ATP and uridine monophosphate.

In the present investigation these observations have been extended. The pea extract was capable of quantitatively converting starch, hexoses and hexose phosphates into carbon dioxide and ethanol. The results indicated that a complete glycolytic system of the Embden–Meyerhof–Parnas type was responsible for these transformations. There was no evidence for the operation of alternate pathways of hexose phosphate degradation in the pea-seed extract.

## EXPERIMENTAL

*Preparation of enzyme extracts.* The crude and partially purified pea extracts were prepared as described previously (Turner, 1957) except that the partially purified extract was dialysed against phosphate buffer at pH 6·6 instead of pH 7. The partially purified extract, which contained approx. 45 mg. of protein/ml., showed no decrease in glycolytic activity after 2 weeks at −15°.

*Substrates and cofactors.* Commercial preparations of glucose and fructose, which gave only one spot when examined on paper chromatograms with butan-1-ol–acetic acid–water (4:1:5, by vol.; Partridge, 1948) as solvent, were used.

ATP, ADP and DPN were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., and glucose 6-phosphate (G 6-P) and 3-PGA from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill. Fructose 6-phosphate (F 6-P) (Neuberg, Lustig & Rothenberg, 1943) and FDP (Neuberg & Lustig, 1942) were also prepared in the laboratory.

G 1-P was standardized by measuring the inorganic phosphate released in 7 min. in N-HCl at 100°. F 6-P, G 6-P, FDP and 3-PGA each moved as single phosphoric ester spots on paper chromatograms developed in propan-1-ol–water–aq. $NH_3$ soln. (6:1:3, by vol.; Hanes & Isherwood, 1949), and were standardized by estimating total organic phosphate. Confirmatory estimations of the fructose component of FDP and F 6-P by the method of Roe & Papadodopoulos (1954) agreed to within 5%.

The yeast nucleotide fraction was prepared as described previously (Turner, 1954, 1957).

### *Analytical methods*

*Identification and estimation of sugars.* Glucose and fructose were separated by chromatography with either propan-1-ol–water–aq. $NH_3$ soln. (16:3:1, by vol.; Turner, 1953) or ethyl acetate–propan-1-ol–water (57:32:13, by vol.; H. G. Wager, unpublished work) as solvent. The individual sugars were detected and estimated by methods previously described (Turner, Turner & Lee, 1957).

*Estimation of phosphoric compounds.* Inorganic orthophosphate was determined by the method of Allen (1940) with the modification of Turner (1957). Phosphoric esters were separated on paper chromatograms (Hanes & Isherwood, 1949) and detected by the method of Wade & Morgan (1953). FDP was estimated by cutting out the area of the spot from the chromatograms and determining the total phosphorus content by the method of Allen (1940). 3-PGA was determined as described by Umbreit, Burris & Stauffer, (1951).

*Aldolase and triose phosphate isomerase activity.* Aldolase activity was demonstrated by the method of Stumpf (1948). Triose phosphate isomerase activity was detected by measuring the ratio of the individual triose phosphates formed from FDP (Stumpf, 1948).

*Determination of ethanol.* Ethanol was distilled from the enzymic digests and estimated by the method of Peel (1951).

### *Enzyme digests*

Enzyme studies were carried out at 30° under anaerobic conditions with Warburg manometers gassed with commercial $O_2$-free $N_2$. The rate of either hexose utilization or $CO_2$ formation was used as a measure of glycolytic activity. A correction factor for $CO_2$ retention in the liquid phase (pH 6·5) was applied (Umbreit *et al.* 1951). Toluene (0·05 ml.) was added to the enzymic digests as a standard procedure. Digest samples were inactivated by the addition of trichloroacetic acid.

## RESULTS

Preliminary observations indicated that the crude pea extracts possessed glycolytic activity. Glucose and fructose were utilized with the simultaneous esterification of inorganic phosphate and production of $CO_2$. The decrease in hexoses was accompanied by increases in hexose monophosphates and FDP, indicating that mechanisms for the formation of ATP were operating. The partially purified pea extract, which was free of glycolytic substrates and cofactors, was used for the more detailed study.

*Cofactor requirement of the partially purified extract.* The partially purified extract, supplemented only with phosphate buffer, did not utilize hexoses or hexose phosphates. The capacity of the system to convert these substrates into $CO_2$ was restored by the addition of $Mg^{2+}$ ions and the yeast nucleotide fraction (Fig. 1).

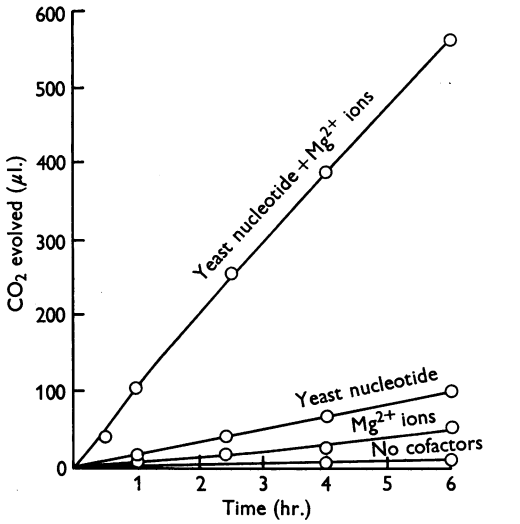


Fig. 1. Cofactor requirements of the partially purified pea extract. The complete system contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); G 1-P, 22 μmoles; fructose, 44 μmoles; $MgCl_2$, 18 μmoles; yeast nucleotide, 3·5 mg. Total vol. 1·8 ml.

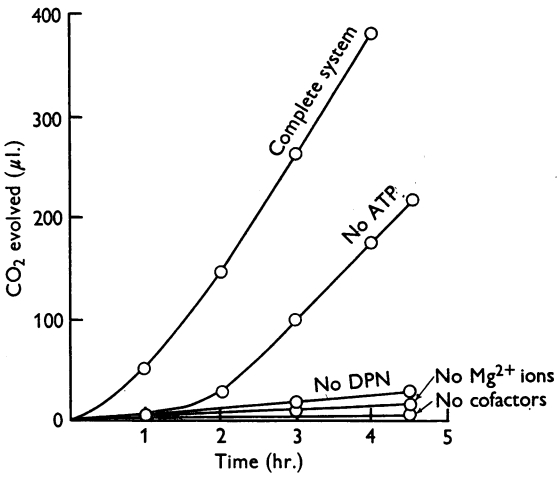


Fig. 2. The requirements for ATP, DPN and $Mg^{2+}$ ions. The complete system contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); fructose, 44 μmoles; G 1-P, 22 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·012 mM; DPN, 0·012 mM. Total vol. 1·8 ml.

A combination of ATP and DPN could replace the yeast nucleotide fraction (Fig. 2). In the absence of $Mg^{2+}$ ions or DPN, glycolytic activity was negligible. The omission of ATP greatly increased the lag period and reduced the maximum activity by about 30%. Adenosine phosphates remaining after the purification procedure may have been sufficient to fulfil partially the requirements of the system. In some experiments the partially purified extract was dialysed against water instead of phosphate buffer, and inorganic phosphate was found to be essential for the utilization of hexoses and hexose phosphates. Arsenate could replace inorganic phosphate when FDP was used as the glycolytic substrate.

*Production of carbon dioxide and ethanol from glycolytic substrates.* Glucose, fructose, G 1-P, G 6-P, F 6-P, FDP, 3-PGA and starch (the extract contained starch phosphorylase) formed $CO_2$ when incubated with the partially purified extract and the appropriate cofactors (Table 1). As shown below, ethanol was also produced, the molar ratio ethanol:$CO_2$ being approx. 1.

With fructose or glucose alone as substrate, glycolytic activity was low (Table 1). The addition of a small amount of FDP initiated maximal steady-rate glycolysis of the hexoses, but the reasons for this are not apparent. The addition of fructose increased the rate of glycolysis of FDP to the level recorded for starch and hexose monophosphates. In separate experiments it was found that arsenate (5 mM) and ADP (10 mM) also increased the rate of FDP glycolysis to the same extent. The rate of $CO_2$ evolution from 3-PGA was considerably lower than the maximum observed rate with hexoses or hexose phosphates as substrates. This, as discussed subsequently, may have been due to an accumulation of acetaldehyde which inhibits pyruvic decarboxylase.

*Hexose to fructose 1:6-diphosphate stage*

When the phosphoric esters of samples taken from enzymic digests of the composition described in Table 1 were examined on paper chromatograms, it was observed that FDP was formed from hexoses or hexose monophosphates. When FDP was incubated with the extract, small amounts of hexose monophosphates and glucose were formed, indicating the presence of phosphatases acting on FDP and glucose monophosphates.

*Fructose 1:6-diphosphate to 3-phosphoglyceric acid stage.* With cyanide as a carbonyl fixative, evidence was obtained for the conversion of FDP into triose phosphate (Table 2). Estimation of the individual triose phosphates showed that dihydroxyacetone phosphate constituted over 95% of the triose

Table 1. *Breakdown of glycolytic substrates and intermediates to carbon dioxide*

Reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); $MgCl_2$, 18 μmoles; ATP, DPN and substrates as indicated. Total vol. 1·8 ml. Glycolytic activity is expressed as an average of the first 4 hr. of steady-rate activity.

| Expt. no. | Substrate | Amount of substrate (μmoles) | ATP (mM) | DPN (mM) | Glycolytic activity (μl. of $CO_2$/hr.) |
|---|---|---|---|---|---|
| 1 | None | — | 1·5 | 0·35 | 0 |
| | Starch* | 66 | 1·5 | 0·35 | 110 |
| | Glucose | 44 | 1·5 | 0·35 | 18 |
| | Glucose | 44 | 0·75 | 0·35 | 96 |
| | FDP | 2·5 | — | — | — |
| | Fructose | 44 | 1·5 | 0·35 | 16 |
| | Fructose | 44 | 0·75 | 0·35 | 113 |
| | FDP | 2·5 | — | — | — |
| | FDP | 2·5 | 0·75 | 0·35 | 18 |
| 2 | G 1-P | 48 | 0·15 | 0·15 | 96 |
| | G 6-P | 40 | 0·15 | 0·15 | 92 |
| | F 6-P | 36 | 0·15 | 0·15 | 76 |
| 3 | FDP | 41 | 0·20 | 0·01 | 67 |
| | FDP | 62 | 0·20 | 0·01 | 64 |
| | FDP | 41 | 0·20 | 0·01 | 116 |
| | Fructose | 22 | — | — | — |
| | 3-PGA | 62 | 0·20 | 0·01 | 72 |

* An additional 14 μmoles of sodium phosphate–potassium phosphate buffer was added in this experiment. Starch concentration is expressed as glucose equivalent.

Table 2. *Evidence for the glycolytic enzymes which convert fructose* 1:6-*diphosphate into* 3-*phosphoglyceric acid*

All reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 $\mu$moles (pH 6·5); FDP, 14·5 $\mu$moles; $MgCl_2$, 18 $\mu$moles; ATP, 0·2 mM; DPN, 0·2 mM; molybdate, 2·5 mM. In addition, the system which formed triose phosphate contained cyanide, 75 mM. The system which formed 3-PGA contained pyruvate, 130 $\mu$moles; fluoride, 10 mM. Total vol. 2 ml.

| Conversion of FDP into triose phosphate | | | Conversion of FDP into 3-PGA | | |
|---|---|---|---|---|---|
| Time (min.) | FDP consumed ($\mu$moles) | Triose phosphate produced ($\mu$moles) | Time (min.) | FDP consumed ($\mu$moles) | 3-PGA produced ($\mu$moles) |
| 90 | 9·4 | 17·8 | 120 | 5·4 | 10·4 |

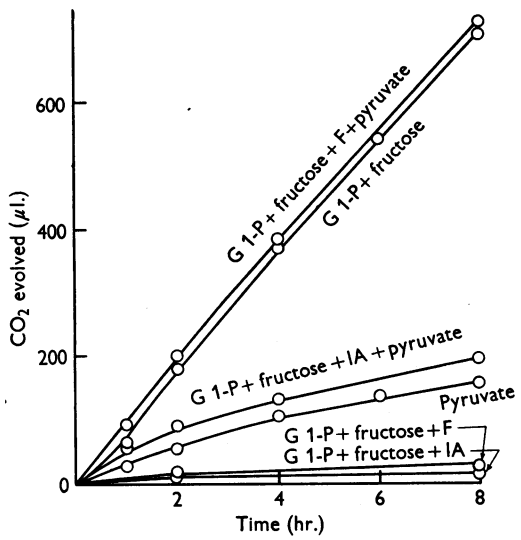


Fig. 3. Pyruvate decarboxylation and evidence for the DPN-linked coupling between triose phosphate dehydrogenase and alcohol dehydrogenase. All experiments contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 $\mu$moles (pH 7); $MgCl_2$, 18 $\mu$moles; yeast nucleotide, 3·5 mg. Additions, as shown, were: G 1-P, 22 $\mu$moles; fructose, 44 $\mu$moles; pyruvate, 108 $\mu$moles; iodoacetate (IA), mM; fluoride (F), mM. Total vol. 1·8 ml.

phosphate formed. Both aldolase and triose phosphate isomerase were therefore functional.

In the presence of fluoride (to inhibit enolase activity) and pyruvate (to allow the DPN-linked coupling of triose phosphate dehydrogenase and alcohol dehydrogenase) the formation of 3-PGA from FDP was demonstrated (Table 2). The increase in organic phosphate resistant to hydrolysis in N-HCl at 100° for 3 hr. was attributed to 3-PGA, since molybdate was added to inhibit the phosphatase acting on FDP. Molybdate has previously been used as an inhibitor of certain plant phosphatases (Bailey, Thomas & Whelan, 1951; Turner & Turner, 1957).

*Pyruvate to ethanol.* The rate of pyruvate decarboxylation by the partially purified extract was relatively low (Fig. 3), and it was suspected that this was due to the accumulation of acetaldehyde. In the presence of a system producing reduced diphosphopyridine nucleotide (DPNH), which allowed the reduction of acetaldehyde to ethanol, the rate of pyruvate decarboxylation was increased four times (Fig. 3). DPNH was generated by the addition of fructose, G 1-P and fluoride; in the presence of fluoride, fructose and G 1-P were broken down to 3-PGA and DPNH was formed. The replacement of fluoride by iodoacetate, which inhibits triose phosphate dehydrogenase (Cori, Slein & Cori, 1948) and therefore DPNH production, eliminated the increased pyruvate decarboxylation.

When excess of fructose and G 1-P were added to a digest containing pyruvate and fluoride, a rapid rate of $CO_2$ production was maintained until the pyruvate was quantitatively decarboxylated. However, in the presence of fluoride and limiting amounts of fructose and G 1-P, the rate of $CO_2$ production from pyruvate declined markedly when the amount of $CO_2$ evolved was quantitatively equivalent to the complete conversion of the added fructose and G 1-P into 3-PGA.

*Inhibitors of glycolysis.* The addition of iodoacetate (mM) and fluoride (mM) resulted in an almost complete inhibition of $CO_2$ production from fructose plus G 1-P (Fig. 3). Similar results were obtained when G 1-P, F 6-P and FDP were supplied individually as substrates. Arsenate (10 mM) completely inhibited the glycolysis of fructose plus G 1-P but increased the rate of glycolysis of FDP.

*Quantitative glycolysis of fructose, glucose* 1-*phosphate and fructose* 1:6-*diphosphate.* The glycolysis of fructose, G 1-P and FDP produced ethanol and $CO_2$ in an approximately 1:1 ratio (Table 3). Approximately 93% of the added G 1-P was recovered as ethanol, $CO_2$ and inorganic phosphate, and 85–90% of fructose and FDP were accounted for as end products. The accumulation of inorganic

Table 3. *Quantitative glycolysis of fructose, glucose 1-phosphate and fructose 1:6-diphosphate to carbon dioxide and ethanol*

Reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); $MgCl_2$, 18 μmoles; ATP, 0·35 mM; DPN, 0·35 mM; substrate as indicated. Total vol. 1·8 ml. Final measurements were made after 25 hr., when the rate of $CO_2$ production had fallen to zero in all experiments.

| Substrate added (μmoles) | | Product recovered (μmoles) $CO_2$ | Ethanol | Inorganic phosphate |
|---|---|---|---|---|
| Fructose (21) + FDP (2·5) | Calc. | 47 | 47 | 5·0 |
| | Found | 40 | 41 | 5·7 |
| G 1-P (25·2) | Calc. | 50·4 | 50·4 | 25·2 |
| | Found | 46 | 49 | 23·4 |
| FDP (25) | Calc. | 50 | 50 | 50 |
| | Found | 41 | 44 | 43·0 |

Table 4. *Adenosine triphosphate:carbon dioxide ratio*

Reaction mixture contained: enzyme, 1·2 ml.; sodium phosphate–potassium phosphate buffer, 29 μmoles; fructose 61 μmoles; FDP, 5 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·36 mM; DPN, 0·018 mM. Total vol. 1·8 ml. The duration of the experiment was 5 hr.

| Substance | Changes in digest (μmoles) | Calc. ATP consumption (μmoles) |
|---|---|---|
| $CO_2$ produced | 15·2 | — |
| Fructose consumed | 18·0 | 18·0 |
| FDP increase | 0·6 | 0·6 |
| FDP converted into $CO_2$ and ethanol | 7·6 | 7·6 |
| ATP production | Calc.* 30·4 ($ATP/CO_2 = 2$) | |
| | Found† 26·2 ($ATP/CO_2 = 1·73$) | |

* Calculated from $CO_2$ production assuming classical glycolysis.

† Calculated from the experimental data assuming that ATP consumption is equal to ATP production.

phosphate rather than ATP was probably due to the action of phosphatases.

*Reversal of glycolysis.* An attempt to bring about a complete reversal of glycolysis by the addition of pyruvate, DPN, ethanol and substrate levels of ATP was unsuccessful. Neither hexose nor hexose monophosphates were formed in detectable amounts. However, incubation of pyruvate with substrate levels of ATP resulted in considerable formation of 3-PGA. Glucose was detected on paper chromatograms after the incubation of 3-PGA with DPN and substrate levels of ethanol and ATP. It was shown previously that glucose was formed from FDP in the presence of the pea extract.

*Energetics of glycolysis: the adenosine triphosphate/carbon dioxide ratio.* In the operation of the Embden–Meyerhof–Parnas glycolytic pathway two high-energy phosphate bonds should be generated for each molecule of $CO_2$ produced. The total ATP production was calculated from the experimental data assuming that ATP consumption was equal to ATP production. In calculating the ATP consumption it was assumed that the disappearance of one molecule of fructose and the appearance of one molecule of FDP, compared with initial levels, each required the utilization of one molecule of ATP. However, the net increase in FDP recorded did not represent the amount produced by the system during the course of the experiment. There was, in addition, the FDP which was formed and then converted into $CO_2$ and ethanol. It was assumed that one molecule of ATP was required for the formation of each molecule of FDP split in this manner. In Table 4 the calculated ATP production is compared with the theoretical production calculated from the $CO_2$ evolved. It was found that 87 % of the theoretical ATP production could be accounted for, giving an $ATP:CO_2$ ratio of 1·73.

## DISCUSSION

The breakdown of starch, hexoses and hexose phosphates to carbon dioxide and ethanol by the partially purified pea extract was consistent with the operation of a glycolytic process. Additional evidence was provided by the conversion of hexoses and hexose monophosphates into FDP, FDP into triose phosphate and 3-PGA, and 3-PGA into carbon dioxide by the extract. The requirement for DPN, ATP and $Mg^{2+}$ ions, and the inhibition by iodoacetate and fluoride, also indicated that glycolysis was operating.

Axelrod & Beevers (1956), in discussing possible alternative pathways of carbohydrate metabolism in plants, suggested that the pentose phosphate cycle could function anaerobically if reduced triphosphopyridine nucleotide generated by the system were oxidized. It has been found that certain intermediates of the pentose phosphate cycle may be converted into ethanol and carbon

dioxide *in vivo* under anaerobic conditions (Axelrod & Beevers, 1956). If the breakdown of hexoses and hexose phosphates by the pentose phosphate cycle occurred anaerobically, the carbon dioxide:ethanol ratio would be greater than 1. The recovery of 93–95% of G 1-P as carbon dioxide and ethanol in a 1:1 ratio indicated that mechanisms other than the Embden–Meyerhof–Parnas glycolytic pathway were not operating to an appreciable extent in the pea extract. The almost complete inhibition of glycolysis by low concentrations of iodoacetate and fluoride supported this conclusion. These observations do not exclude the possibility that additional mechanisms of hexose phosphate breakdown may operate in the intact pea seed.

There have been a number of reports of certain intermediary glycolytic conversions catalysed by cell-free plant extracts, and of these the system of Stumpf (1950) is of particular interest. Stumpf found that FDP was converted into carbon dioxide, provided that arsenate was added, but there was no evidence that ATP was produced. During glycolysis of hexoses and hexose monophosphates by the pea extract in the present investigation, the theoretical production of two molecules of ATP per molecule of carbon dioxide was approached. This indicated that both 3-phosphoglyceric acid kinase and pyruvic kinase produced high-energy phosphate bonds. The ATP formed was then available for the initial phosphorylations involved in the glycolysis of starch, hexoses and hexose monophosphates. These processes allowed the cyclic interconversion of ATP and ADP.

The addition of fructose increased the rate of glycolysis of FDP by the pea extract to the rate observed with starch or hexose monophosphates. This suggested that the level of ADP, which is required for the action of 3-phosphoglyceric acid kinase and pyruvic kinase, may have been limiting. This was supported by the observations that arsenate, which eliminates 3-phosphoglyceric acid kinase from the glycolytic sequence (Warburg & Christian, 1939), and ADP also increased the rate of FDP glycolysis.

There was some evidence that the low rate of production of carbon dioxide from pyruvate and 3-PGA by the pea extract was due to an accumulation of acetaldehyde. Acetaldehyde is known to inhibit pyruvic decarboxylase (Lohmann & Schuster, 1937). When enolase was inhibited by fluoride, the addition of fructose plus G 1-P resulted in an increase in the rate of pyruvate decarboxylation by the pea extract. It is probable that, under these conditions, alcohol dehydrogenase and triose phosphate dehydrogenase were coupled by DPN so that DPNH produced by the latter enzyme was available for acetaldehyde reduction.

## SUMMARY

1. A partially purified extract from pea seeds, when supplemented with cofactor levels of adenosine triphosphate, diphosphopyridine nucleotide and $Mg^{2+}$ ions, catalysed the conversion of starch, hexoses, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate into carbon dioxide and ethanol.

2. mM-Iodoacetate and -fluoride inhibited glycolysis by the pea extract.

3. Approximately 93% of glucose 1-phosphate and 85–90% of fructose and fructose 1:6-diphosphate were converted into carbon dioxide and ethanol. Within experimental error the ratio carbon dioxide:ethanol was 1.

4. The following intermediary glycolytic transformations were demonstrated: hexoses and hexose monophosphates to fructose 1:6-diphosphate; fructose 1:6-diphosphate to triose phosphate and to 3-phosphoglyceric acid; 3-phosphoglyceric acid and pyruvate to carbon dioxide.

5. Evidence was obtained for the operation of cycles involving the oxido-reduction of diphosphopyridine nucleotide and the interconversion of adenosine triphosphate and adenosine diphosphate.

6. The ratio of adenosine triphosphate formed to carbon dioxide produced was calculated from the experimental data. A value of 1·73, compared with a theoretical figure of 2, was obtained.

7. Reversibility of the glycolytic process was demonstrated in two stages. Pyruvate was converted into 3-phosphoglyceric acid and 3-phosphoglyceric acid into glucose.

8. The evidence indicated that a complete Embden–Meyerhof–Parnas glycolytic system was functioning. Alternative pathways of hexose degradation were not operating to a significant extent.

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# The Metabolism of Adrenocorticotrophic Hormone and Ascorbic Acid in the Chick

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Depletion of adrenal ascorbic acid follows the administration of adrenocorticotrophic hormone in the rat or guinea pig but not in the chick (Jailer & Boaz, 1950), quail (Zarrow & Baldini, 1952) or duckling (Zarrow & Zarrow, 1950). These authors found that continued injections of adrenocorticotrophic hormone or a 'stress agent' such as stilboestrol did nevertheless produce adrenal hypertrophy in birds. They therefore concluded that the synthesis and release of adrenocortical hormones could occur without an effect on the adrenal ascorbic acid. This paper describes a further investigation of the influence of adrenocorticotrophic hormone and stress agents on ascorbic acid metabolism in the chick.

## METHODS

*Animals.* Cockerels, 6–10 weeks old, obtained from local breeders and kept in wire cages in a chicken shed for about a week before experiment, were fed on a commercial chick crumb diet mixed with 5% of dried-grass meal. The birds used were Light Sussex × Rhode-Island Red, Light Sussex × North-Holland Blue and North-Holland Blue strains. In each individual experiment only one strain was used.

Male Wistar rats, laboratory bred, of 200–300 g. body wt. were used.

Male guinea pigs, of approx. 250 g. body wt., were obtained from Allington Farm, Ministry of Supply, Porton, Wilts, and before being placed on experiment were fed with a fish-meal diet (20% of protein) (Harris, Constable, Howard & Leader, 1956) supplemented by 50 mg. of ascorbic acid/guinea pig/day (orally). When the guinea pigs reached a body weight of 300 g., enough casein (Glaxo Laboratories Ltd., Greenford, Middlesex) was added to the diet to increase the protein content to 55%; the diet was damped immediately before use.

*Adrenocorticotrophic hormone (ACTH).* Lyophilized ACTH (Armour and Co. Ltd., Chicago, Ill., U.S.A.) was injected as a solution in saline (25 i.u./2·5 ml. of 0·9% sodium chloride soln.) when a single dose was given. Since a gelatin medium is said to enhance ACTH activity when given over long periods (Wolfson, 1953) a 16% gelatin solution of the hormone [25 i.u. dissolved in 0·5 ml. of saline, followed by addition of 2 ml. of 20% (w/v) gelatin] was used when several doses were to be administered.

*Analytical methods.* All animals were killed swiftly by dislocation of the cervical vertebrae. This was followed by decapitation and exsanguination. Whole blood from the jugular vein was collected carefully into a receptable containing a few crystals of sodium oxalate. The organs to be analysed were rapidly excised and estimations carried out immediately. Ascorbic acid was estimated in the adrenals and plasma by the method of Mindlin & Butler (1937) and in whole blood and plasma by that of Roe & Kuether (1943); adrenal cholesterol by the ferric chloride method of Zlatkis, Zak & Boyle (1953) as described by Knobil, Hagney, Wilder & Briggs (1954); liver glycogen by the method of Good, Kramer & Somogyi (1933) as modified by Somogyi (1937) and blood glucose as described by King (1951).