(curve *C*, Fig. 4). It is obvious that for valid comparisons of reaction rate at different temperatures the initial slope of the reaction curve must be obtained. When this is done it is still found that the velocity of reaction reaches a maximum somewhere between 20 and 30° and that an 'optimum' temperature does not result from the onset of inactivation.

The experiments in which *Q*-enzyme was kept at 30° for some time before adding starch and testing activity confirm this lability. Thus in 2 hr. only 6% of the original activity remained. It is noteworthy that the decay of activity at 30° in the solution which contained starch throughout was much less rapid, a result which suggests that starch or its reaction product tends to stabilize the enzyme.

SUMMARY

1. Potato *Q*-enzyme has been prepared in crystalline form and with high activity by a method involving precipitation with ethanol at low temperature and low ionic strength.
2. Electrophoretic analysis has been used to follow the course of the purification.
3. Activation of the enzyme by ions has been confirmed, and the effect of temperature on the activity of the enzyme has been examined.

The authors are indebted to Prof. M. Stacey, F.R.S., for his interest in this work, and to the Royal Society and the Dunlop Rubber Co. Ltd., for grants for equipment. One of them (A. D. P.) wishes to record his thanks to the University of Birmingham for the award of an A. E. Hills research scholarship.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Barker, S. A., Bourne, E. J. & Peat, S. (1949). *J. chem. Soc.* p. 1705.
 Barker, S. A., Bourne, E. J., Wilkinson, I. A. & Peat, S. (1950). *J. chem. Soc.* p. 84.
 Bourne, E. J., Macey, A. & Peat, S. (1945). *J. chem. Soc.* p. 882.
 Bourne, E. J. & Peat, S. (1945). *J. chem. Soc.* p. 877.
 Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. & Uroma, E. (1950). *J. Amer. chem. Soc.* **72**, 465.
 Cohn, E. J., Strong, L. E., Hughes, W. L. jun., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
 Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 835.
 Gilbert, G. A. & Patrick, A. D. (1950). *Nature, Lond.*, **165**, 573, 878.
 Gilbert, G. A. & Swallow, A. J. (1949). *J. chem. Soc.* p. 2849.
 Green, D. E. & Stumpf, P. K. (1942). *J. biol. Chem.* **142**, 355.
 Halsall, T. G., Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 3200.
 Hanes, C. S. (1940a). *Proc. roy. Soc. B*, **128**, 421.
 Hanes, C. S. (1940b). *Proc. roy. Soc. B*, **129**, 174.
 Haworth, W. N., Hirst, E. L. & Isherwood, F. A. (1937). *J. chem. Soc.* p. 577.
 Haworth, W. N., Peat, S. & Bourne, E. J. (1944). *Nature, Lond.*, **154**, 236.
 Kerr, R. W. (1950). *Chemistry and Industry of Starch*, 2nd ed. New York: Academic Press.

Enzymes of the Potato Concerned in the Synthesis of Starch

2. THE SEPARATION OF PHOSPHORYLASE

BY G. A. GILBERT AND A. D. PATRICK

Department of Chemistry, The University, Edgbaston, Birmingham

(Received 10 April 1951)

Several accounts have been published of attempts to purify the starch phosphorylase of the potato. The original technique of Hanes (1940), in which the enzyme was precipitated from potato juice with

ammonium sulphate, has been modified by, among others, Green & Stumpf (1942), Meyer & de Traz (1944), Weibull & Tiselius (1945), Bourne & Peat (1945) and Barker, Bourne, Wilkinson & Peat (1950).

The degree of purity which can be reached by these methods is probably about 5 %, with respect to protein, according to an electrophoretic analysis carried out by Weibull & Tiselius (1945). The electrophoretic analysis would not, however, have differentiated proteins with the same mobility as the enzyme, but a similar estimate from ultracentrifugation experiments by these authors lent support to the figure. The protein impurity is probably mostly inert, but Barker *et al.* (1950) have shown that their preparation still retains considerable phosphatase activity, and that traces of *Q*-enzyme and amylases may be present. A threefold greater purification is reported by Sumner, Chou & Bever (1950) for the phosphorylase of jack bean after a treatment including precipitation from concentrated acetone solution, but only a low yield is claimed.

As described in Part 1 of this series (Gilbert & Patrick, 1952), *Q*-enzyme can be isolated from potato juice in good yield, and in satisfactory purity, by a method of protein purification developed by Cohn *et al.* (1946) and Cohn *et al.* (1950), based on fractionation by ethanol precipitation. Green & Stumpf (1942) and Meyer & de Traz (1944) concluded that ethanol causes complete deactivation of potato phosphorylase even at 0°, but in contradiction to this conclusion it was found that, under the conditions adopted for the preparation of *Q*-enzyme, phosphorylase was stable in the presence of considerable concentrations of ethanol, and could be prepared from the ethanol solution remaining after the precipitation of *Q*-enzyme from potato juice. This residual solution was therefore used as a starting material for the following experiments.

METHODS

Potato juice (100 ml.) was precipitated successively at 11 and 14.9 % ethanol concentration as described for the preparation of *Q*-enzyme in Part 1. The supernatant was then adjusted at -5° to a concentration of ethanol of 17.7 % by adding 0.087 ml. of 50 % (v/v) ethanol-citrate solution (0.01 M-disodium hydrogen citrate, NaOH to pH 6.0)/ml. of supernatant, with the precautions of slow addition and thorough but gentle stirring previously described. After removal by centrifugation at -5°, the precipitate was dissolved at 0° in 20 ml. of 0.01 M-citrate solution of pH 6.0 and then reprecipitated at -5° and 20 % ethanol concentration by adding 0.67 ml. of the 50 % ethanol-citrate precipitant/ml. The precipitate *A* was dissolved at 0° in 5 ml. of 0.05 M-citrate solution of pH 6.0 and freeze-dried.

The powder (about 100 mg.) was extracted, with gentle but frequent stirring, for 30 min. at -5° with 25 ml. of 20 % ethanol-citrate solution (pH 6.0, 0.01 M). After centrifugation at -5°, the insoluble residue was extracted at -2° with stirring for 30 min. with 50 ml. of 11 % ethanol solution containing 0.01 M-sodium acetate (HOAc to pH 6.0) (this solution representing a considerable decrease in ionic strength in comparison with the previously used citrate solution of the same molarity). After centrifugation at -2°,

the residue, *B*, was extracted for 30 min. with stirring at 0° with 50 ml. of freshly prepared sodium acetate solution (0.01 M, pH 6.0) containing 0.000167 M-zinc acetate. The extracted residue, *C*, was dissolved in 50 ml. of 0.01 M-citrate solution of pH 6.0 and precipitated at 0° from 22 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ by the addition of 0.79 ml. of 50 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ (pH to 6.0 with NH_3) to each ml. of solution. The precipitate was discarded and the supernatant brought to a concentration of 35 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ by the addition of 0.87 ml. of 50 % solution/ml. to give precipitate *D*. This precipitate was dissolved in 5 ml. of 0.05 M-citrate buffer solution of pH 6.0 and freeze-dried.

Modification. A higher recovery of the phosphorylase of the potato juice was obtained when the concentration of ethanol for the initial precipitation of the phosphorylase was raised from 17.7 % ethanol to 21 %, and the precipitate then washed with 30 ml. of 21 % ethanol solution containing 0.01 M-citrate of pH 6.0. The effect on the purity of the final product has not yet been studied.

Measurement of enzyme activity. (1) Phosphorylase activity was measured by the method of Green & Stumpf (1942), after slight modification of their conditions, as described in Part 1. The unit used is the weight in mg. of elementary phosphorus produced/min./ml. of test solution for a concentration of protein in the test solution of 1 mg./ml.

(2) A portion of the same solution of precipitate *D* (in 0.01 M-citrate solution, pH 6.0) that was tested for its phosphorylase activity, was tested for *Q*-enzyme and amylase activity by the method of Barker, Bourne & Peat (1949). The amylose test solution (14 ml.) contained 0.05 mg. protein. Protein N in the solution of *D* was determined by precipitation of the protein with trichloroacetic acid, followed by washing to remove $(\text{NH}_4)_2\text{SO}_4$ and Kjeldahl estimation according to Falconer & Taylor (1946). A factor of 6.25 was used to convert weight of nitrogen to weight of protein. Samples of the digest were examined after 2 and 20 hr. Control solutions were incubated (a) with water instead of enzyme solution, (b) with water instead of amylose solution.

(3) Phosphatase activity was estimated using, (a) sodium glycerophosphate or (b) glucose-1-phosphate as substrate.

(a) Enzyme solution (5 ml.) was added to 10 ml. of a buffer solution containing 0.15 M-sodium glycerophosphate and 0.2 M-disodium hydrogen citrate (pH to 6.5 with NaOH), and the solution incubated at 20°. A control solution contained water instead of enzyme solution. Free phosphate in these solutions was estimated periodically by the method of Allen (1940).

(b) Enzyme solution (12 ml.) was added to a solution prepared from 0.1 M-glucose-1-phosphate dissolved in 0.5 M-citrate-NaOH buffer of pH 6.0 (12 ml.) and 0.1 % A.R. soluble starch (4 ml.). The test solution was incubated at 20°. At intervals, 5 ml. samples were withdrawn and tested for liberated glucose by the method of Shaffer & Hartmann (1921). A known weight of glucose (about 0.2 mg.) was added to each 5 ml. sample before this test.

RESULTS

The absolute activities of the residues and precipitates, lettered as under Methods, obtained during the course of the fractionation are given in Table 1.

Table 1. *Activities of phosphorylase preparations*

Preparation	Activity (mg. P/min./ml./ mg. protein/ml.)	Yield relative to ppt. A
A	0.15	1
B	0.29	0.9
C	0.61	0.7
D	0.98	0.6

Fig. 1. Electrophoresis of potato phosphorylase after precipitation from potato juice with ethanol (precipitate A). An arrow indicates the centre of the phosphorylase boundary.

Fig. 2. Philpot-schlieren photograph of sectional electrophoresis U-tube, illustrating the separation of the component (arrow) of precipitate A with phosphorylase activity (ascending boundary A), and of the components without activity (descending boundary B).

Preparation A contains about 70 % of the phosphorylase originally in the potato juice, and about 20–30 % of the original Q-enzyme. It can be seen that precipitate D is about seven times more active with respect to unit weight of protein than preparation A.

In the test for amylases carried out on precipitate D, no increase in reducing power of the amylose test solution was detected even after incubation for 20 hr. The optical density (6800 Å.) of the amylose solution fell from 1.08 to 1.04 in 20 hr. and in the control solution from 1.05 to 1.02. There was thus no indication in these tests of the presence of either

amylases or Q-enzyme in the phosphorylase preparation.

Electrophoresis of precipitate A. Electrophoretic analysis of precipitate A was carried out in 0.1M-sodium citrate-NaOH buffer at pH 7.0 after dialysis at 0° for 18 hr. The protein migrated towards the anode and was resolved into at least four components during 138 min. (Fig. 1). The fastest of these components, which has been indicated by an arrow in the figure, represented about 6 % of the total protein, the slowest, which hardly moved, about 60 %. By adjusting the positions of the boundaries and sliding the sections of the Tiselius cell, a portion of the fast component was isolated in an electrophoretically pure state from the ascending boundary (Fig. 2). When tested for phosphorylase activity this isolated fraction was found to liberate 0.11 mg. phosphorus/ml. of test solution in 12 min. compared with 0.0024 mg. liberated by the corresponding fraction isolated from the descending boundary which had been freed only from this component. The fast component therefore contained the whole of the phosphorylase.

Fig. 3. The residual phosphorylase activity of residue B after extraction with dilute solutions of zinc acetate at pH 6.0.

The effect of extraction with zinc acetate solution on the phosphorylase activity of residue B. In the course of determining whether zinc salts could be used to effect any purification of phosphorylase preparations, residue B was extracted with 0.01M-sodium acetate solution, pH 6.0 (50 ml.), containing various quantities of zinc acetate. Determination was then made of the protein content and the activity of the extracted residues and of the solutions. It was discovered that if the concentration of zinc ion was above about $3.3 \times 10^{-5} \text{M}$ a considerable loss of activity occurred from the residue, but that between this concentration and $1.7 \times 10^{-4} \text{M}$ -zinc a constant decrease of about 20 % took place as illustrated in

Fig. 3. After the extraction procedure, only the residues were active and no activity was detected in any of the zinc solutions.

In order to throw light on these results, the activity of residue *B* from the stage before the treatment with zinc was measured in a normal test solution and also in a test solution containing 0.02M-zinc acetate. The rate of liberation of phosphorus proved to be the same, showing that the 20% loss in activity was not due to any traces of zinc retained in the precipitate during the treatment. (The citrate in the test solution prevented precipitation of the protein by the zinc.)

Fig. 4. The hydrolysis of sodium glycerophosphate by partially purified potato phosphorylase (precipitate *A*).

In spite of the loss in total activity incurred, it was decided to include the zinc treatment in the fractionation scheme since there was a net increase in activity/mg. protein (Table 1).

Tests for phosphatase. No phosphatase activity was detected in any of the precipitates or residues when these were incubated with glucose-1-phosphate, and only a normal synthesis of amylose occurred, without a measurable release of reducing sugar. However, when incubated with glycerophosphate, precipitate *A* was found to have considerable phosphatase activity. A solution of the precipitate, concentrated 2.5 times with respect to the potato juice from which it was derived, caused a 4.4% hydrolysis of 0.1M-sodium glycerophosphate to free phosphate in 290 min. This rate of hydrolysis was doubled by the addition of magnesium sulphate to 0.01M. An induction period occurred at the beginning of the reaction (Fig. 4).

Extraction with 11% ethanol (to give residue *B*) removed almost all the phosphatase, only 0.2% hydrolysis of 0.1M-sodium glycerophosphate then occurring in 240 min. The last trace of phosphatase

activity was removed (none being detected even with 0.01M-Mg⁺⁺ in the test solution) during extraction with 0.000167M-zinc acetate solution in the preparation of residue *C*.

Freeze-drying of precipitate D. At least 92% of the phosphorylase activity of this precipitate was retained during freeze-drying from solution in 0.05M-citrate buffer solution of pH 6.0. These are the conditions recommended by Barker *et al.* (1950) except for the use of a lower salt concentration.

DISCUSSION

Fractional precipitation and extraction of potato juice with ethanol, zinc acetate and ammonium sulphate has led to the preparation in reasonable yield of very active phosphorylase. This would seem to have about nine times the activity per unit weight of the preparations discussed by Weibull & Tiselius (1945). Even so, the electrophoretic analysis of precipitate *A*, and the electrophoretic and ultracentrifugation experiments of Weibull & Tiselius on their preparation, suggest that an increase in purity of at least twice remains to be achieved.

The decrease of one-fifth in the activity of phosphorylase during extraction with very dilute solutions of zinc acetate is not yet understood. It could arise from the presence of two rather similar enzymes, only one of which is inactivated by zinc, or from the displacement of an essential cation by zinc, but it cannot be due simply to the retention of traces of zinc by the extracted residue.

Amylase activity was not detected in the purified phosphorylase. The test described, however, cannot be regarded as a satisfactory criterion of purity for phosphorylase required for studies involving amylose of high molecular weight, and it becomes desirable to use measurements of molecular weight directly to prove complete freedom from amylase.

SUMMARY

1. Potato phosphorylase has been prepared by a method involving precipitation from potato juice with ethanol followed by extraction with zinc acetate and precipitation with ammonium sulphate.
2. The phosphorylase preparation has an activity about nine times greater than that of preparations previously described.
3. No phosphatase, amylase or *Q*-enzyme activity has been detected in the product.

The authors are indebted to Prof. M. Stacey, F.R.S., for his interest in this work, and to the Royal Society and the Dunlop Rubber Co. Ltd., for grants for equipment. One of them (A. D. P.) wishes to record his thanks to the University of Birmingham for the award of an A. E. Hills research scholarship.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Barker, S. A., Bourne, E. J. & Peat, S. (1949). *J. chem. Soc.* p. 1705.
 Barker, S. A., Bourne, E. J., Wilkinson, I. A. & Peat, S. (1950). *J. chem. Soc.* p. 84.
 Bourne, E. J. & Peat, S. (1945). *J. chem. Soc.* p. 877.
 Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. & Uroma, E. (1950). *J. Amer. chem. Soc.* **72**, 465.
 Cohn, E. J., Strong, L. E., Hughes, W. L. jun., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
 Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 835.
 Gilbert, G. A. & Patrick, A. D. (1952). *Biochem. J.* **51**, 181.
 Green, D. E. & Stumpf, P. K. (1942). *J. biol. Chem.* **142**, 355.
 Hanes, C. S. (1940). *Proc. roy. Soc. B*, **129**, 174.
 Meyer, K. H. & Traz, C. de (1944). *Helv. chim. Acta*, **27**, 840.
 Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **45**, 377.
 Sumner, J. B., Chou, T. C. & Bever, A. T. (1950). *Arch. Biochem.* **26**, 1.
 Weibull, C. & Tiselius, A. (1945). *Ark. Kemi Min. Geol. A*, **19**, 1.

The Component Acids of the Milk Fat of a Grey Atlantic Seal

By M. L. MEARA

Department of Industrial Chemistry, University of Liverpool

(Received 5 October 1951)

The milk fats of many land animals are characterized by the presence of fatty acids of lower molecular weight than those obtaining in their respective depot fats. This phenomenon is most marked in the case of the milk fats of the ruminants which contain appreciable amounts, especially when expressed as a molar percentage, of the lower even-membered saturated acids from butyric to myristic, which are either absent or occur only in small amounts in their respective depot fats. It is not so pronounced, however, in the case of the milk fats of other herbivora, e.g. horse and ass, while sow milk fat has been shown (De la Mare & Shorland, 1944) to resemble closely that of a normal pig depot fat. The Reichert and Polenske values recorded for milk fats of a number of other animals (e.g. cat, dog and mouse) also indicate the absence therefrom of short-chain acids.

Little, however, is known of the composition of the milk fats of marine animals. Klem (1935) has recorded data for various species of whales, and indicated that the fat content of the milk ranged from 19 to 46 %, thus far exceeding that found in the milk of land animals (usually 3–6 %). He also noted that the mean unsaturation of the milk fat of a blue whale was considerably higher (iodine value 171.8) than that of the fat from any other site in the same animal (iodine values 94.0–102.8), though these latter values are considerably lower than the values obtained for fat obtained from the same sites in healthy males and non-lactating females of the same

species (iodine values 120–140). He further showed that the blue whale milk fat contained the following acids: myristic 8.4, palmitic 16.8, stearic 1.8, unsaturated acids C_{14} 1.2 (–2.0H), C_{16} 6.2 (–2.0H), C_{18} 26.8 (–3.3H), C_{20} 25.9 (–8.5H), C_{22} 12.9 (–11.0H) % (w/w). Similarly, Schmidt-Nielsen & Frog (1933) showed that there are no acids of lower molecular weight than lauric acid present in the milk fat of the finner whale.

An opportunity for further study of the milk fat of a marine animal was made possible by the kindness of Prof. E. C. Amoroso, Dr S. J. Folley, F.R.S., and Dr S. J. Rowland, who placed a small quantity of the milk of a grey (Atlantic) seal (*Halichoerus grypus*), caught off the Pembrokeshire coast in 1950, at the disposal of this laboratory.

METHODS AND RESULTS

The milk was pale cream in colour, of a thick viscous consistency and had a pronounced fishy odour. On extraction of 235 g. milk with light petroleum (b.p. 40–60°) (emulsions readily broken by addition of ethanol) 118 g. (50.0 %, w/w) of a pale oil, saponification equivalent 295.9, iodine value 146.2, unsaponifiable matter 1.5 %, was obtained. The fat (101 g.) gave 95.2 g. mixed fatty acids, after saponification and acidification of the soaps. The steam distillate from the mixed fatty acids required 1.8 ml. 0.1 N-KOH to neutralize the steam volatile acids, this being equivalent to 0.03 g. decanoic acid (or a proportionally smaller amount of the lower steam-volatile acids) indicating the virtual absence of this group of fatty acids.