Paper ionophoresis of inositol phosphates, with a note on the acid hydrolysates of phytic acid

The use of paper ionophoresis for the fractionation of inositol phosphates has some advantages over anion-exchange column[1] and paper chromatographic[2] techniques. In particular the ionophoretic fractionation is simple to operate and especially suited for micro-quantitative work. The main shortcoming of the method is that the hexa- and penta-phosphates of inositol migrate at similar rates and are therefore determined together.

The ionophoresis was carried out on filter paper supported between plate-glass sheets, using an apparatus essentially the same as that described by FOSTER[3]. Plate-glass sheets measuring 57 × 25 cm were used because rather long migrations were necessary to effect the separation of the esters. Strips of acid-washed[4] Whatman No. 3 filter paper 71 × 8 cm were found to be suitable for single runs which, for quantitative work, were preferable to multiple runs on wide strips of paper. Solution containing 10–150 μg P was delivered from a micro-pipette to a point midway along a line 10 cm from one end of the paper, care being taken to see that the wetted area did not exceed 0.5 cm diameter. The ionophoresis was carried out in buffer made from 0.2 *N* acetic acid and 0.2 *N* sodium acetate in the ratio 10 vols: 1 vol (pH 3.65). It was convenient to be able to adjust the voltage of the current supplied by a power pack unit so that the apparatus could be left overnight. In order to locate the phosphate esters, the paper was dried and sprayed with an acid-molybdate reagent (5 ml 60% w/w $HClO_4$, 20 ml *N* HCl, 25 ml 4% w/w ammonium molybdate, water to 100 ml) similar to that used by HANES AND ISHERWOOD[4]. After spraying, the paper was heated to 85° C in an oven for 8 minutes and then exposed to ultra-violet radiation for 2–4 minutes as described by BANDURSKI AND AXELROD[5]. Whereas inorganic ortho-phosphate gives a yellow colour, clearly visible after the initial spraying, the organic phosphorus spots (and inorganic ortho-phosphate) appear at the end of the treatment as blue regions which reach peak intensity after standing in diffuse daylight for 2 hours.

With the aid of excellent specimens of inositol hexa-, penta- and tetra-phosphates kindly supplied by Professor J. COURTOIS and with inositol mono-phosphate prepared from sodium phytate* by POSTERNAK's method[6], it was shown that mono-, tetra- and penta-phosphates could be separated in micro-gram quantities. The hexa- and penta-phosphates migrated at similar rates and could not be separated from each other. Using partially hydrolysed phytic acid (see below) two additional organic phosphorus-containing spots were found lying between the inositol mono- and tetra-phosphate spots with the slower moving one coincident with the inorganic ortho-phosphate spot. Assuming that the two unidentified spots were due to di- and tri-phosphate esters and that the tri-ester migrated further than the di-ester, then, when the hexa- and penta-phosphates are allowed to migrate 50 cm (220 volts for 16 hours at room temperature) the other esters will be located at the following distances from the origin:

Mono-	21.0 cm
Di- and inorg. ortho-	32.0 cm
Tri-	42.0 cm
Tetra-	47.5 cm
(Penta- and hexa-	50.0 cm)

Overloading the paper must be avoided in order to obtain satisfactory resolution of the tetra- and penta- plus hexa-phosphate esters.

Hydrolysis of phytic acid (1, 2, 3, 5/4, 6 inositol hexa-phosphate) at pH 3.0

Commercial sodium phytate* was purified by precipitating the phytate as ferric phytate at pH 1.0 (HCl) with $FeCl_3$. The precipitate was washed with large volumes of distilled water. Iron was removed as $Fe(OH)_3$ by adding excess NaOH to an aqueous suspension of the ferric phytate. The whole operation was repeated twice. Finally, hydrogen-saturated Zeokarb 215 was used to bring the pH below 3.0 and the solution was back-titrated to pH 3.0 with NaOH. The resulting solution contained 5.3 mg/ml of organic phosphorus and only trace amounts of inorganic phosphate. Ten ml portions of this stock solution were hydrolysed in sealed glass tubes at 120 ± 1° C for various periods of time ranging from 78 to 240 minutes to yield a series of partially dephosphorylated phytic acid solutions (Fig. 1) *cf.* FLEURY[7].

Suitable aliquots (5–30 μl) of the partially hydrolysed phytic acid solutions were subjected to paper ionophoresis as described. After spraying with the acid-molybdate reagent the yellow zones containing inorganic and inositol di-phosphate phosphorus were cut out with clear margins of 1.0 to 1.5 cm and allowed to dry prior to analysis. The remainder of each paper was treated as described above to reveal the organic phosphorus spots which were cut out. Each phosphorus-

* The sodium phytate was a gift kindly presented by Ciba Laboratories, Ltd.

containing section of the paper was extracted with 10 ml N NH_4OH by standing for 6 hours. Aliquots of the resulting solutions were transferred to micro-Kjeldahl flasks and concentrated to small volume. 0.5 ml 3:2 H_2SO_4: 60% $HClO_4$ was added and the solutions were digested for 3–4 minutes. Total phosphorus was determined by a modified BERENBLUM AND CHAIN[8] method as described by BARTLEY[9]. Inorganic phosphate was determined in the appropriate non-digested solutions so that figures for inositol di-phosphate ester phosphorus were obtained by difference (total — inorganic).

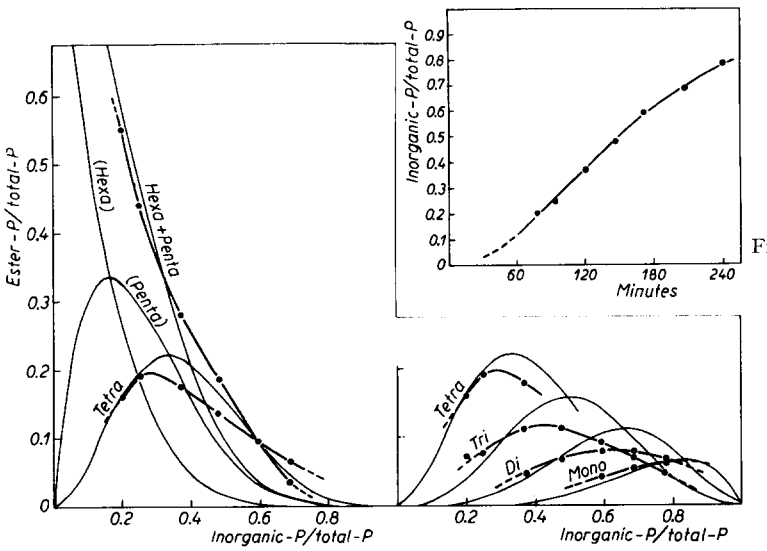

Fig. 1.

Fig. 2.

Fig. 1. Inorganic phosphorus release curve for phytic acid at pH 3.0 (120° C).

Fig. 2. Experimental and theoretical curves showing the ester-phosphorus distribution during the hydrolysis of phytic acid at pH 3.0 (120° C).

———— Theoretical curves

Six consecutive first order reactions. One value for k.

$$\begin{cases} \text{Inorg. P} = 1 - e^{-kt} \\ \text{Hexa-P} = e^{-6kt} \\ \text{Penta-P} = 5e^{-5kt}(1 - e^{-kt}) \\ \text{Tetra-P} = 10e^{-4kt}(1 - e^{-kt})^2 \\ \text{Tri-P} = 10e^{-3kt}(1 - e^{-kt})^3 \\ \text{Di-P} = 5e^{-2kt}(1 - e^{-kt})^4 \\ \text{Mono-P} = e^{-kt}(1 - e^{-kt})^5 \end{cases}$$

-●—●- Experimental values.

In Fig. 2 the experimental results are recorded as a family of curves showing the changes in the ester-phosphorus distribution (hexa- plus penta-, tetra-, tri-, di- and mono-phosphates) as the hydrolysis proceeds. Whatever the mechanism of the hydrolysis, first order kinetics are probably followed as in other similar solvolytic reactions[10]. It is, however, unlikely that there is a single value for the rate constant in the successive stages of the dephosphorylation. This is substantiated by examination of Fig. 2 in which the experimental results are compared with a family of theoretical curves showing the ester-phosphorus distribution which would arise from six consecutive first order reactions with a single rate constant. From a qualitative comparison of the experimental and theoretical families of curves it is concluded that, although values for the rate constants in the successive stages of the dephosphorylation are all of similar magnitude, the tetra-, tri- and di-phosphates are slightly less stable than the hexa- (and/or penta-) phosphate. Furthermore, the comparative forms of the experimental and theoretical distribution curves for the tetra-, tri- and di-phosphate phosphorus indicate that some of the isomers which are produced are probably more resistant than others to hydrolysis. This is most clearly seen in the tetra-phosphate curves in which, during the late stages of the hydrolysis, the experimental phosphorus values do not decrease as rapidly as the theoretical values. A similar trend is shown by the tri- and di-phosphate curves. Any fuller interpretation of the data is unjustified because there is

no guarantee that the phytic acid used in the experiments was free from small amounts of inositol penta-phosphate which would be difficult to detect by chemical analysis. The fact that in the early stages of the hydrolysis the experimental values for hexa- plus penta-phosphate phosphorus are less than the theoretical values may possibly indicate that some inositol penta-phosphate was present in the original phytic acid.

The writer wishes to thank Dr. R. K. SCHOFIELD and Mr. M. J. R. HEALY for their help with the interpretation of the data.

P. W. ARNOLD

Rothamsted Experimental Station, Harpenden, Herts. (England)

1 D. H. SMITH AND F. E. CLARK, *Soil Sci.*, 72 (1951) 353.
2 G. ANDERSON, *Nature*, 175 (1955) 863.
3 A. B. FOSTER, *Chemistry & Industry*, (1952) 1050.
4 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
5 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
6 M. H. MCCORMICK AND H. E. CARTER, *Biochem. Preparations*, 2 (1952) 65.
7 P. FLEURY, *Compt. rend.*, 221 (1945) 416.
8 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
9 W. BARTLEY, *Biochem. J.*, 54 (1953) 677.
10 P. W. C. BARNARD *et al.*, *Chemistry and Industry*, (1955) 760.

Received December 7th, 1955

Functions of ribonucleic acid in liver cytoplasm

There is evidence that cytoplasmic ribonucleic acid (RNA) plays an important role in protein synthesis[1,2]. That this may not be its only role is suggested by the following observations, made in the course of a study[3] of hormonal influence on the incorporation of labelled precursors into the protein and RNA of liver cytoplasm.

Adrenalectomized rats (males of about 220 g body weight) maintained on saline, and control rats kept on the same food intake, were given 8 μc of orotic acid-6-^{14}C by intraperitoneal injection. After 2 hours, at which time the radioactivity of cytoplasmic ribonucleic acid had not reached a "plateau" (*cf* [4]), the livers were removed and homogenized in 0.25 *M* sucrose solution. Differential centrifugation was then performed, essentially as previously described[5]. The fractions thus isolated were treated with cold trichloracetic acid solution, defatted, dried, and ground to a powder suitable for the determination of radioactivity with a thin-window counter.

Similar experiments were performed with DL-leucine-1-^{14}C in place of orotic acid. The dose was 20 μc, given 1 ½ hours before autopsy.

In the case of the mitochondrial fraction, and of the overlying "fluffy layer" (which was analysed separately), the only significant effect of adrenalectomy was a reduction in the amount of leucine incorporated into the protein of the mitochondrial fraction (the value being calculated as percentage recovery of injected isotope). The amount of orotic acid incorporated into the RNA of the crude "nuclear" fraction tended to be low after adrenalectomy.

In the case of the microsomal fraction, of the supernatant fraction, and of a "sub-microsome" fraction derived from the latter by ultracentrifugation, the effects of adrenalectomy were striking (Table I). With the microsomal fraction the incorporation of orotic acid was reduced, whereas with the ultracentrifugal fraction and particularly with the whole supernatant fraction the incorporation was markedly increased. The RNA of the supernatant fraction is known to increase in amount after adrenalectomy[5], but nevertheless its actual specific activity was definitely increased in the present experiments with orotic acid.

As further shown in Table I, the incorporation of leucine was significantly lowered by adrenalectomy in the case of the microsomal fraction, and tended to be low in the case of the supernatant and ultracentrifugal fractions.

With the microsomal fraction, which is believed to be of particular importance in protein synthesis[1], the data of Table I thus suggest that there is a decrease in the rate of synthesis both of protein and of RNA after adrenalectomy. Since no such correlation is evident with the supernatant fraction, the RNA of this fraction probably has, at least in part, some function unconnected with protein synthesis. The marked divergence between the microsomal fraction and the supernatant fraction, with respect to the effect of adrenalectomy on the incorporation of orotic acid, would be difficult to explain merely in terms of an effect of adrenalectomy on "pool" size, or