Heterogeneity of the calcium-dependent phosphatidylinositol phosphodiesterase in rat brain

Keisuke HIRASAWA, Robin F. IRVINE and Rex M. C. DAWSON

Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

(Received 17 March 1982/Accepted 28 April 1982)

1. The Ca^{2+} -dependent phosphatidylinositol phosphodiesterase (phospholipase C-type) from the cytosolic supernatant of rat brain was active against exogenous [^{32}P]-phosphatidylinositol from pH 5.0 to pH 8.5. However, the activity in the range pH 7.0–8.5 could not be recovered after precipitation with $(\text{NH}_4)_2\text{SO}_4$; most of the enzyme activity was recovered in the 30–50% fraction and showed a single sharp pH optimum at 5.5. 2. The cytosolic supernatant was analysed by isoelectric focusing on acrylamide gels, and assay at pH 5.5. Four peaks of phosphodiesterase activity were found at pI ranges 7.4–7.2, 6.0–5.8, 4.8–4.4 and 4.2–3.8. 3. The cytosolic supernatant was also applied to a chromatofocusing column, and again assayed at pH 5.5. Four peaks were eluted: minor, but consistent, activity at the beginning of the elution with a pI of near 7.2 or above; a second peak at pH 6.0–5.85; a third broad peak with a wide range pH 5.3–4.2; and a fourth peak, which was eluted by washing the column with 1 M-NaCl, suggesting an isoenzyme with a pI below 4.0 (supported by the result of the isoelectric focusing). 4. If all the chromatofocusing fractions were assayed at pH 7.0 or 8.0 (at 1 mM- Ca^{2+}), only a single sharp peak was detected, with a pI of 4.6–4.8. This peak disappeared on $(\text{NH}_4)_2\text{SO}_4$ fractionation (30–50%) of the cytosolic supernatant, whereas the four peaks with activity at pH 5.5 were virtually unaffected. 5. The four activities (assayed at pH 5.5) separated by chromatofocusing produced inositol 1:2-cyclic monophosphate, inositol 1-monophosphate and diacylglycerol as enzymic products. 6. We conclude that the Ca^{2+} -dependent phosphatidylinositol phosphodiesterase exhibits considerable heterogeneity, both with respect to pH optima of activity, and its isoelectric properties.

The high turnover rate of phosphatidylinositol observed in many tissues, especially during physiological activity (Dawson, 1954; Michell, 1975; Irvine *et al.*, 1982), is generally accepted to be due to the hydrolysis of this phospholipid by the Ca^{2+} -dependent phosphatidylinositol phosphodiesterase, which produces inositol 1:2-cyclic phosphate, inositol 1-phosphate and diacylglycerol (Dawson *et al.*, 1971). This enzyme has been demonstrated in various mammalian tissues, for example sheep pancreas (Dawson, 1959), rat liver (Kemp *et al.*, 1961), guinea-pig intestinal mucosa (Atherton & Hawthorne, 1968) and brain (Thompson, 1967).

Some evidence for at least two distinct pH optima of enzyme activity has been presented in lymphocytes and rat brain if the enzyme is assayed at 1 mM- Ca^{2+} , but only one peak is observed if the [Ca^{2+}] is buffered to 1 μM (Allan & Michell, 1974; Hirasawa *et al.*, 1981). Kemp *et al.* (1961) have also

reported some evidence for two pH optima in the liver enzyme, a major one at 5.7 and a minor one at 6.9. On the other hand, only one peak of activity was observed even with [Ca^{2+}] at 1–2 mM, at pH 5.5–5.8 in rat brain (Thompson, 1967), guinea-pig intestinal mucosa (Atherton & Hawthorne, 1968), ox brain (Keough & Thompson, 1972) and rabbit smooth muscle (Abdel-Latif *et al.*, 1980), and at pH 7 for the enzyme purified from rat liver (Takenawa & Nagai, 1981). Although some of these inconsistencies can be resolved on the basis of Ca^{2+} /substrate concentration ratios (Hirasawa *et al.*, 1981), there is still a dichotomy between apparently identical assays on different tissues, for example, the results of Hirasawa *et al.* (1981) compared with those of Abdel-Latif *et al.* (1980) or of Atherton & Hawthorne (1968), or even the same tissue [the results of Thompson (1967) compared with those of Hirasawa *et al.* (1981)]. Here the only explanation

can be a difference in enzyme preparation, and in the present paper we confirm that this is so; the activity at an alkaline pH is lost if the enzyme is submitted to an $(\text{NH}_4)_2\text{SO}_4$ fractionation as employed by Abdel-Latif *et al.* (1980), Thompson (1967), Atherton & Hawthorne (1968) and Takenawa & Nagai (1981).

We have also examined the heterogeneity of the phospholipase by standard isoelectric focusing, and by a new chromatofocusing technique with an ampholyte-containing pH gradient buffer that has recently become commercially available. As well as providing information on the heterogeneity of the phosphatidylinositol phosphodiesterase, these techniques have shed further light on the effect of $(\text{NH}_4)_2\text{SO}_4$ precipitation on the pH-versus-activity profile of the enzyme.

Materials and methods

Preparation of ^{32}P - and non-labelled phosphatidylinositol

[^{32}P]Phosphatidylinositol was prepared as described by Hazlewood & Dawson (1975) and Irvine *et al.* (1978). Non-radioactive phosphatidylinositol was purified from baker's yeast by the same method.

Assay system for the activity of Ca^{2+} -dependent phosphatidylinositol phosphodiesterase

Unless specified otherwise, the assay mixture contained 50 mM-Tris/maleate buffer, pH 5.5, 129 nmol of a mixture of ^{32}P - and non-labelled phosphatidylinositol, 1 mM- CaCl_2 and the enzyme fraction, in a final volume of 0.5 ml.

^{32}P - and non-labelled phosphatidylinositol mixed to the desired concentration was dispersed in the buffer, by shaking. The reaction mixture was incubated for 30 min at 37°C, and the reaction was terminated by three different methods. (a) When total water-soluble products were required, 0.05 ml of 5% bovine serum albumin followed by 0.5 ml of 20% trichloroacetic acid were added to the reaction medium and radioactivity in the supernatant was measured (Irvine *et al.*, 1979). (b) When lysophosphatidylinositol production was measured, the reaction was terminated by the addition of water-saturated n-butanol (1 ml) (Bjerve *et al.*, 1974). The butanol phase was dried and applied on t.l.c. plates; the plates were developed with chloroform/methanol/conc. NH_3 /water, (65:35:2:3, by vol.). (c) For separation of individual water-soluble products (inositol 1:2-cyclic phosphate, inositol 1-monophosphate and glycerophosphoinositol) the reaction was terminated by the addition of excess chloroform/methanol (1:1, v/v) and the water-soluble radioactivity was separated by ionophoresis at pH 3.6 (Dawson & Clarke, 1972).

Enzyme preparation

Female albino rats weighing approx. 200 g were used. The rats were killed by decapitation, the cerebral hemispheres homogenized in 9 vol. of 0.25 M-sucrose, or, for chromatofocusing, in 50 mM-imidazole/HCl buffer, pH 7.4, containing 0.25 M-sucrose and 1 mM-dithiothreitol. The homogenate was centrifuged at 100 000 g for 60 min at 4°C. For some experiments the cytosolic supernatant was fractionated by the addition of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to 0–30, 30–50 and 50–80% saturation, as described by Thompson (1967). Unless stated otherwise, the precipitates were resuspended in 0.25 M-sucrose in 5 mM-Tris/HCl buffer, pH 7.2, and dialysed against the same buffer to remove $(\text{NH}_4)_2\text{SO}_4$.

Isoelectric focusing of proteins on polyacrylamide gels

Isoelectric focusing was carried out in 5% polyacrylamide gels [mixture of acrylamide and methylbisacrylamide (323:10, w/v)] containing 5% (v/v) ampholyte (Ampholine; LKB, Bromma, Sweden; mixture of pH interval range 2.5–11), 0.025% (v/v) $\text{NNN}'\text{N}'$ -tetramethylethylenediamine (BDH) and $1.8 \times 10^{-4}\%$ (w/v) riboflavin. Before making the thin-layer-plate gel (0.1 cm $\times$ 12.5 cm $\times$ 12.8 cm), the gel mixture was degassed, the gel solution polymerized under a u.v. lamp for approx. 3 h, and left in a moist atmosphere at room temperature overnight.

Thick paper ends were soaked in anode (1 M- H_3PO_4) and cathode (1 M-NaOH) solutions and placed on the gel at each end, 9.5 cm apart. The plate was prefocused at 500 V for 15 min using an LKB Basic Unit and LKB power supplier no. 2103. The sample was loaded on thick filter paper (Whatman 3 mm) 0.3 cm inside from the cathode. A constant power supply of 10 W was applied for 2 h, and then the gel was sliced with a razor blade into sixteen 5 mm lengths. The slices of gel were incubated in the enzymic assay reaction mixture for 20 h and then bovine serum albumin and trichloroacetic acid were added as described above. Another 16 slices were eluted in water from a gel run parallel to the one above to measure the pH, and a further gel from a parallel run was stained with 0.2% (w/v) Coomassie Brilliant Blue. Destaining was performed by frequent changing of a solution [water/ethanol/acetic acid (50:3:1, by vol.)] surrounding the gel.

Chromatofocusing columns

Chromatofocusing is a technique for separating proteins on the basis of their isoelectric points, by sequential elution from a column using an ampholyte-containing buffer; the pH of the eluent gradually decreases, and proteins leave the column when the pH of the eluent is the same as their isoelectric point.

The basic principles and procedures of this technique have recently been published as a booklet (Chromatofocusing with Polybuffer and PBE) by Pharmacia Fine Chemicals, Uppsala, Sweden. We have modified the recommended methods as follows. The column (C10/40; Pharmacia Fine Chemicals) was packed with 22 ml of Polybuffer exchanger PBE94 (Pharmacia Fine Chemicals), bed height 28 cm, and equilibrated by running through 400 ml of the starting buffer (50 mM-imidazole/HCl buffer, pH 7.4, containing 1 mM-dithiothreitol and 10 mg of Polyethyleneglycol 4000/ml). The enzyme (cytosolic supernatant) with 10 mg of Polyethyleneglycol 4000/ml added was applied to the column and eluted with a pH gradient solution prepared by a 4-fold dilution of Polybuffer 74/HCl, pH 4.0 (to give a 0.025 pH unit change/ml of eluent), containing 1 mM-dithiothreitol and 10 mg of Polyethyleneglycol 4000/ml. After eluting with 170 ml of elution buffer, the eluent reached a pH of 4.0, and then the elution buffer was switched to 1 M-NaCl solution to recover protein with a pI value below 4.0. All through the experiment the flow rate was $35 \text{ cm} \cdot \text{h}^{-1}$ and fractions (6.0 ml) were collected in 1 ml of the collecting buffer, 50 mM-imidazole/HCl, pH 7.4. This collecting buffer prevented the denaturation and precipitation of the fractionated proteins that resulted from them being kept at their isoelectric points.

Results

Effect of the $(\text{NH}_4)_2\text{SO}_4$ fractionation of cytosolic supernatant on the pH-activity profile

The activity of the Ca^{2+} -dependent phosphatidylinositol phosphodiesterase in a cytosolic brain supernatant showed a broad pH range at 5.0–8.5 (Hirasawa *et al.*, 1981).

When $(\text{NH}_4)_2\text{SO}_4$ fractionation (30–50%) of the brain supernatant was carried out as described by Thompson (1967), the phosphodiesterase preparation obtained showed an optimum at pH 5.6 with little activity above pH 7 (Fig. 1). Activity at alkaline pH could be detected in neither the 0–30% nor 50–80% saturation fractions after dialysis against 5 mM-Tris/maleate buffer, pH 7.2 (Fig. 1). These findings suggest that the Ca^{2+} -dependent phosphatidylinositol phosphodiesterase could have at least two enzyme forms with different stabilities under the conditions of $(\text{NH}_4)_2\text{SO}_4$ precipitation. Similar results were obtained when the experiments were repeated with a supernatant preparation from beef brain.

Isoelectric focusing on polyacrylamide gel

The cytosolic supernatant was separated by isoelectric focusing (range pH 7.7–3.5), as used by many workers for analysis of isoenzymes (for example, Warner *et al.*, 1978; Ingelman-Sundberg &

Fig. 1. Effect of pH on the hydrolysis of $[\text{32P}]$ phosphatidylinositol by the cytosolic supernatant and its $(\text{NH}_4)_2\text{SO}_4$ fractions

The incubation system is described in the Materials and methods section with the pH being varied with a series of 50 mM-Tris/maleate buffers. The water-soluble $[\text{32P}]$ phosphate esters released were measured in a trichloroacetic acid supernatant. Curves show the pH activity profiles of the cytosolic supernatant enzyme (●), and $(\text{NH}_4)_2\text{SO}_4$ fractions (0–30%) (■), (30–50%) (▲) and (50–80%) (△).

Fig. 2. Isoelectric focusing of the cytosolic supernatant on a polyacrylamide gel

The conditions are described in the Materials and methods section. The sliced gels were incubated in the reaction mixture, and the pH of the mixture was not altered more than ± 0.2 after the addition of the gel. The $[\text{32P}]$ phosphatidylinositol hydrolysis (●) was measured as trichloroacetic acid-soluble radioactivity (see the Materials and methods section). The pH gradients (○) were measured on elution of the sliced gel with water. The stained gel illustrated was run in parallel with that assayed for enzyme activity using the same cytosolic supernatant, and is therefore an exact match with the pH and activity profiles.

Gustafsson, 1977). If assayed at pH 5.5 the activity of Ca^{2+} -dependent phosphatidylinositol phosphodiesterase showed four main peaks at pI about 7.4–7.2, 6.0–5.8, 4.8–4.4 and 4.2–3.8 (Fig. 2). These activities were determined by incubation of the sliced gel with radioactive substrate (see the Materials and methods section).

Inositol 1:2-cyclic phosphate and inositol 1-phosphate were produced by each of the four peak fractions. The predominance of the peak at pI 5.8 may be partly because of the necessity for prolonged incubation of the gels (20h); this fraction may be more stable under these conditions (compare the results in the next section).

Chromatofocusing of the cytosolic supernatant

Chromatofocusing separates proteins on the basis of their isoelectric points (see the Materials and methods section) and in theory therefore should give a similar pattern to isoelectric focusing on poly-

acrylamide gels. The pH gradient of the eluent was linear, and pH intervals of ± 0.05 could be obtained. By doubling the concentration of Ampholine buffer to twice as much as that recommended, we obtain highly reproducible resolutions of enzyme fractions between different experiments. Also, the addition of 10mg of Polyethyleneglycol 4000/ml helped ensure consistent results, perhaps by preventing proteins aggregating at their isoelectric points on the column.

The first small peak (fractions 3–6) of the Ca^{2+} -dependent phosphatidylinositol phosphodiesterase activity on chromatofocusing (Fig. 3a) indicated that some of the enzyme had an isoelectric point at 7.0 or above. This probably corresponds to the isoelectric focusing peak at pI 7.2 (Fig. 2). The activity was small, but was always present, and was often resolved into two peaks showing distinct activities when the enzyme was assayed at pH 5.5 and 7.0. In Fig. 4(a), the pH-activity profile of this first peak obtained after concentration of the enzyme

Fig. 3. *Chromatofocusing of the rat brain supernatant, and the $(\text{NH}_4)_2\text{SO}_4$ (30–50%) fraction* (a) and (b) show the elution patterns of the cytosolic supernatant and the $(\text{NH}_4)_2\text{SO}_4$ (30–50%) fraction from this respectively. —, A_{280} ; the activity is presented when assayed at pH 5.5 (●), pH 7.0 (△) and pH 8.0 (▲) in the presence of 1 mM- Ca^{2+} . ---- shows the pH measurement of the eluent (for experimental details see the Materials and methods section).

is shown; there are two optima at pH 5.5 and 7.5. The second peak (Fractions 11–13) appeared in the range pH 6.0–5.85 and showed a pH optimum of only 5.5 (Fig. 4*b*). The products of the activity were inositol 1:2-cyclic phosphate and inositol 1-phosphate (Table 1). The third peak (Fractions 16–25)

showed a wide pI range 5.3–4.2 when assayed at pH 5.5 but only one sharp peak at pI 4.6 when assayed at pH 7.0 and pH 8.0. Fraction 21 gave a similar activity curve (Fig. 4*c*) to the original cytosolic supernatant shown in Fig. 1 and the enzymic products are shown in Table 1. The EDTA-insensitive lysophosphatidylinositol-producing activity (Hirasawa *et al.*, 1981) had a pI around 4.35–4.25 (Table 1).

A fourth phosphatidylinositol phosphodiesterase peak (Fractions 31–35) had an isoelectric point below 4.2, or was bound to the column strongly; it was not eluted by the Ampholine buffer, but was moved by 1 M-NaCl. There is a possibility that this fourth peak could be a mixture of the three previous peaks which have stuck to the column. However, the evidence from the isoelectric focusing on polyacrylamide-gel experiments suggested that a fourth peak of enzyme activity exists with a pI range 3.8–3.65 (see above). Also in further experiments (results not shown) employing a narrow range (pH 4.5–2.5) isoelectric focusing on polyacrylamide, there was enzyme activity at pH 3.85–3.6. This fourth chromatofocusing peak showed a major pH optimum at 5.5 (Fig. 4*d*).

Chromatofocusing of (NH₄)₂SO₄ (30–50%) fraction of cytosolic supernatant

The procedures of (NH₄)₂SO₄ fractionation were followed as described by Thompson (1967). The precipitate (30–50%) was dissolved in the starting buffer and, after dialysis against the same buffer for 6h, the sample was fractionated on the chromatofocusing column (Fig. 3*b*). The activity of the eluate assayed at pH 5.5 indicated that there was no significant change between the chromatofocusing of cytosolic supernatant (Fig. 3*a*) and that of the (NH₄)₂SO₄ fraction (Fig. 3*b*). However, assay at pH 7 or 8 revealed that the sharp activity peak with a pI of 4.6 (Fractions 18–23) had disappeared during the (NH₄)₂SO₄ fractionation.

Fig. 4. Effects of pH on the phosphatidylinositol phosphodiesterase activity of the peak fractions obtained on chromatofocusing of the cytosolic supernatant

The hydrolysis of [³²P]phosphatidylinositol (O) is shown for: the concentrated pool (see Table 1) of fraction nos. 2–6 (a); fraction no. 12 (b); fraction no. 21 (c); fraction no. 33 (d). These enzyme fractions were first eluted on a small Sephadex G-75 column (7 cm × 16 cm) with a 0.25 M-sucrose solution containing 1 mM-dithiothreitol, to remove the imidazole and ampholyte buffers, and the hydrolysis determined over a 15 min incubation period.

Table 1. The products of the peaks of phosphatidylinositol-hydrolysing activity (Fig. 3*a*) separated from cytosolic supernatant of rat brain

The Fraction nos. refer to those in Fig. 3*a*). Phosphatidylinositol hydrolysis and subsequent separation of water-soluble products was as described in the Materials and methods section. The data are expressed as the percentages of the total water-soluble radioactivity released by each fraction.

	Fraction no. ...	2–6*		12	21			26†	33
		5.5	7.5		5.5	7.0	8.0		
Inositol 1:2-cyclic phosphate	Assay pH ...	64.4	47.4	44.8	62.1	52.1	42.0	0.0	53.0
Inositol 1-phosphate		28.0	52.6	55.1	29.2	41.2	50.8	0.0	40.8
Glycerophosphoinositol		2.0	0.1	0.0	0.0	0.0	0.0	0.0	1.0
Lysophosphatidylinositol		—	—	—	—	—	—	99.0	—

* Fraction nos. 2–6 were pooled in dialysis tubing, and the volume was reduced to approximately one-sixth by covering the tubing with solid Polyethyleneglycol 4000.

† Assayed with 2 mM-EDTA present.

Discussion

These experiments have resolved the apparent disagreement between the reports of Thompson (1967) or Keough & Thompson (1972) and our previous observations (Hirasawa *et al.*, 1981) regarding the pH-activity profile of cerebral phosphatidylinositol phosphodiesterase. Thus if the brain supernatant fraction is subject to $(\text{NH}_4)_2\text{SO}_4$ fractionation before enzyme assay, as was carried out by the earlier workers, the activity at alkaline pH values is practically eliminated. It seems likely that this is also so for many other tissues where a single pH optimum at 5.5–5.9 has been reported for the enzyme after $(\text{NH}_4)_2\text{SO}_4$ fractionation (Atherton & Hawthorne, 1968; Abdel-Latif *et al.*, 1980). Thus it is probable that many tissues will show pronounced phosphodiesterase activity active against phosphatidylinositol at an alkaline pH if they are examined before $(\text{NH}_4)_2\text{SO}_4$ fractionation. In this respect we have recently extended our observations to sheep pancreas supernatant fraction, where strong activity is seen up to pH 8.5 (Dawson *et al.*, 1982) and we have also obtained evidence that a similar situation exists in rat liver and kidney.

However, it is still not clear whether the isoenzyme (if it is a true isoenzyme) that is active at alkaline pH was denatured, or whether a cofactor for the activity in that pH range was separated from the enzyme (or denatured). Recently Takenawa & Nagai (1981) have succeeded in purifying a homogeneous (pH 7.0) activity from rat liver. They assayed at pH 7.0 from the beginning of the purification steps, and also used $(\text{NH}_4)_2\text{SO}_4$ fractionation (33–45% saturation), which might have contributed to the low recovery from the initial preparation. The final purified enzyme in a yield of 2% showed a pH optimum at only 7.0, so presumably they have isolated only this fraction.

In conclusion, chromatofocusing has proved to be a valuable aid to probing the heterogeneity of the present enzyme, but the functions and distributions both between and within the cells of the various enzyme fractions with differing isoelectric points warrant further investigation. That the fractions revealed are not artefacts of the method is indicated by the broadly similar patterns obtained for chromatofocusing and electrofocusing. However, chromatofocusing probably gives a much better indication of the quantitative spread of activity since the incubation lasted 15 min compared with the 20 h necessary to assay the electrofocusing-gel slices. The

chemical nature of the heterogeneity (for example, whether these are true isoenzymes or whether they are the same polypeptide with other molecules attached) is not established from the present data.

Dr. D. B. A. Symons and Mrs. C. Clarkson are thanked for their generous assistance in this investigation. We also thank Miss N. Hemington for preparing the [^{32}P]phosphatidylinositol.

References

- Abdel-Latif, A. A., Luke, B. & Smith, J. P. (1980) *Biochim. Biophys. Acta* **614**, 425–434
- Allan, D. & Michell, R. H. (1974) *Biochem. J.* **142**, 591–597
- Atherton, R. S. & Hawthorne, J. N. (1968) *Eur. J. Biochem.* **4**, 68–75
- Bjerve, K. S., Daae, L. N. W. & Bremer, J. (1974) *Anal. Biochem.* **58**, 238–245
- Dawson, R. M. C. (1954) *Biochim. Biophys. Acta* **14**, 374–379
- Dawson, R. M. C. (1959) *Biochim. Biophys. Acta* **33**, 68–77
- Dawson, R. M. C. & Clarke, N. (1972) *Biochem. J.* **127**, 113–118
- Dawson, R. M. C., Freinkel, N., Jungalwala, F. B. & Clarke, N. (1971) *Biochem. J.* **122**, 605–607
- Dawson, R. M. C., Irvine, R. F., Hirasawa, K. & Hemington, N. L. (1982) *Biochim. Biophys. Acta* **710**, 212–220
- Hazlewood, G. P. & Dawson, R. M. C. (1975) *J. Gen. Microbiol.* **89**, 163–174
- Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1981) *Eur. J. Biochem.* **120**, 53–58
- Ingelman-Sundberg, M. & Gustafsson, J.-Å. (1977) *FEBS Lett.* **74**, 103–106
- Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1978) *Biochem. J.* **176**, 475–484
- Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1979) *Eur. J. Biochem.* **99**, 525–530
- Irvine, R. F., Dawson, R. M. C. & Freinkel, N. (1982) *Contemporary Metabolism* (Freinkel, N., ed.), vol. 2, pp. 301–342, Plenum Press, New York
- Kemp, P., Hübscher, G. & Hawthorne, J. N. (1961) *Biochem. J.* **79**, 193–200
- Keough, K. M. W. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270**, 324–336
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Takenawa, T. & Nagai, Y. (1981) *J. Biol. Chem.* **256**, 6769–6775
- Thompson, W. (1967) *Can. J. Biochem.* **45**, 853–861
- Warner, M., Lamarca, M. V. & Neims, L. H. (1978) *Drug Metab. Dispos.* **6**, 353–362