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DISSOCIATION BETWEEN ORNITHINE DECARBOXYLASE ACTIVITY AND ARYL HYDROCARBON HYDROXYLASE INDUCTION IN CELL CULTURES TREATED WITH BENZ[*a*]ANTHRACENE AND INHIBITORS OF ORNITHINE DECARBOXYLASE

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Abstract—The induction of aryl hydrocarbon hydroxylase and ornithine decarboxylase by benz[*a*]-anthracene in the presence or absence of ornithine decarboxylase inhibitors was studied in three different cell culture systems. An almost complete abolishment of ornithine decarboxylase activity by 1,3-diamino-2-propanol or α-difluoromethyl ornithine before the addition of the inducer did not affect appreciably the induction of aryl hydrocarbon hydroxylase by benz[*a*]anthracene in human embryo, HeLa and Reuber H-II-4-E cells in culture. These results suggest that the induction of aryl hydrocarbon hydroxylase does not require ornithine decarboxylase activity *per se* and can be expressed in the absence of continuous polyamine synthesis.

The microsomal monooxygenase complex of mammalian cells metabolizes a variety of drugs, pesticides and carcinogens. Aryl hydrocarbon (benzo[*a*]-pyrene) hydroxylase (AHH, EC 1.14.14.1), one part of this complex, is found in various tissues of several species [1, 2], and in cells in culture (see Ref. 3).

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the rate-limiting enzyme for polyamine synthesis and is in key position in polyamine metabolism, increased synthesis of polyamines (putrescine, spermine and spermidine) being correlated with an increased rate of proliferation in a range of tissues and cell types (see Ref. 4). It is suggested that in response to microsomal enzyme-inducing compounds (e.g. polycyclic aromatic hydrocarbons and barbiturates), elevated ODC activity, drug-induced liver hypertrophy and microsomal enzyme induction form a tight temporal sequence of causally related events [5–8]. A close correlation between induced ODC and AHH activities has recently been reported in inbred AHH-responsive and non-responsive mice treated with inducers of the microsomal monooxygenase system [9].

Many inhibitors of ODC have been introduced during the last few years. The administration of various diamines such as 1,3-diaminopropane will reduce ODC activity in various tissues [10–12] and in cells in culture [14–15]. 1,3-Diamino-2-propanol, a close analogue of 1,3-diamino-propane, has been found to be equally or more effective in depressing ODC activity than the parent compound [16]. A newly-discovered irreversible inhibitor of ODC, DL-α-difluoromethyl ornithine (DFMO, RMI 71.172) [17] markedly inhibits ODC activity in cultured cells [18, 19] and also slows down cell proliferation and inhibits DNA replication [20, 21].

One report from this laboratory shows that peroral administration of 1,3-diamino-2-propanol to rats and mice inhibits the activity of hepatic microsomal drug-metabolizing enzymes [22]. To further evaluate the relationship between ODC and AHH induction, we studied the effects of 1,3-diamino-2-propanol (DAP) and DFMO on benz[*a*]anthracene (BA)-induced ODC and AHH activities in three different cell culture systems.

MATERIALS AND METHODS

Chemicals. DL-α-difluoromethyl ornithine was a generous gift from Dr. D. J. Wilkins (Centre de Recherche Merrell International, Strasbourg, France). Other chemicals were purchased as follows: 1,3-diamino-2-propanol and benz[*a*]anthracene from Fluka AG (Buchs, Switzerland), DL-[1-^{14}C]ornithine (sp. act. 58 mCi/mmole, CO_2-Met and PCS (Phase Combining System) from the Radiochemical Centre (Amersham, U.K.).

Cell cultures. The HeLa cells were generously given to us by Professor A. Vaheri (Helsinki University) and the Reuber H-II-4-E cells by Dr. D. W. Nebert (National Institutes of Health, Bethesda, MD). The human embryo cells were from a fetus obtained during routine hysterotomy performed for sociomedical reasons on an otherwise healthy woman. The culture was prepared as described earlier [23]. The cells were grown in 75 cm^2 plastic bottles (Nunc, Roskild, Denmark) in Basal Medium Eagle (Flow Laboratories, Irvine, U.K.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Glasgow, U.K.), 10 μg/ml of gentamicin and 25 IU/ml of nystatin.

Toxicity of the inhibitors. Normal cell growth and viability was observed over a 48-hr period in the presence of different concentrations of the inhibitors. DAP was found to be non-toxic to human embryo

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cells, HeLa cells, and H-4-II-E cells at concentrations below 10 mM, 5 mM and 1 mM, respectively. DFMO was non-toxic at concentrations below 10 mM, and concentrations of 1–2 mM were used during the experiments.

Induction with benz[a]anthracene. For the experiments, the cells were plated at a concentration of 3×10^6/90 mm petri dish (Sterilin Ltd., Teddington, U.K.). Stock solutions of BA were made up in dimethylsulphoxide (DMSO) and added to the culture media at various times after starting the cells in culture. The inhibitors of ODC were always added to the culture media 24 hr before addition of BA. DMSO at concentration of 0.5% did not affect the parameters under investigation. The cells were harvested by rinsing the surface of the dish with ice-cold 0.1 M phosphate-buffered saline (pH 7.1), scraping with a rubber rod and washing twice with centrifugation at 500 *g*. The washed pellet was frozen at −40° until assayed.

Analytical methods. ODC activity was measured as the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine using a modification of the method of Beaven, Wilcox and Terpsta [24]. The cells were disrupted by sonication in 300 μl of 100 mM Tris–HCl (pH 7.1) and centrifuged at 10,000 *g* for 30 min. A 110 μl aliquot of the supernatant was used for the assay. The final concentrations in the incubation mixtures were: EDTA, 4 mM; dithiothreitol, 4 mM; pyridoxal phosphate, 0.4 mM; L-ornithine, 20 mM; and 0.45 μCi DL-[1-^{14}C]ornithine in a volume of 120 μl. A 1.5 ml polypropylene Eppendorf vial containing the reaction mixture was placed inside a 20 ml screw-cap glass liquid scintillation counting vial. After a 60 min incubation in a water bath at 37°, the reaction was terminated by the addition of 250 μl of trichloroacetic acid. The incubation was continued for 30 min to trap all the $^{14}CO_2$ released. The $^{14}CO_2$ given off was collected on a piece of Whatman No. 1 filter paper impregnated with 100 μl CO_2-Met-ethyleneglycol (1:1) placed on the bottom of the vial. After removing the Eppendorf vials, 7 ml of PCS–xylene scintillation cocktail was added to the glass vials and the radioactivity was determined. The specific activity of ODC is expressed in nmoles CO_2 released per mg of protein per 60 min.

AHH was assayed eseentially as described by Nebert and Gelboin [25], and its specific activity expressed in nmoles 3-OH-benzo[*a*]pyrene formed per mg protein per 60 min. Cell protein was determined by the method of Lowry *et al.* [26], using bovine serum albumin as a standard.

RESULTS

Effect of DAP on BA-induced ODC and AHH activities. Table 1 shows the response of confluent human embryo cells to 10 μM BA at various times after addition of the inducing compound. The cells were plated at a concentration of 3×10^6/90 mm petri dish. On day 4 after seeding, DAP was added to the culture medium of some cells at the concentrations indicated. By this time the cells had formed a confluent monolayer. Twenty-four hours later some of the cells were induced with 10 μM BA and harvested at the time-points indicated. Induction with BA resulted in a biphasic increase in ODC activity, the maximal stimulation (about 4-fold) occurring 2 hr after addition of the compound. The activity of AHH steadily increased during incubation with BA, the maximum occurring 18 hr after induction. Pretreatment of the cultures with DAP resulted in a concentration-dependent decrease in ODC activity, 2.5 mM of DAP inhibiting the ODC activity by more than 99% at every time-point after induction. DAP, however, had no apparent effect on the extent of AHH induction. Markedly lower levels of basal ODC activity were observed in the confluent H-4-II-E cells (Table 2). The addition of BA produced a 10-fold induction in AHH activity, but failed to

Table 1. Effect of DAP on AHH and ODC activities in human embryo cells after treatment with BA

	Enzyme activities at specific time-points after induction							
	2 hours		6 hours		18 hours		24 hours	
	ODC*	AHH†	ODC	AHH	ODC	AHH	ODC	AHH
Control	1.10±0.09‡	0.15±0.02	5.18±1.80	0.17±0.04	5.07±0.45	0.09±0.02	1.43±0.32	0.21±0.05
BA	4.90±0.29	0.26±0.04	2.80±1.10	0.58±0.12	7.51±0.32	1.03±0.09	1.00±0.26	1.44±0.16
BA+DAP 50 μM	1.50±0.66	0.29±0.15	1.50±0.26	0.76±0.31	5.06±0.24	0.76±0.24	0.50±0.08	1.02±0.30
BA+DAP 500 μM	0.07±0.04	0.27±0.05	0.32±0.33	0.85±0.16	1.73±0.43	1.42±0.33	0.30±0.07	1.23±0.07
BA+DAP 2.5 mM	0.04±0.07	0.19±0.02	0.02±0.04	0.76±0.11	0.01±0.01	1.19±0.11	0.03±0.00	1.03±0.08
DAP 2.5 mM	0.10±0.05	0.19±0.02	0.00	0.22±0.12	0.31±0.53	0.17±0.07	0.01±0.00	0.28±0.02

* Expressed as nmoles CO_2 released/mg protein/60 min.
† Expressed as nmoles 3-OH-benzo[*a*]pyrene formed/mg protein/60 min.
‡ Values are means ± S.D. for 6 dishes.
BA = benz[*a*]anthracene, DAP = 1,3-diamino-2-propanol.

Table 2. Effect of DAP on AHH and ODC activities in H-4-II-E cells treated with BA

	Enzyme activities at specific time-points after induction							
	2 hours		12 hours		18 hours		24 hours	
	ODC*	AHH†	ODC	AHH	ODC	AHH	ODC	AHH
Control	0.20±0.18‡	0.41±0.14	0.15±0.04	0.31±0.04	0.22±0.06	0.34±0.04	0.10±0.11	0.72±0.12
BA	0.06±0.00	0.74±0.03	0.07±0.00	5.89±0.45	0.15±0.10	5.23±0.78	0.15±0.11	7.26±0.12
BA+DAP 50 uM	0.13±0.00	0.68±0.08	0.14±0.13	4.19±0.90	0.07±0.04	5.25±0.82	0.06±0.03	8.25±0.58
BA+DAP 100 uM	0.04±0.02	0.62±0.06	0.19±0.19	4.56±0.64	0.12±0.04	6.67±0.41	0.04±0.01	6.36±0.19
BA+DAP 500 uM	0.05±0.00	0.71±0.03	0.08±0.09	4.45±0.50	0.16±0.10	6.47±0.32	0.04±0.02	9.16±0.71
DAP 500 uM	0.04±0.02	0.54±0.05	0.02±0.02	0.39±0.09	0.21±0.08	0.65±0.26	0.02±0.02	0.84±0.23

* Expressed as nmoles CO_2 released/mg protein/60 min.
† Expressed in nmoles 3-OH-benzo[*a*]pyrene formed/mg protein/60 min.
‡ Values are means ± S.D. for 6 dishes.
BA = benz[*a*]anthracene, DAP = 1,3-diamino-2-propanol.

induce the activity of ODC. Exposure of the cells to 500 μM DAP inhibited ODC activity by 30–78%, but the corresponding AHH activities were of the same magnitude or slightly higher than in the cells treated with BA alone.

Effect of DFMO on BA-induced ODC and AHH activities in human embryo cells. Figure 1 shows that the addition of 1 mM DFMO to the human embryo cells in culture resulted in an almost 100% reduction in ODC activity, but had no significant effect on AHH levels after a 24-hr treatment with 10 μM BA. Probably due to the relatively long incubation period with the inducing compound (24 hr), no elevation in ODC activity was observed in response to BA.

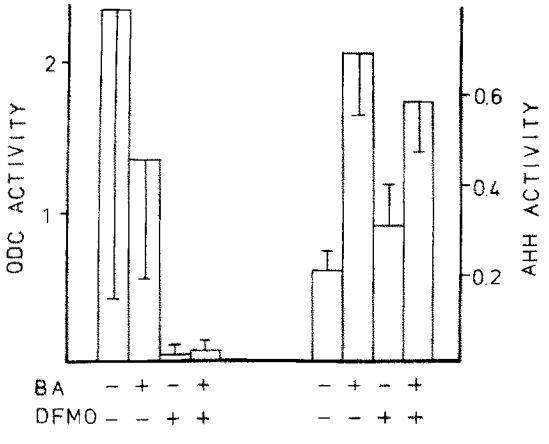

Fig. 1. Effect of DFMO on BA-induced ODC and AHH activities in human embryo cells. Cells (3×10^6) were plated on 90 mm petri dishes. On day 3 after seeding the cells, 1 mM DFMO dissolved in water was added to the culture medium of some cells. Twenty-four hours later 10 μM BA was added, and the cells were harvested after a 24-hr incubation period with the inducing agent. The activity of AHH is expressed as nmoles 3-OH-benzo[*a*]pyrene formed/mg protein/60 min and that of ODC nmoles CO_2 released/mg protein/60 min.

Effect of DFMO on ODC and AHH activities in growing and stationary HeLa and H-4-II-E cells. As seen in Fig. 2, the basal ODC level was markedly higher in the growing HeLa cells than in the confluent cells. Again, BA failed to induce ODC activity either during growth or in the stationary phase. The inducibility of AHH by BA was somewhat lower during the growth phase than in the stationary phase (6-fold and 11-fold induction, respectively). Addition of 2 mM DFMO to the culture medium immediately after dilution of the cells resulted in a more than 99% reduction in ODC activity, but had no effect on AHH activity. In the stationary cells, 2 mM DFMO caused about a 74% reduction in ODC activity, whereas the inducibility of AHH was not affected.

The ODC activity of the H-4-II-E cells was also low in the high-density cultures, but relatively low in the exponentially growing cells (Fig. 3). Addition of 10 μM BA to the culture medium induced the activity of ODC slightly but insignificantly in both the growing and the stationary cells. One mM DFMO produced an 88% reduction in induced ODC activity in the growing cells. In contrast with previous results, DFMO had an inhibiting effect on BA-induced activity in the growing cells.

DISCUSSION

The ability of polycyclic aromatic hydrocarbons to induce AHH segregates in the manner of an autosomal dominant gene which codes for a cytosol protein, the induction receptor [27, 28]. It has been postulated that several enzymes, including AHH and ODC, are induced via the same receptor for polycyclic aromatic compounds [29]. Thus changes in the activities of ODC may strongly modulate the activity of AHH.

A number of studies show that the activity of ODC diminishes when animal tissues or cultured cells are exposed to natural polyamines or certain other dia-

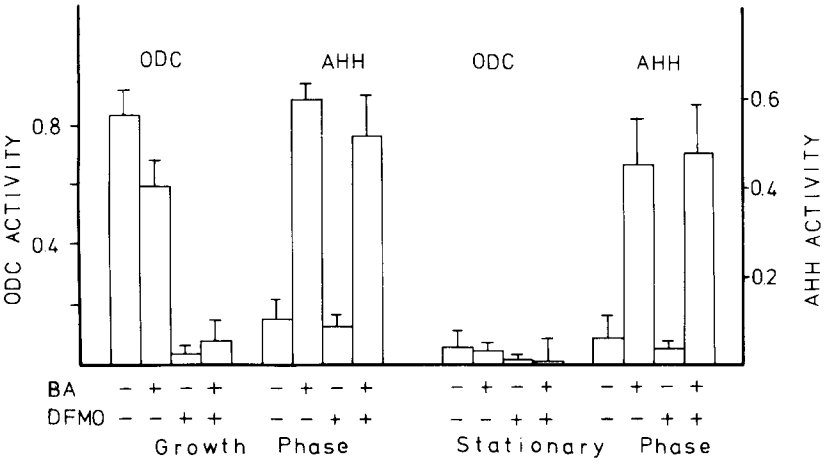

Fig. 2. Effect of DFMO on ODC and AHH activities in growing and stationary HeLa cells. Cells (3×10^6) were plated on a 90 mm petri dish. Two mM DFMO was added simultaneously, or after the cells had formed a monolayer (day 3 after seeding). The cells were induced with BA 24 hr after addition of the inhibitor and harvested 24 hr later. The activities of ODC and AHH are expressed as in Fig. 1.

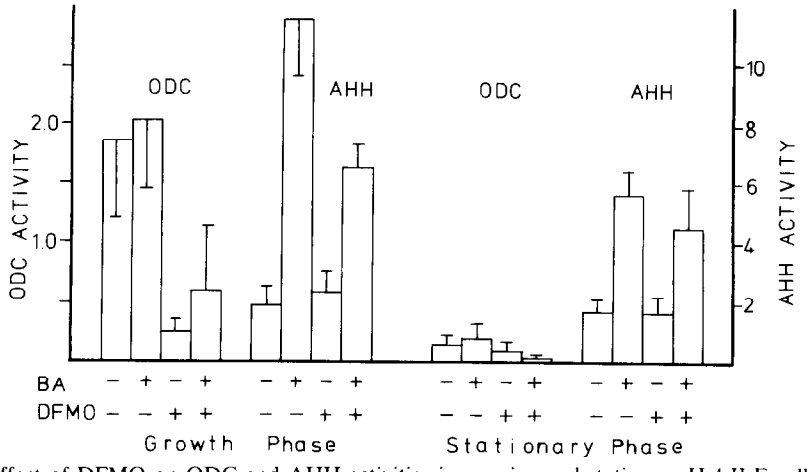

Fig. 3. Effect of DFMO on ODC and AHH activities in growing and stationary H-4-II-E cells. One mM DFMO was added to the growth medium of some of the cells immediately after subculturing, or 72 hr later. The cells were induced 24 hr after the addition of DFMO.

mines not normally present in the living cell [30–34]. The mechanism by which exogenous diamines suppress the expression of ODC activity is not well understood at present, but there is evidence to indicate that this effect may involve post-transcriptional and post-translational sites of action [34, 35]. It is interesting that in many experimental systems the synthesis of RNA and protein remain relatively normal in spite of severe polyamine depletion [36, 37].

The results presented show that, at least in cells in culture, a virtually complete disappearance of ODC activity has no apparent effect on the activity and induction of AHH, although in some cases, a near-complete inhibition of ODC activity resulted in a slightly enhanced induction of AHH, a result totally unexpected. It is noticeable that the dissociation between ODC activity and AHH induction can be seen in cells with widely variable basal and induced AHH activities, such as human embryo cells and H-4-II-E cells. The effect does not seem to be dependent on the basal ODC activity, since cells with high activity (human embryo cells and HeLa cells in the growth phase) and with low activity (H-4-II-E cells or HeLa cells in the stationary phase) behave very similarly.

As suggested by Kano and Nebert [38], the apparent lack of any relationship between AHH and ODC induction in cell cultures can be interpreted in different ways: (1) differences between polycyclic aromatic hydrocarbon-treated and control cultures are obscured by the abnormally elevated basal ODC activity; (2) ODC induction is not an essential prerequisite during the enhancement of drug-metabolizing enzyme activities; and (3) although a very low ODC basal level will give rise to an obligatory striking increase in ODC activity, high basal levels are sufficient to obscure such stimuli as AHH induc-

tion, and therefore no further enhancement of ODC activity is required. In the experimental system used here, however, the inhibitors of ODC were added to the culture media 24 hr before induction with BA. This means that the basal ODC level was already suppressed at the point of the addition of BA. It may, however, be possible that even the markedly reduced ODC activity is sufficient for the AHH induction process.

The results obtained are inconsistent with previous studies suggesting a close relationship between ODC and AHH induction in intact animals [5–9, 22]. It is possible that our earlier finding, the ability of DAP to inhibit rat and mouse liver drug-metabolizing enzymes, could be attributed to the general toxic effect of the compound. In cell cultures the toxicity of the ODC inhibitors can be controlled, and one can thus avoid misleading results due to this effect.

In any case, further studies are needed to reveal the role of ODC and polyamines in Ah locus-associated AHH induction in living cells.

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REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. H. V. Gelboin, *Adv. Cancer Res.* **10**, 1 (1967).
3. J. P. Whitlock, *Pharmac. Ther.* **4**, 587 (1979).
4. J. Jänne, H. Pösö and A. Raina, *Biochim. biophys. Acta* **473**, 241 (1978).
5. M. Costa, E. R. Costa, C.-A. Manen, J. G. Sipes and D. H. Russell, *Molec. Pharmac.* **12**, 871 (1976).
6. C. V. Byus, M. Costa, J. G. Sipes, B. B. Brodie and D. H. Russell, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1241 (1976).
7. D. H. Russell, C. V. Byus and C.-A. Manen, *Life Sci.* **19**, 1278 (1976).
8. C.-A. Manen, M. Costa, J. G. Sipes and D. H. Russell, *Biochem. Pharmac.* **27**, 219 (1978).
9. D. W. Nebert, N. M. Jensen, J. W. Perry and T. Oka, *J. biol. Chem.* **255**, 6836 (1980).
10. H. Pösö and J. Jänne, *Biochem. biophys. Res. Commun.* **69**, 885 (1976).
11. S. K. Guha and J. Jänne, *Biochem. biophys. Res. Commun.* **75**, 136 (1977).
12. A. E. Pegg, S. Conover and A. Wrona, *Biochem. J.* **170**, 651 (1978).
13. D. R. Bethell and A. E. Pegg, *Biochem. J.* **180**, 87 (1979).
14. J. S. Heller, K. Y. Chen, D. A. Kyriakidis, W. F. Fong and E. S. Canellakis, *J. cell. Physiol.* **96**, 225 (1978).
15. P. P. McCann, C. Tardif, J.-M. Hornsperger and P. Böhlen *J. cell. Physiol.* **99**, 183 (1979).
16. K. Piik, H. Pösö and J. Jänne, *FEBS Lett.* **87**, 307 (1978).
17. B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara and J. P. Vevert, *J. Am. chem. Soc.* **100**, 2551 (1978).
18. P. S. Mamont, M.-C. Duchesne, J. Grove and P. Bey, *Biochem. biophys. Res. Commun.* **81**, 58 (1978).
19. H. C. Isom and A. E. Pegg, *Biochim. biophys. Acta* **564**, 402 (1979).
20. N. Seiler, C. Danzin, N. J. Prakash and J. Koch-Weser, in *Enzyme-Activated Irreversible Inhibitors* (Eds N. Seiler, M. J. Jung and J. Koch-Weser), p. 55. Elsevier-North Holland Biomedical Press, Amsterdam (1978).
21. P. S. Mamont, P. Böhlen, P. P. McCann, P. Bey, F. Schuber and C. Tardif, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1626 (1976).
22. H. Raunio and O. Pelkonen, *Biochem. biophys. Res. Commun,* **91**, 658 (1979).
23. O. Pelkonen, P. Korhonen and N. T. Kärki, *Life Sci.* **16**, 1403 (1975).
24. M. A. Beaven, G. Wilcox and G. K. Terpsta, *Analyt. Biochem.* **84**, 638 (1978).
25. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. P. E. Thomas, R. E. Kouri and J. J. Hutton, *Biochem. Genet.* **6**, 157 (1972).
28. A. P. Poland, E. Glover, J. R. Robinson and D. W. Nebert, *J. biol. Chem.* **249**, 5599 (1974).
29. D. W. Nebert and T. Oka, *Proc. X Int. Cong. Biochem.* p. 386, (1976).
30. J. E. Kay and V. J. Lindsay, *Biochem. J.* **132**, 791 (1973).
31. J. Jänne and E. Hölttä, *Biochem. biophys. Res. Commun.* **61**, 449 (1974).
32. P. P. McCann, C. Tardif and P. S. Mamont, *Biochem. biophys. Res. Commun.* **75**, 948 (1977).
33. P. S. Sunkara, N. R. Pota and K. Nishioka, *Biochem. biophys. Res. Commun.* **74**, 1125 (1977).
34. W. F. Fong, J. S. Heller and E. S. Canellakis, *Biochim. biophys. Acta* **428**, 456 (1976).
35. J. S. Heller, W. F. Fong and E. S. Canellakis, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1858 (1976).
36. R. H. Fillingame, C. M. Jorstad and D. R. Morris, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4042 (1975).
37. H. Pösö and J. Jänne, *Biochem. J.* **158**, 485 (1976).
38. I. Kano and D. W. Nebert, *Molec. Pharmac.* **20**, 172 (1981).