Differential effect of phenobarbital and β-naphthoflavone on the mRNAs coding for cytochrome P450 and NADPH cytochrome P450 reductase

Elizabeth A. Shephard, Ian R. Phillips, Susan F. Pike, Alan Ashworth and Brian R. Rabin

Department of Biochemistry, University College London, Gower Street, London, WC1E 6BT, England

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The induction in rat liver of a specific variant(s) of cytochrome P450 (PB-P450) by phenobarbital and its repression by β-naphthoflavone occur through corresponding changes in the levels of mRNA coding for the protein(s). The level of translatable mRNA coding for NADPH-cytochrome P450 reductase in rat liver increases on treatment with phenobarbital but not β-naphthoflavone.

Cytochrome P450 *NADPH cyt. P450 reductase* *mRNA* *Immunoprecipitation* *Phenobarbital* *β-Naphthoflavone*

1. INTRODUCTION

Cytochromes P450 and NADPH cytochrome P450 reductase are two major components of the mixed function monooxygenase systems of liver microsomal membranes. The enzyme systems are involved in the metabolism of xenobiotics, including drugs and carcinogens, as well as endogenous substrates such as steroid hormones [1–6]. A variety of xenobiotics increase the level of total cytochromes P450, the actual variants of cytochromes P450 induced depending on the xenobiotic used [7–9]. Radioimmunoassays [8,10] for a specific variant have shown that phenobarbital (PB) increases the levels of the major PB-inducible cytochrome P450 variant (PB P450) and NADPH cytochrome P450 reductase 43- and 2.6-fold, respectively. However, treatment of animals with β-naphthoflavone (β-NF) (an inducer of different cytochrome P450 variants) [8] resulted in a 55% decrease in the amount of PB P450 and had no effect on the amount of NADPH cytochrome P450 reductase. Induction of PB P450 by PB involves an increase in the amount of translatable mRNA coding for this protein [11]. Here, we investigate the mechanism of repression of PB P450 protein by β-NF and the molecular basis of the differential effect of PB and β-NF on the expression of NADPH cytochrome P450 *reductase*.

Abbreviations: PB, phenobarbital; β-NF, β-naphthoflavone; PB P450, major inducible cytochrome P450 variant of M_r 52 000

2. MATERIALS AND METHODS

2.1. *Animals*

Male Sprague-Dawley rats (180–200 g body wt) bred at University College Animal facility were used in these experiments. Treatment of animals with sodium phenobarbital or β-naphthoflavone is in [8].

2.2. *Isolation of RNA*

Total rat liver RNA was isolated from the livers of PB-treated, β-NF-treated and control animals as in [12]. Poly(A)$^+$-containing RNA was isolated by a modification of the method in [13]. Total RNA was adjusted to 1 mg/ml in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS, 0.5 M NaCl (buffer A) and heated at 68°C for 2 min and chromatographed on a column of oligo(dT)-

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cellulose (PL Biochemicals, type 7). The entire eluate was reloaded onto the column which was then washed with buffer A until the A_{260} returned to zero. Poly(A)$^+$ RNA was eluted with 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS. Fractions containing poly(A)$^+$ RNA were adjusted to 0.5 M with respect to NaCl, treated at 68°C for 2 min and rechromatographed on oligo(dT)-cellulose. The column was washed and poly(A)$^+$ RNA was eluted as above. Poly(A)$^+$ RNA was adjusted to 0.25 M with respect to sodium acetate (pH 6.0), mixed with 2.5 vol. absolute ethanol and stored at −20°C overnight. Poly(A)$^+$ RNA was pelleted at 16 000 × *g* for 10 min at −10°C, resuspended in water and reprecipitated as above. Final RNA pellets were washed twice with 90% ethanol, dried under a stream of nitrogen, and resuspended in water at ~4.5 mg/ml.

2.3. *Preparation of polyribosomes*

Polyribosomes were prepared from rat liver as in [11].

2.4. *Translation in vitro*

Polyribosomes, total RNA and poly(A)$^+$ RNA were translated in vitro using a rabbit reticulocyte lysate (Amersham International, Bucks) as in [11]. Total translation products were analysed on SDS–10% polyacrylamide gels as in [11].

2.5. *Immunoprecipitation*

Immunoprecipitation of translation products was as in [11] except that translation products (see figure legends for cpm used) were made 1% (w/v) with respect to both sodium deoxycholate and Triton X-100, centrifuged at 10 000 × *g* for 10 min and diluted to 900 μl with a solution containing 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100/150 mM NaCl/5 mM EDTA/50 mM Tris–HCl (pH 7.4)/0.02% (w/v) NaN_3/2 mM methionine/1 mM phenylmethanesulphonyl fluoride before addition of the appropriate antibody. Immunoprecipitated polypeptides were analysed by SDS–polyacrylamide gel electrophoresis and quantitated as in [11].

2.6. *Purification of proteins and preparation of antibodies*

The major PB-inducible cytochrome P450 (PB P450; M_r 52 000), and NADPH cyt. P450 reductase (M_r 76 000) were isolated from the livers of PB-treated rats as in [14]. Antibodies to these two proteins were raised in rabbits as in [8].

3. RESULTS

3.1. *Effect of phenobarbital and β-naphthoflavone treatment on products of translation in vitro*

Poly(A)$^+$ RNA isolated from the livers of PB,β-NF and untreated animals were translated in vitro in a rabbit reticulocyte cell free system. The spectrum of proteins synthesized by each RNA sample was analysed by SDS–polyacrylamide gel electrophoresis and fluorography. The overall patterns of protein synthesis directed by poly(A)$^+$ mRNA

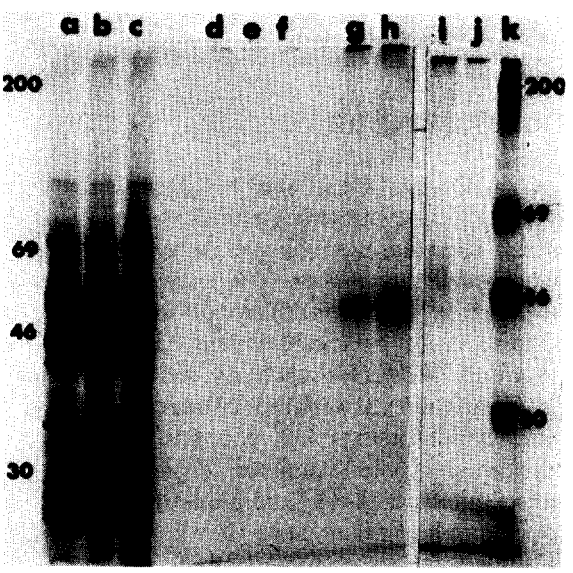


Fig. 1. SDS–polyacrylamide gel electrophoresis demonstrating the differential effects of PB and β-NF on translatable PB P450 mRNA. Total translation products (tracks a–c) contained 4 × 10^4 acid-precipitable cpm. For immunoprecipitation analysis (tracks d–j) 6 × 10^5 (tracks d–f,h) 4 × 10^5 (track g) or 4 × 10^6 (tracks i,j) acid-precipitable cpm of translation products were incubated with 10 μl non-immune serum (track d) or 10 μl of anti(PB P450) serum (tracks e–j) and processed as in section 2.5. Translation products were from poly(A)$^+$ RNA isolated from β-NF- (tracks a,e,j), PB- (tracks b,d,g,h) or un-treated (tracks c,f,i) rats. Numbers give M_r of marker proteins (× 10^{-3}) (k). Samples i–k were electrophoresed on a separate gel to samples a–h.

isolated from PB-β-NF and un-treated animals are very similar (fig. 1a–c). As shown in [11] PB treatment causes an increase in the translation of an M_r 52 000 polypeptide (fig. 1b), which corresponds to the major inducible cytochrome P450 (PB P450) [8,11]. However, β-NF treatment increased the translation of an M_r 54 000 polypeptide corresponding to the major β-NF-inducible cyt. P450 [8]. In addition, β-NF treatment caused a decrease visually in the quantity of an M_r 52 000 polypeptide (fig. 1a, cf. fig. 1c). This result suggested that the observed decrease in the amount of PB P450 in microsomal membranes following β-NF treatment [8] may involve a decrease in the amount of the mRNA coding for PB P450. To investigate this possibility, we quantified the amount of PB P450 mRNA present in RNA isolated from PB-β-NF- and un-treated rats.

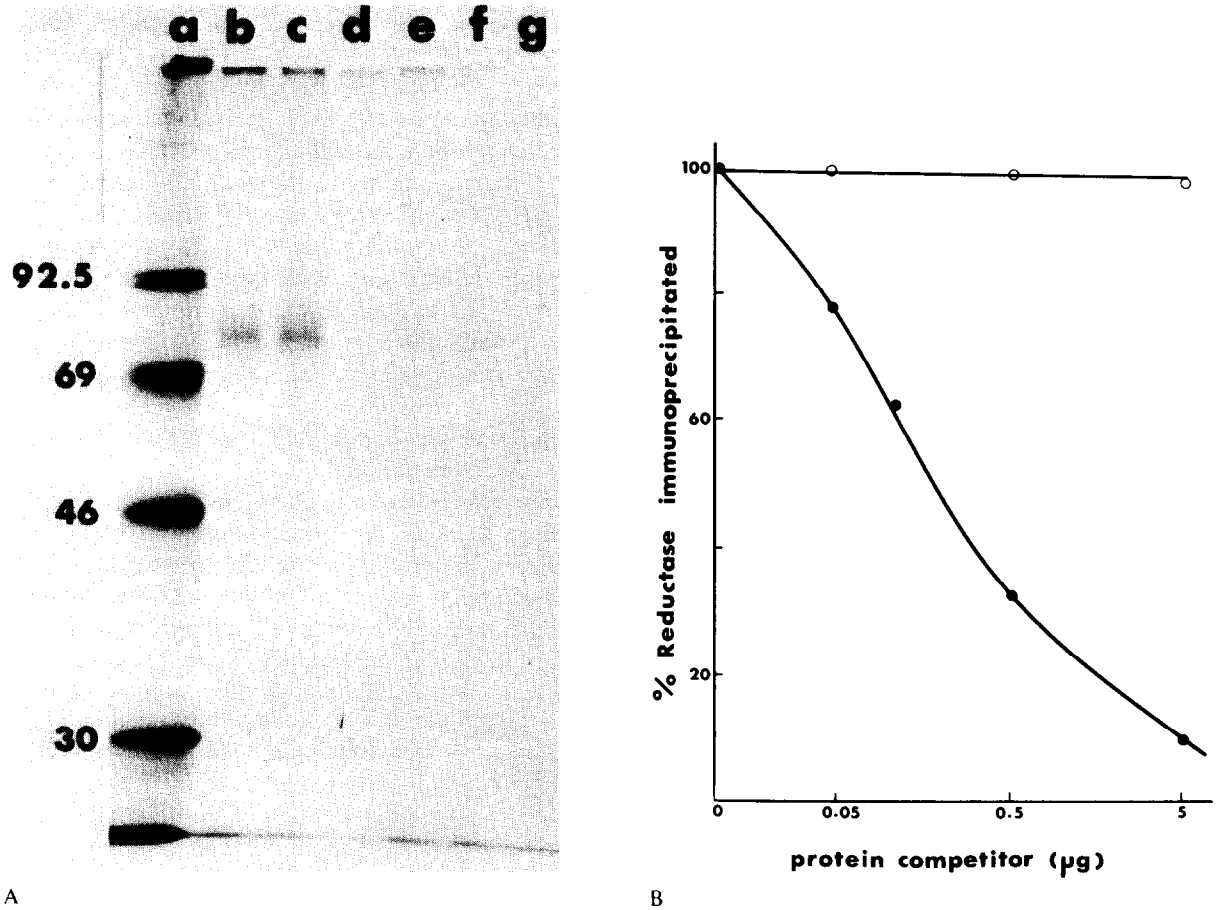
3.2. *Quantification of PB P450 mRNA in poly(A)$^+$ RNA*

We have shown by immunoprecipitation using antibodies specific for PB P450 that PB treatment causes an ~20-fold increase in the amount of translatable mRNA coding for this protein [11]. We have now extended these studies to analyse the effect of a different xenobiotic on the amount of translatable PB P450 mRNA. Anti-(PB P450) antiserum precipitated PB P450 from the translation products of poly(A)$^+$ RNA isolated from PB-treated rats (fig. 1g,h). No PB P450 was visibly precipitated from the translation products of poly(A)$^+$ RNA isolated from control (fig. 1f) or β-NF-treated (fig. 1c) rats. To ensure the accurate quantitation of the small amounts of translatable PB P450 mRNA present in the livers of control and β-NF-treated animals the quantity of translation products used in the immunoprecipitation was increased from 6×10^5–4×10^6 acid-precipitable cpm (fig. 1i,j). PB P450 mRNA was quantified by determining the radioactivity incorporated into the M_r 52 000 immunoprecipitated polypeptide and expressing this as a percentage of the total acid-precipitable radioactivity [11]. PB P450 represented 0.63%, 0.029% and 0.015% of the proteins synthesized by poly(A)$^+$ RNA isolated from the livers of PB-treated, untreated, or β-NF-treated rats, respectively. It should be noted that PB P450 mRNA represented 0.029% of poly(A)$^+$ RNA isolated from control animals whether 6×10^5 cpm or 4×10^6 cpm were included in the immunoprecipitation reaction. However, when only 6×10^5 cpm were analysed it was not possible to detect any PB P450 in the translation products of poly(A)$^+$ RNA isolated from β-NF-treated rats. The results demonstrate that the amount of translatable PB P450 poly(A)$^+$ mRNA increased 22-fold on treatment with PB but decreased by ~53% on treatment with β-NF. This decrease in mRNA is very similar to the decrease we found in PB P450 in the liver microsomal membranes of β-NF-treated rats [8].

3.3. *Quantification of NADPH cytochrome P450 reductase mRNA*

We have also investigated the different effects of PB and β-NF on the amount of translatable mRNA coding for NADPH cytochrome P450 reductase. It was first necessary to establish the specificity of the immunoprecipitation technique for NADPH cytochrome P450 reductase. A single polypeptide of M_r 76 000, the same as that of purified NADPH cytochrome P450 reductase [15], was immunoprecipitated by anti-(NADPH cytochrome P450 reductase) serum from the products of translation in vitro of liver polyribosomes isolated from both PB- (fig. 2A,b,c) and un-treated (fig. 2A,e,f) rats. This polypeptide was not immunoprecipitated by non-immune serum (fig. 2A,d,g) or by PB P450 antiserum (fig. 1). The specificity of the immunoprecipitation reaction was further demonstrated by the ability of purified NADPH cytochrome P450 reductase to compete with the M_r 76 000 translation product from binding sites on the NADPH cytochrome P450 reductase antibody (fig. 2B). No competition was observed with increasing amounts of PB P450.

Having established that anti-(NADPH cytochrome P450 reductase) serum precipitates only NADPH cytochrome P450 reductase from the products of translation in vitro, the amount of translatable mRNA coding for this protein was quantitated in liver poly(A)$^+$ RNA isolated from PB-, β-NF- and un-treated rats as described for PB P450 mRNA. NADPH cytochrome P450 reductase was visibly immunoprecipitated from the translation products of all three RNA samples (fig. 3). The mRNA coding for this protein represented 0.062% of mRNA isolated from PB-treated animals (fig. 3A,b) and 0.03% of the mRNA



A B

Fig. 2. Determination of the specificity of the immunoprecipitation of NADPH cytochrome P450 reductase from the products of translation in vitro. (A) Translation products (3×10^6 acid-precipitable cpm) of polyribosomes isolated from the livers of PB-treated (tracks b–d) or control (tracks e–g) rats were incubated with 10 μl non-immune serum (tracks d,g) or 10 μl NADPH cytochrome P450 reductase antiserum (tracks b,c,e,f). Samples were processed as in section 2.5. Numbers give M_r of marker proteins ($\times 10^{-3}$) (a). (B) Translation products (4×10^6 acid-precipitable cpm) of polyribosomes isolated from the livers of PB-treated rats were mixed with the stated quantities of purified NADPH cytochrome P450 reductase (●) or purified PB P450 (○). Samples were incubated with 10 μl NADPH cytochrome P450 reductase antiserum and processed as in section 2.5. Regions of the gel corresponding to M_r 76 000 were excised and radioactivity was determined. Results are expressed as % of the radioactivity immunoprecipitated in the absence of any competitor.

isolated from control (fig. 3A,a) or β-NF-treated animals (fig. 3A,c). The amount of translatable mRNA coding for NADPH cytochrome P450 reductase was therefore increased ~2-fold following PB-treatment and remained unchanged when rats were treated with β-NF.

To eliminate the possibility that the differential effect of PB and β-NF on the amount of mRNA coding for NADPH cytochrome P450 reductase could be due to differences in the recovery of poly(A)$^+$ RNA (for example, due to differences in poly(A) tail-length) the experiment was repeated on the products of translation in vitro of total RNA isolated from PB-, β-NF- and un-treated animals (fig. 3B). Essentially the same result was obtained and NADPH cytochrome P450 reductase mRNA represented 0.063%, 0.028% and 0.027% of total RNA isolated from PB-treated (fig. 3B,b), control (fig. 3B,c) or β-NF-treated (fig. 3B,a) animals, respectively.

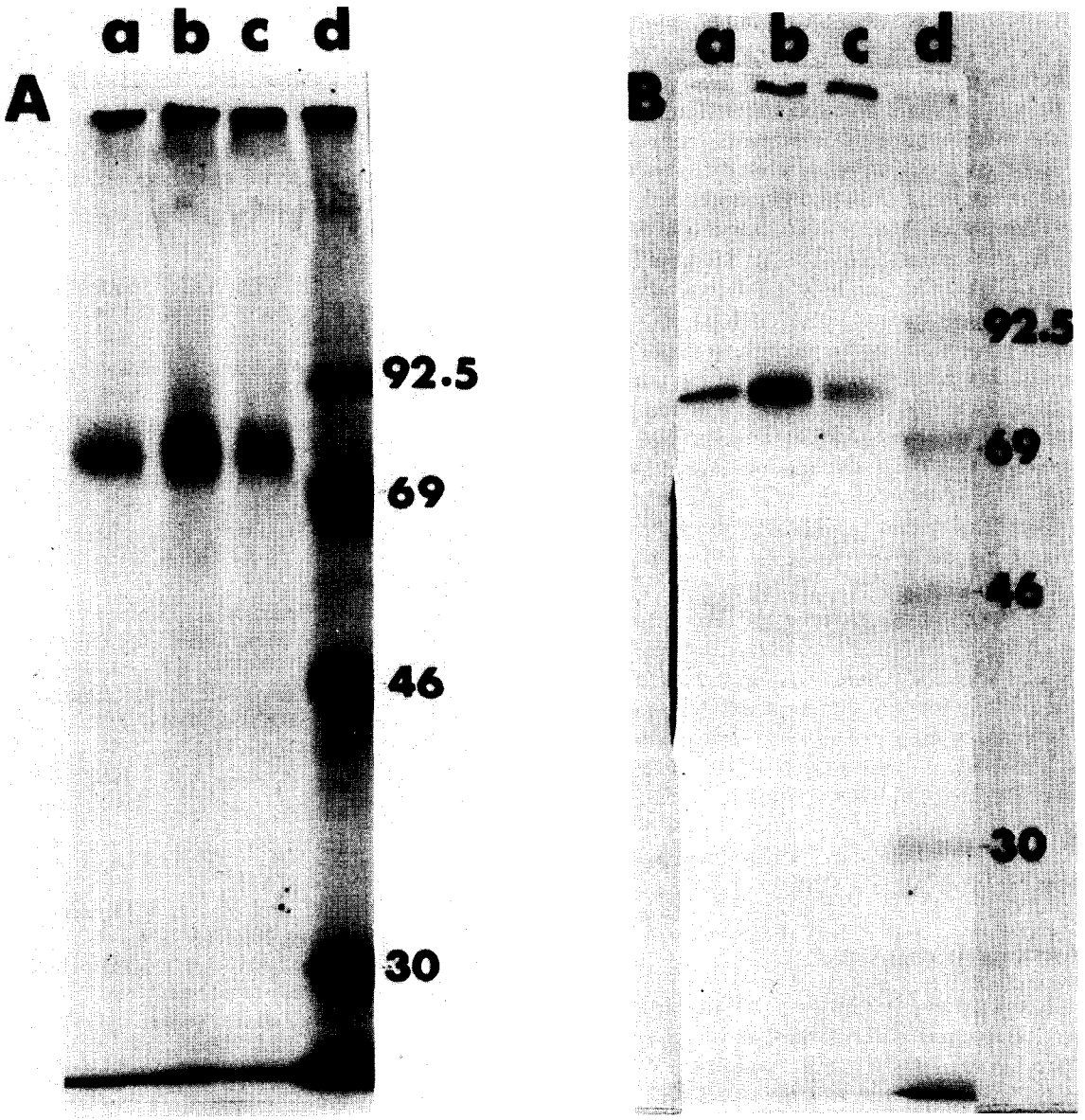


Fig. 3. SDS–polyacrylamide gel-electrophoresis demonstrating the differential effect of PB and β-NF on translatable NADPH cytochrome P450 reductase mRNA. Translation products (5×10^6 acid-precipitable cpm) of poly(A)$^+$ RNA (A) or total RNA (B) isolated from the livers of β-NF- (a), PB- (b) or un-treated (c) rats were incubated with 10 μl NADPH cytochrome P450 reductase antiserum. Samples were processed as in section 2.5. Numbers give M_r of the marker proteins ($\times 10^{-3}$) (d).

4. DISCUSSION

Treatment with β-NF decreases the amount of PB P450 in microsomal membranes [8]. Here, we demonstrate that β-NF treatment causes a corresponding decrease in the level of the translatable mRNA coding for this protein. This suggests that the decrease of PB P450, in response to β-NF is not due entirely to a passive 'dilution' of the protein caused by a proliferation of microsomal mem-

branes following xenobiotic treatment, but it involves a direct effect on PB P450 gene expression. The decrease of PB P450 mRNA by β-NF is unlikely to be due to some non-specific difference between the mRNA population isolated from β-NF-treated and control rats because no difference was found in the amount of NADPH cytochrome P450 mRNA present in these mRNA preparations.

The results demonstrate that the increase in the amount of NADPH cytochrome P450 reductase in microsomal membranes [10] caused by PB-treatment is mediated by an increase in the amount of translatable mRNA coding for this protein. An increase in NADPH cytochrome P450 reductase mRNA in response to PB-treatment was also reported in [16,17]. β-NF had no effect on the amount of NADPH cytochrome P450 reductase mRNA, indicating that in addition to inducing different cytochrome P450 variants, PB and β-NF have different effects on the expression of the genes for NADPH cytochrome P450 reductase.

The finding that PB increases the mRNAs coding for PB P450 and NADPH cytochrome P450 reductase to very different extents suggests that differences may exist in the molecular processes involved in the induction of these two mRNAs. However, in microsomal membranes, the molecular ratio of total cytochrome P450:NADPH cytochrome P450 reductase is not changed by PB-treatment [10].

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REFERENCES

[1] Conney, A.H. (1967) Pharmacol. Rev. 19, 317–366.
[2] Gelboin, H.V. (1967) Adv. Cancer Res. 10, 1–81.
[3] Estabrook, R.W. and Lindenlaub, E. eds (1979) The Induction of Drug Metabolism, Schattauer-Verlag, Stuttgart.
[4] Guengerich, F.P. (1979) Pharmac. Ther. 6, 99–121.
[5] Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gilette, J.R. and O'Brien, P.J. eds (1980) Microsomes, Drug Oxidations and Chemical Carcinogenesis, vol. 1 and 2, Academic Press, New York.
[6] Lu, A.Y.H. and West, S.B. (1980) Pharmacol. Rev. 31, 277–295.
[7] Thomas, P.E., Reik, L.M., Ryan, D. and Levin, W. (1981) J. Biol. Chem. 256, 1044–1052.
[8] Phillips, I.R., Shephard, E.A., Bayney, R.M., Pike, S.F., Rabin, B.R., Heath, R. and Carter, N. (1983) submitted.
[9] Pickett, C.B., Jeter, R.L., Morin, J. and Lu, A.Y.H. (1981) J. Biol. Chem. 265, 8815–8820.
[10] Shephard, E.A., Phillips, I.R., Bayney, R.M., Pike, S.F. and Rabin, B.R. (1983) submitted.
[11] Phillips, I.R., Shephard, E.A., Mitani, F. and Rabin, B.R. (1981) Biochem. J. 196, 839–851.
[12] Chirgwin, J.M., Przybla, A.E., MacDonald, R.J. and Rutter, W.G. (1979) Biochemistry 18, 5294–5299.
[13] Aviv, H. and Leder, J. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[14] Guengerich, F.P. and Martin, V.M. (1980) Arch. Biochem. Biophys. 205, 365–379.
[15] Shephard, E.A., Pike, S.F., Rabin, B.R. and Phillips, I.R. (1983) submitted.
[16] Gonzalez, F.J. and Kasper, C.B. (1980) Biochemistry 19, 1790–1796.
[17] Gonzalez, F.J. and Kasper, C.B. (1982) J. Biol. Chem. 257, 5962–5968.