Affinity purification of cellular retinoic acid-binding protein on 14-carboxy-13-*cis*-retinamide–Sephacrose 4B

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A biologically active bifunctional retinoid, ethyl 14-carboxyretinoate, has been synthesized and shown to bind cellular retinoic acid (RA)-binding protein (CRABP) via its free carboxy group. We describe herein the synthesis of 14-carboxy-13-*cis*-retinamide–Sephacrose 4B, which is an affinity matrix bearing an all-*trans*-RA moiety, and thus was used to purify and characterize CRABP from chick-embryo skin. An amide bond was first formed between the free carboxy group of the retinoid and a primary amino group of aminohexyl-Sephacrose 4B, by reaction with carbodi-imide, and the ester group of the resin-bound retinoid was then hydrolysed in an alkaline medium. Polyacrylamide-gel electrophoresis and f.p.l.c. Superose column-chromatographic analysis demonstrated that the affinity-purified CRABP (M_r 15000) was close to electrophoretic homogeneity (> 90%) and specifically interacts with RA. By using affinity gel chromatography, conversion of holo-CRABP into apo-CRABP by treatment with *p*-hydroxymercuribenzoate and a possible involvement of a thiol group in RA binding to CRABP were established. This affinity procedure provides several advantages: (i) 14-carboxy-13-*cis*-retinamide–Sephacrose exhibited high efficiency and selectivity for RA-binding protein (i.e. retinol- or fatty-acid-binding proteins did not bind); (ii) the presence of the amide linkage between the ligand and the matrix makes this affinity resin relatively stable to cytosolic enzymes; and (iii) other RA-binding proteins, e.g. nuclear receptor(s), may be purified.

INTRODUCTION

Retinoids control cell differentiation and are transported by specific retinoid-binding proteins (Lotan, 1980; Chytil & Ong, 1984). Cellular retinol- and retinoic acid-binding proteins (CRBP and CRABP respectively) are present in many tissues (Bashor *et al.*, 1973; Sani & Hill, 1974; Ong & Chytil, 1978) and appear to be involved in the metabolism and/or biological functions of retinoids (Roberts & Sporn, 1984). CRBP and CRABP have been isolated and characterized from tissue extracts by the following analytical methods: sucrose-gradient centrifugation (Sani & Hill, 1974), h.p.l.c. analysis using ion-exchange or size-exclusion chromatography (Bonelli & DeLuca, 1985; Shubeita *et al.*, 1986), polyacrylamide gel electrophoresis (Siegenthaler & Saurat, 1987) and dextran-charcoal adsorption (Trown *et al.*, 1980). A radioimmunoassay using polyclonal antibodies has been described for the quantification of these binding proteins (Ong *et al.*, 1982). Recently, nuclear receptors for RA (RA-inducible trans-activators) have been identified by recombinant-DNA techniques (Giguerre *et al.*, 1987; Brand *et al.*, 1988).

The high affinity of CRABP for retinoic acid (RA) suggested that this retinoid immobilized on an insoluble matrix might function as an affinity adsorbent for the purification and characterization of CRABP. The synthesis and biological evaluation of several terminal

bifunctional retinoids has been reported by Shealy *et al.* (1988). As expected, those bifunctional analogues in which both terminal groups are ester or amide do not bind to CRABP. However, the bifunctional retinoids that have a free 14-carboxy group either *trans* (as in RA) or *cis* (as in 13-*cis*-RA) bind effectively to CRABP (Shealy *et al.*, 1988). The binding affinity of these retinoids for CRABP suggested their potential use as affinity adsorbents. It appeared *a priori* that the bifunctional retinoid ethyl 14-carboxyretinoate (Scheme 1, structure I) might be coupled to aminohexyl (AH)-Sephacrose 4B through the 14-carboxy group. Alkaline hydrolysis of the protecting ester group of the coupled retinoid then provide an all-*trans*-RA unit attached covalently to the resin through an amide linkage formed between the 14-carboxy group of I and a primary amino group of AH-Sephacrose 4B. The affinity resin has been obtained as postulated and has been shown to provide a rapid and efficient procedure for purifying CRABP from chick-embryo skin and other tissues.

EXPERIMENTAL

Materials

DEAE-Sephadex (A-50), Sephadex G-100 and AH-Sephacrose 4B were purchased from Pharmacia, Piscataway, NJ, U.S.A. Affi-Gel Blue (100–200 mesh), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide

Abbreviations used: CRBP, cellular retinol-binding protein; RA, retinoic acid; CRABP, cellular retinoic acid-binding protein; PHMB, *p*-hydroxymercuribenzoate; DTT, dithiothreitol; f.p.l.c., fast protein liquid chromatography; AH-, aminohexyl; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride.

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Scheme 1. Synthesis and structure of the affinity resin

Ethyl 14-carboxyretinoate (I) was coupled to AH-Sepharose 4B by reaction with carbodi-imide to form II and then hydrolysed by alkali to yield the affinity matrix, 14-carboxy-13-*cis*-retinamide-Sepharose 4B (III). Details are given in the text.

hydrochloride (EDC) and electrophoresis reagents were from Bio-Rad Laboratories, Richmond, CA, U.S.A. [11,12-³H]Retinoic acid (3.6 Ci/mmol) was supplied by the National Cancer Institute, Bethesda, MD, U.S.A. [15-³H]Retinol (1.66 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. *p*-Hydroxymercuribenzoate (PHMB) and dithiothreitol (DTT) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of analytical grade. Locally supplied fertilized eggs were used as a source for embryonic-chick skin.

Synthesis of 14-carboxy-13-*cis*-retinamide-Sepharose 4B

Scheme 1 shows for synthesis of 14-carboxy-13-*cis*-retinamide coupled to Sepharose 4B. Ethyl 14-carboxyretinoate (Shealy *et al.*, 1988) (30 mg, purity 95%) was dissolved in 1.5 ml of dioxan. The ligand solution was adjusted to 30% dioxan with distilled water and the pH was adjusted to 4.5 with 1 M-HCl. AH-Sepharose 4B (2.5 g) was swelled in 0.5 M-NaCl for 15 min and washed repeatedly with water. A gel suspension (10 ml) was made in water with the pH adjusted to 4.5. The ligand solution was mixed with the gel suspension to give a final 2:1 ratio (ligand/gel). The coupling reaction was started by slowly adding dropwise a solution (100 mg/ml) of

EDC over a period of 15 min to give a final concentration of 0.1 M. The mixture was continuously rotated, the pH of slurry was maintained between 4.5 and 5.0 for the first 2 h, and the mixture was incubated overnight at room temperature. Any remaining activated sites on Sepharose were blocked by a further 3 h reaction with carbodi-imide. The gel was thoroughly washed on a sintered-glass funnel with 30% dioxan in water and with 30% dioxan in sodium phosphate buffer, pH 7.2. The ester protecting group of 14-carboethoxy-13-*cis*-retinamide attached to Sepharose 4B (structure II) was then hydrolysed by alkali. Solid KOH was added to the gel suspension to achieve a 1:1.25 molar ratio of ligand/KOH and the reaction was allowed to proceed for 2 h at 37 °C, followed by 8–10 h at room temperature. Finally, the resin was washed extensively with water and equilibrated with 0.05 M-phosphate buffer, pH 7.2, containing 0.01% NaN₃ for storage at 4 °C. The structure (III) of 14-carboxy-13-*cis*-retinamide-Sepharose 4B is shown in Scheme 1. All steps were performed under subdued light.

Preparation of cytosol

The embryonic skin taken from 12–13 day-old chick embryos was homogenized in 3 vol. of TE buffer (30 mM-Tris/HCl/1 mM-EDTA, pH 7.4), and the cytosolic frac-

tion was obtained by ultracentrifugation at 105000 *g* for 60 min at 4 °C. To achieve higher recovery and purification of CRABP, the cytosol was subjected to DEAE-Sephadex (A-50), Sephadex G-100 or Affi-Gel Blue chromatography as described previously (Sani & Banerjee, 1978; Sani *et al.*, 1980*a*). In another experiment, the cytosol samples were treated with 10 mM-PHMB for 60 min at 4 °C, followed by incubation with 50 mM-DTT for 45 min. This protocol, as reported previously, caused the dissociation of RA-CRABP complexes and thus converted existing holo-CRABP into apo-CRABP (Shubeita *et al.*, 1986; Sani *et al.*, 1980*a,b*). Cytosolic protein was determined by the method of Lowry *et al.* (1951).

Purification of CRABP on affinity resin

A Bio-Rad Econo column (1 cm × 10 cm) was packed with 7.5 ml of 14-carboxy-13-*cis*-retinamide-Sepharose 4B gel and equilibrated with 0.05 M-sodium phosphate buffer, pH 7.4. Partially purified cytosol samples (0.5–2 ml, 20 mg/ml) were loaded on the affinity column and washed with 50 ml of phosphate buffer containing 50 mM-NaCl (flow rate 1–2 ml/h). The elution was performed with 0.5 M-NaCl in 0.05 M-phosphate buffer, pH 6.0, and 0.5 ml fractions were collected. In pilot experiments, the elution profile of CRABP was monitored by using phosphate buffer containing 10 mM-PHMB or 0.5 mM-NaSCN. The concentration of CRABP in various fractions was determined after incubation with [³H]RA by the dextran-charcoal method described below. All steps were done at 4 °C and in subdued light. After each affinity-chromatographic experiment, the resin was regenerated by washing with 1 M-NaCl in phosphate buffer, pH 7.4. The affinity column can be recycled at least six times without apparent loss of CRABP-binding capacity.

Measurement and analysis of CRABP

The concentration of CRABP in partially purified and affinity-purified preparations was determined by the dextran-charcoal assay. Aliquots (0.3 ml) were incubated with 150 pmol of [³H]RA overnight at 4 °C. Non-specific binding was measured in the presence of 200-fold molar excess of the ligand. Bound radioligand was separated from the free ligand by adding an equal volume of 2.5 % (w/v) dextran (T-70)-coated charcoal in phosphate buffer, pH 7.4, containing 0.1 % gelatin. After 10 min, the charcoal suspension was centrifuged, filtered through 0.22 µm-pore-size filters (Gelman Acrodisc) and the radioactivity determined.

To determine the molecular mass of affinity-purified protein, samples (10–25 µg), labelled with 150 pmol of [³H]RA in the absence or presence of 200-fold excess of ligand or pretreated with 2 mM-PHMB and 10 mM-DTT as described above, were applied to a gel-filtration column (Superose H-12, 10/30) equilibrated with 30 mM-phosphate buffer, pH 7.4, containing 0.15 M-NaCl and attached to an automated f.p.l.c. system (flow rate 1 ml/min). Fractions (1 ml) were collected and the radioactivity was determined. Affinity-purified CRABP preparations were also analysed by vertical-10 % (w/v)-polyacrylamide-slab-gel electrophoresis in the presence of SDS according to Laemmli's (1971) method. Cytochrome *c* (*M_r* 12400) or lysozyme (*M_r* 14400) was used as an external standard.

Fig. 1. Retention of CRABP on 14-carboxy-13-*cis*-retinamide-Sepharose 4

Partially purified chick-embryo cytosol samples (15 ml, 1 mg/ml in phosphate buffer, pH 7.4) were passed through the affinity column, and the flow-through fractions (0.5 ml) were incubated with [³H]RA to measure the non-retained CRABP levels by the dextran-charcoal assay as described in the text. Non-specific binding was determined and subtracted by parallel incubations in the presence of a 200-fold molar excess of RA. Curve A, untreated cytosol; curve B, cytosol pretreated with PHMB/DTT; curve C, cytosol prelabelled with 150 pmol of [³H]RA before it was applied to the column. Results are expressed as the percentage of [³H]RA binding of the unfractionated cytosol; 100 % specific binding: curves A and B, 2500 and 3000 d.p.m. respectively; curve C, 1900 d.p.m.; non-specific binding was 150–300 d.p.m.

RESULTS

A terminal bifunctional retinoid, ethyl 14-carboxy-retinoate (Scheme 1, structure I), prepared by Shealy *et al.* (1988), was used to synthesize an affinity resin for the purification and characterization of CRABP. This retinoid is an analogue of both ethyl all-*trans*-retinoate and 13-*cis*-RA. Through the action of a water-soluble carbodi-imide, an amide bond was formed between the free carboxy group of the retinoid and a primary amino group of AH-Sepharose 4B. The Sepharose-bound retinoid (structure II, Scheme 1) comprised both a 13-*cis*-retinamide and an ethyl ester of all-*trans*-RA; the ester group was then hydrolysed in an alkaline medium. Thus a matrix was obtained in which all-*trans*-RA groups are attached covalently to the resin through an amide bond at position 14 (Scheme 1, structure III). With its free 14-carboxy group, this affinity resin (Fig. 1) would, therefore, act as a high-affinity ligand for CRABP. Although we have found that formation of all-*trans*-retinamides from all-*trans*-RA using carbodi-imides has resulted in less than 5 % isomerization of the 13-*cis*-isomers during the

Fig. 2. Chromatography of CRABP on an affinity column

Conditions were as described in the text. The arrow indicates the shift to the elution buffer (0.5 M-NaCl/0.05 M-sodium phosphate, pH 6.0). ●, $[^3\text{H}]\text{RA-CRABP}$ radioactivity; ○, A_{280} .

present coupling reaction, some isomerization at the $\text{C}^{13}=\text{C}^{14}$ double bond could have occurred. However, this may not affect the CRABP-binding capacity of the resin, since both 13-*cis*- and all-*trans*-RA bind CRABP with approximately equal affinity (Sani & Hill, 1976).

Retention capacity of the affinity gel for CRABP

The effectiveness of 14-carboxy-13-*cis*-retinamide-Sepharose 4B as an affinity matrix for CRABP binding was determined with partially purified cytosol preparations from chick-embryo skin. Partial purification of cytosol by gel-filtration, ion-exchange or Affi-Gel Blue chromatography was required before the affinity step for the removal of interfering proteins like serum albumin, which may interact with RA. Partially purified cytosol (15 ml; 1 mg/ml) was applied to a mini affinity column (1 ml) at a flow rate of 5 ml/h, and the flow-through fractions were assayed for $[^3\text{H}]\text{RA-CRABP}$ activity by the dextran-charcoal assay. The concentration of CRABP in the initial 1–15 flow-through fractions was reduced to 15% of the totally loaded CRABP, indicating that the remaining 85% was retained on the affinity column (Fig. 1, curve A). As more of the cytosol preparation was loaded on the column, the CRABP activity in the later fractions (16–25) increased up to 55%, suggesting that the CRABP-binding sites of the affinity resin were approaching a saturated state. Furthermore, if the cytosol was pretreated with PHMB and DTT (to dissociate RA from the protein complex and to regenerate the RA-binding site on CRABP), about 5% more CRABP (apo form) was retained by the affinity resin, as indicated by lower CRABP levels in the effluent fractions 1–15 (Fig. 1, curve B). In order to verify the requirement of apo-CRABP for binding to the resin, the cytosol was labelled with $[^3\text{H}]\text{RA}$ before application to

the affinity column. Almost all of the $[^3\text{H}]\text{RA-CRABP}$ applied to the column was recovered in the initial flow-through fractions (Fig. 1, curve C). Thus the capacity of the affinity resin to bind CRABP depended on the ligand (RA), which, if prebound to protein (holo form), blocked its adsorption to the affinity column. This observation also suggested that CRABP (apo form) was bound selectively with high efficiency to 14-carboxy-13-*cis*-retinamide gel. CRBP samples prelabelled with $[^3\text{H}]\text{-retinol}$ were not retained by the affinity column (results not shown).

Elution of CRABP from the affinity column

Several methods were tested for eluting the bound CRABP (from chick-embryo skin) from the affinity column. Elution with phosphate buffer, pH 7.4, containing 10 mM-PHMB was inefficient. However, 30–40% of bound CRABP could be eluted with 0.5 M-NaCl/phosphate buffer, pH 7.4. Most of the CRABP bound to the affinity resin was satisfactorily eluted by lowering the pH of the 0.5 M-NaCl/phosphate buffer from 7.4 to 6.0, the best yields obtained being between 0.3 and 0.9 mg of CRABP. An alternative elution method utilizing a chaotropic salt, 0.5 M-NaSCN, was also efficient; however, some loss of activity was noted (results not shown).

The elution profiles of proteins and CRABP measured after affinity chromatography of partially purified cytosol (20 mg/ml) are shown in Fig. 2. The $[^3\text{H}]\text{RA}$ -binding activity in flow-through fractions possibly represented pre-existing holo-CRABP in the cytosol. Most of the other proteins were eluted from the column during sample application and subsequent washing with phosphate buffer, pH 7.4, containing 50 mM-NaCl. Up to 75 μg of CRABP was adsorbed per ml of affinity gel. Eluate fractions containing the maximum CRABP levels were

Table 1. Purification of CRABP from chick-embryo skin preparations

Portions (0.3–0.5 ml) of CRABP were incubated with 150 pmol of [3 H]RA in the absence or presence of 200-fold molar excess of RA overnight at 4 °C and specific binding was determined by the dextran–charcoal assay (0.25 % dextran/2.5 % Norit A).

Preparation	Total protein (mg)	$10^{-3} \times$ [3 H]RA–CRABP radioactivity (d.p.m./mg)	Purification (fold)
Cytosol	690	16.8*	1
DEAE-Sephadex (A-50)	228	30.7	1.8
Affi-Gel Blue	189	52.4	3.1
Sephadex G-100	36	98.5	5.9
Affinity resin	0.54	8450.6	503

* Value represents the specific binding of CRABP corrected for non-specific binding of [3 H]RA to other proteins in cytosol.

Fig. 3. Characteristics of the affinity-purified CRABP

Samples of purified CRABP (10–25 μ g) were analysed by an f.p.l.c. Superose column and (inset) by SDS/polyacrylamide-gel electrophoresis to determine the purity and M_r (lane 1, partially purified cytosol; lane 2, affinity-purified CRABP). Protein markers used were cytochrome *c* (M_r 12400) and lysozyme (M_r 14400). ●, [3 H]RA; ○, 200-fold molar excess of RA; △, 2 mM-PHMB.

pooled and stored frozen at –20 °C for further analysis. Data from a typical purification procedure are presented in Table 1. The affinity-purified CRABP pool contained a 500-fold higher concentration of CRABP than did the whole cytosol, and the recovery of binding protein was about 15 %. The first step involving DEAE-Sephadex, Sephadex G-100 or Affi-Gel Blue chromatography was quite effective in removing certain contaminating proteins, such as serum albumin, so that the recovery of CRABP from the affinity column was higher.

Characteristics of CRABP from the affinity column

The M_r as well as the specificity of the affinity-purified

CRABP from chick embryo was assessed on a Superose H-12 (gel-filtration) column attached to an f.p.l.c. system (Fig. 3). A sharp peak of [3 H]RA–CRABP radioactivity was observed in fractions 14–16, and its apparent native M_r was 15000. This radioactive peak was abolished in the presence of 200-fold molar excess of RA or by pretreatment of the cytosol with PHMB (Fig. 3). This finding confirmed the ligand specificity of CRABP.

The purity of CRABP from the affinity column was tested on discontinuous polyacrylamide slab gels under denaturing conditions. As shown in the inset to Fig. 3, purified CRABP gave a major band, thus reflecting the high degree of purification (about 90 %). By using external standards, an M_r of 15000 was obtained for the denatured protein. A minor band of M_r about 50000 was also observed in a few of the affinity-purified preparations.

DISCUSSION

In the present paper we have described the synthesis and application of 14-carboxy-13-*cis*-retinamide–Sepharose 4B, which is an affinity matrix bearing all-*trans*-RA moiety, and the use of this affinity resin to purify and characterize CRABP from chick-embryo skin. This affinity procedure provides several advantages: (i) 14-carboxy-13-*cis*-retinamide–Sepharose has high efficiency and selectivity for the RA-binding protein, (ii) the presence of an amide linkage between the ligand and the matrix makes this affinity resin relatively stable against cytosolic enzymes, and (iii) the affinity resin is easy to prepare.

Available procedures to isolate and determine CRBP and CRABP in tissue extracts include the classical sucrose-gradient analysis, h.p.l.c. size-exclusion chromatography and gel electrophoresis (Sani & Hill, 1974; Bonelli & DeLuca, 1985; Siegenthaler & Saurat, 1987). These methods require the treatment and/or removal of free radioactive retinoid before analysis. The principal advantage of the present procedure of CRABP purification is the specificity of the affinity-chromatographic step using 14-carboxy-13-*cis*-retinamide–Sepharose 4B. In addition to high recovery and rapidity of purification, this affinity procedure affords CRABP having a purity of more than 90 %. Since the affinity material is specifically

designed for CRABP, other proteins with similar M_r values and charges, such as CRBP and fatty-acid-binding protein, do not bind to the affinity matrix.

The purification procedure described here was used to prepare about 1 mg of CRABP from 60–70 g of chick-embryo skin. In this two-step protocol it is important to remove other RA-binding proteins, such as serum albumin, before the affinity step to achieve higher recovery and purity of CRABP. The degree of purification and recovery of CRABP by this method is comparable with those obtained by previously reported methods consisting of several separation steps (Ong & Chytil, 1978; Bonelli & DeLuca, 1985; Sani & Banerjee, 1978). As judged from the results of polyacrylamide-gel electrophoresis and f.p.l.c. Superose analysis, the affinity-purified CRABP is close to electrophoretic homogeneity. It co-migrates specifically with bound [3 H]RA on a Superose column, and its M_r corresponds to 15000 reported by this and other laboratories (Sani & Banerjee, 1978; Ong & Chytil, 1978). The conversion of holo-CRABP into apo-CRABP by PHMB treatment (Shubeita *et al.*, 1986; Sani *et al.*, 1980a,b) and a possible involvement of an SH group in RA binding to CRABP are also confirmed by this affinity-chromatographic procedure.

In conclusion, a rapid and efficient affinity-chromatographic method is now available which allows purification of CRABP from tissue extracts. Purification of the nuclear receptor(s) for RA (Giguere *et al.*, 1987; Brand *et al.*, 1988) by the use of this affinity gel is now required. Availability of pure CRABP and, possibly, nuclear receptor(s) by this procedure will be useful in studies dealing with the biological functions of these binding proteins.

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REFERENCES

- Bashor, M. M., Toft, D. O. & Chytil, F. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3483–3487
- Bonelli, F. C. & DeLuca, L. M. (1985) *Anal. Biochem.* **147**, 251–257
- Brand, N., Petkovich, M., Krust, A., Chambon, P., Thè, H., Marchio, A., Tiollais, P. & Dejean, A. (1988). *Nature (London)* **332**, 850–853
- Chytil, F. & Ong, D. E. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. & Goodman, D. S., eds.), vol. 2, pp. 90–125, Academic Press, New York
- Giguere, V., Ong, E. S., Segui, P. & Evans, R. M. (1987) *Nature (London)* **330**, 624–629
- Laemmli, U. K. (1971) *Nature (London)* **227**, 680–685
- Lotan, R. (1980) *Biochim. Biophys. Acta* **604**, 33–91
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275
- Ong, D. E. & Chytil, F. (1978) *J. Biol. Chem.* **253**, 4551–4554
- Ong, D. E., Crow, J. A. & Chytil, F. (1982) *J. Biol. Chem.* **257**, 13385–13389
- Roberts, A. B. & Sporn, M. B. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. & Goodman, D. S., eds.), vol. 2, pp. 210–286, Academic Press, New York
- Sani, B. P. & Banerjee, C. K. (1978) *Biochem. J.* **173**, 643–649
- Sani, B. P. & Hill, D. L. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1276–1282
- Sani, B. P. & Hill, D. L. (1976) *Cancer Res.* **36**, 409–413
- Sani, B. P., Condon, S. M., Brockman, R. R., Weiland, L. H. & Schutt, A. J. (1980a) *Cancer* **45**, 1199–1209
- Sani, B. P., Condon, S. M. & Banerjee, C. K. (1980b) *Biochem. Biophys. Acta* **624**, 226–236
- Shealy, F. Y., Krauth, C. A., Riordan, J. M. & Sani, B. P. (1988) *J. Med. Chem.* **31**, 1124–1130
- Shubeita, H. E., Patel, M. D. & McCormick, A. M. (1986) *Arch. Biochem. Biophys.* **247**, 280–288
- Siegenthaler, G. & Saurat, J. H. (1987) *Eur. J. Biochem.* **166**, 209–214
- Trown, P. W., Palleroni, A. V., Bohoslawec, O., Richelo, B. N., Halpern, J., Gizzi, N., Geiger, R., Lewinski, C., Machlin, L., Jetten, A. & Jetten, M. E. R. (1980) *Cancer Res.* **40**, 212–220

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