Biochimica et Biophysica Acta, 758 (1983) 58–69
Elsevier

BBA 21481

SUBCELLULAR LOCALIZATION OF DIAMINE OXIDASE IN RABBIT KIDNEY CORTEX

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(Received December 13th, 1982)

Key words: Subcellular localization; Diamine oxide; (Rabbit kidney cortex)

The intracellular localization of diamine oxidase (EC 1.4.3.6) in rabbit kidney cortex was studied. The distribution of diamine oxidase in the subcellular fractions, obtained by modifying the classical method of Wattiaux-De Coninck, S., Rutgeerts, M.T. and Wattiaux, R. (Biochim. Biophys. Acta (1965) 105, 446–459) demonstrated that this activity is concentrated (> 60%) in the microsomal fraction. Biochemical and morphological data indicate a 20–30% contamination of this fraction by plasma membrane and brush border fragments. Subfractionation of the microsomes, obtained by centrifuging in a continuous sucrose-Ficoll gradient (d 1.038–1.064) for 75 min, showed that diamine oxidase is concentrated in membrane deriving from the endoplasmic reticulum. In fact the bulk of diamine oxidase activity was recovered in a subfraction of the gradient which was shown both biochemically and morphologically to derive from the endoplasmic reticulum. The possible significance of this result is discussed.

Introduction

Diamine oxidase (histaminase; amine:O_2 oxidoreductase deaminating, EC 1.4.3.6) catalyzes the oxidative deamination of histimine and of aliphatic diamines, such as cadaverine and putrescine [1,2], according to the following reaction:

$$NH_2\text{-}(CH_2)_n\text{-}NH_2 + O_2 + H_2O \rightleftarrows NH_2\text{-}(CH_2)_{n-1}\text{-}CHO + NH_3 + H_2O_2$$

The enzyme belongs to the wide class of amine oxidases which were separated into two types, monoamine oxidases and diamine oxidases by Zeller [1] according to their substrate specificity. However it is now preferable to distinguish the amine oxidases according to their prosthetic groups: FAD-amine oxidases or 'true' monoamine oxidases and copper-amine oxidases or diamine oxidase-type [3].

Copper-amine oxidase are inhibited by carbonyl reagents, and guanidine derivatives [4,5], while FAD-amine oxidases are not.

Despite the fact that diamine oxidase was discovered more than 50 years ago by Best [6] and is present in various normal and neoplastic tissues [7,8] its physiological role still remains obscure, although recent data suggest that it may represent a mechanism of control of intracellular polyamine levels [9,10].

The mammalian kidney contains a significant amount of diamine oxidase [11–13], which has been immunohistochemically localized in proximal convoluted tubules of pig kidney [14–16].

An important clue of the understanding of the physiological role of diamine oxidase may come from its intracellular localization. The aim of this work was to study the intracellular localization of the diamine oxidase in rabbit kidney cortex. This material was chosen since it contains a large amount of proximal convoluted tubules [17] and higher diamine oxidase activity than the same tissue from other species [12].

Materials and Methods

Animals

Adult rabbits of both sexes, weighing 2.5–3 kg, starved for 20–24 h were killed by cervical dislocation. The kidneys were immediately excised and washed in ice-cold 0.25 M sucrose. The cortex tissue was obtained by dissecting transverse sections of the decapsulated kidney and was immediately weighed. All manipulations were carried out at 0–4°C.

Tissue fractionation

(A) In a first series of experiments, tissue from both kidneys (8–10 g) was homogenized in 0.25 M sucrose, containing 1 mM EDTA, by 4–5 up-and-down strokes with a motor-driven (1000 rpm) loose-fitting teflon pestle (clearance 250 μm) of a Thomas homogenizer (A.H. Thomas Co; Philadelphia, USA).

The homogenate (20%, w/v) was filtered through two layers of surgical gauze and centrifuged at $600 \times g$ for 10 min at 4°C.

The pellet was washed four times by rehomogenizing and recentrifuging it in the above-mentioned conditions.

The last pellet was resuspended in 0.25 M sucrose/1 mM EDTA (1 g of the original kidney cortex/ml), yielding the 1 : 1 nuclear fraction.

Supernatants obtained from centrifugations were pooled and made up to volume to yield the 1 : 10 cytoplasmic extract.

Fractionation of the cytoplasmic extract was performed according to Wattiaux-De Coninck et al. [18] with some modifications: four particulate fractions were isolated using a Spinco-Beckman model L5-50 ultracentrifuge operating with a 50 Ti rotor: a heavy mitochondrial fraction ($3300 \times g$, 10 min); a light mitochondrial fraction ($25\,000 \times g$, 10 min); a fluffy layer fraction corresponding to the pink, loosely-packed layer which overlies the dark brown, tightly-packed, $25\,000 \times g$ pellet: this layer was carefully separated from the light mitochondrial pellet, gently resuspended in 0.25 M sucrose/1 mM EDTA and spun down at $25\,000 \times g$ for 10 min; finally a microsomal fraction ($105\,000 \times g$ 60 min) and a soluble fraction i.e. the final supernatant were obtained. Each particulate fraction was washed once in 10 ml of 0.25 M sucrose/1 mM EDTA and resuspended 1 : 1 in the same medium.

(B) In a second series of experiments, homogenization was performed according to Jakobsson [19] in 3 mM Tris-HCl buffer pH 7.5, with a Thomas glass-teflon homogenizer (pestle clearance 250 μm), using 6–7 strokes at 650 rpm.

After homogenization the sucrose concentration was brought to 0.25 M by adding 1 M sucrose to the homogenate. The homogenate (20%, w/v) was filtered through two layers of surgical gauze and centrifuged at $10\,000 \times g$ for 20 min in a 50 Ti Spinco-Beckman rotor to get rid of cell debris, nuclei, mitochondria, lysosomes and peroxisomes. The $10\,000 \times g$ supernatant was aspirated off with a Pasteur pipette, avoiding contamination with the pellet, particularly with the fluffy white layer overlapping the pellet, most probably constituted by large plasma membrane fragments.

The microsomal fraction was obtained by centrifuging the $10\,000 \times g$ supernatant over a cushion of 1.6 M sucrose (5 ml postmitochondrial supernatant over 3.5 ml 1.6 M sucrose) at $105\,000 \times g$ for 30 min [19].

The material concentrated at the 0.25–1.6 M sucrose interphases was removed from the centrifuge tube by aspirating it with a pipette avoiding contamination by the supernatant and was diluted with 3 mM Tris-HCl buffer, pH 7.5, to a tissue concentration of 0.3–0.4 g wet weight/ml and to a sucrose concentration of 6% ($d_0 = 1.030$). This fraction, referred to as total 'cushin' microsomes, was subfractionated on continuous linear sucrose-Ficoll gradients, prepared according to Jakobsson [19]. These gradients consisted of 10% (w/v) sucrose throughout and ranged from 0% Ficoll (Pharmacia, Uppsala, Sweden) at the top to 8% (w/v) Ficoll at the bottom; their density ranged from 1.038 to 1.064.

At the top of the gradient, whose total volume was 28 ml, 8 ml of the total 'cushion' microsomes were layered. The gradients were centrifuged at $50\,000 \times g$ for 75 min, in 38.5 ml centrifuge tubes of a SW 27 Spinco-Beckman rotor.

Fractions were collected by puncturing the bottom of the tube just above the edge of the pellet and collecting dropwise for subfractions.

Subfraction 1 consisted of the bottom 9 ml of the gradient, subfraction 2 consisted of the next 7

ml, subfraction 3 consisted of the following 9 ml and subfraction 4 of the top 11 ml. Pellets and subfractions from three parallel gradients were always pooled. The pellets, resuspended in 10 ml of 0.25 M sucrose, as well as the subfractions, were centrifuged at 150 000 × *g* (50 Ti Spinco-Beckman rotor) for 120 min. The final pellets obtained were resuspended (2 mg protein/ml) in ice-cold 0.25 M sucrose/1 mM EDTA and immediately assayed for protein and enzymes.

Recoveries of protein content and enzymatic activities were calculated with respect to total 'cushion' microsomes, centrifuged (150 000 × *g* for 120 min) and resuspended as the gradient subfractions.

Chemical assays

Proteins were determined according to Lowry et al. [20], using bovine serum albumin as standard. Inorganic phosphate was assayed by the method of Fiske and SubbaRow [21].

Enzyme assays

Cytochrome oxidase (EC 1.9.3.1.) was measured as described by Warton and Tzagoloff [22]; calculations were performed according to Rieske [23].

Acid phosphatase (EC 3.1.3.2) was assayed on frozen and thawed fractions, in the presence of 0.1% Triton X-100 [18] with *p*-nitrophenylphosphate as substrate [24].

Catalase (EC 1.11.1.6.) was measured by the spectrophotometric method of Lück [25].

Alakaline phosphatase (EC 3.1.3.1.) was assayed by the method of Shibko and Tappel [26].

For measuring $(Na^+ + K^+)$-ATPase (EC 3.6.1.4.), the different fractions were made 0.1% with respect to sodium deoxycholate [27] and assayed according to Bonting [28]. The $(Na^+ + K^+)$-ATPase activity was calculated as the difference in activity obtained from incubations in the presence of Na^+, K^+ and Mg^{2+} and in the presence of Mg^{2+} alone.

The $(Na^+ + K^+)$-ATPase activity was almost completely inhibited by the addition of 1 mM ouabain to the incubation medium. Glucose-6-phosphatase (EC 3.1.3.9.) was assayed according to Harper [29].

NADH-cytochrome *c* reductase (EC 1.6.2.1.) and NADPH-cytochrome *c* reductase (EC 1.6.2.3.) activities were measured by the method of Dallner [30]. The extinction coefficient used for calculation was $18.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [31].

Monoamine oxidase (EC 1.4.3.4.) and diamine oxidase (EC 1.4.3.6.) activities were assayed in dialyzed fractions as previously reported [32]. Blanks were performed in the presence of 10^{-3} M aminoguanidine (for diamine oxidase), or 10^{-3} M pargyline (for monoamine oxidase), or in the absence of substrate.

Enzyme units. 1 unit of enzymatic activity is the amount of enzyme which changes 1 μmol of substrate per min, excepts for catalase which is expressed as μg of enzyme per g of kidney cortex with reference to the catalytic constant of a standard sample of purified enzyme (bovine liver, Boheringer) measured in the same conditions. For monoamine oxidase and for diamine oxidase activities 1 unit corresponds to the amount of enzyme which oxidizes 1 nmol of substrate per min.

Electron microscopy

Fractions were fixed in suspension by adding an equal amount of 5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, and centrifuged to obtain tight pellets. Small pieces were postfixed with 1% OsO_4 in the same buffer, dehydrated with graded ethanol and embedded in Epon.

Ultrathin sections were cut on a LKB Ultratome III, stained with uranyl acetate and lead citrate and examined with a Philips E 400 electron microscope.

Chemicals

Cytochrome *c*, NADH, NADPH, horse-radish peroxidase, bovine liver catalase were from Boheringer. Tris-ATP, glucose 6-phosphate, histamine dihydrochloride, cadaverine dihydrochloride, putrescine dihydrochloride, tyramine hydrochloride, *p*-nitrophenylphosphate, disodium salt, ouabain and *o*-dianisidine base were from Fluka. *o*-dianisidine hydrochloride was obtained by recrystallizing *o*-dianisidine base, as described by Bergmeyer and Bernt [33]. Benzylamine hydrochloride, aminoguanidine hemisulfate and pargyline hydrochloride were from Sigma.

All other chemicals were of reagent grade and used without further purification.

TABLE I

INTRACELLULAR DISTRIBUTION OF PROTEIN AND ENZYMES IN RABBIT KIDNEY CORTEX

Absolute values are given as mg/g wet weight of kidney cortex for protein and as U/g for enzymes. E, cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; F, fluffy layer fraction; P, microsomal fraction; S, final supernatant. Number of experiments in parentheses. All values are given as mean ±S.D.

		E+N	N	M	L	F	P	S	Recovery (%)
		(absolute values)			(percentage values)				
Protein	(5)	127.00± 16.0	18.61±3.6	24.10±6.3	3.20±0.3	7.14±0.9	12.25±3.8	35.68±4.0	100.89± 5.1
Cytochrome oxidase	(5)	53.73± 15.5	13.64±2.7	50.17±2.7	19.29±6.0	3.91±1.1	1.66±0.7	0	88.67± 4.3
Monoamine oxidase (tyramine)	(5)	87.35± 9.1	9.90±2.9	50.07±2.5	16.46±6.6	10.53±1.2	11.00±1.7	0	97.88± 8.0
Monoamine oxidase (benzylamine)	(5)	145.29± 30.6	10.36±2.8	59.36±4.3	16.17±7.4	9.97±2.6	10.03±3.5	0	104.16± 9.8
Catalase	(5)	838.95±144.9	10.61±3.8	17.91±2.6	13.84±0.2	2.15±0.9	1.43±0.4	48.22±5.5	94.16± 9.8
Acid phosphatase	(5)	0.97± 0.1	18.45±2.0	13.90±2.0	11.00±3.1	12.21±2.5	11.50±1.2	26.26±3.3	93.32± 3.7
Alkaline phosphatase	(5)	10.20± 1.6	15.78±4.3	16.66±2.0	2.94±0.5	23.50±1.4	31.15±1.4	6.66±2.2	101.69±14.1
$(Na^+ + K^+)$-ATPase	(5)	28.13± 1.6	9.92±1.4	28.17±5.1	1.70±0.5	23.57±2.3	21.21±2.3	10.61±1.3	94.57± 3.4
Glucose-6-phosphatase	(5)	3.63± 1.1	13.44±1.9	19.70±1.6	5.12±1.3	22.05±4.1	36.75±8.1	1.12±0.3	98.18±10.1
NADPH cytochrome *c* reductase	(3)	4.14± 0.5	10.10±1.1	31.10±2.5	4.40±0.7	15.80±2.4	25.30±3.4	8.50±1.5	95.20± 7.0
NADH cytochrome *c* reductase	(3)	17.40± 2.3	21.50±2.3	29.10±3.7	9.70±1.2	10.50±2.1	25.20±3.4	8.45±1.3	104.50± 6.6
Diamine oxidase (histamine)	(7)	17.60± 5.2	10.91±3.1	8.15±2.0	1.70±0.7	14.65±2.7	67.68±7.7	0	103.10± 6.4
Diamine oxidase (cadaverine)	(7)	15.98± 3.1	13.12±3.5	7.92±1.9	2.87±1.1	13.20±3.1	62.03±6.8	0	99.14± 7.3

Results

Table I shows the percentage distribution and recovery of protein, marker enzymes and diamine oxidase in the fractions obtained according to Wattiaux-De Coninck et al. [18].

Absolute values are the sum of the values obtained separately on the nuclear fraction and cytoplasmic extract, taken as being representative of the whole tissue [34].

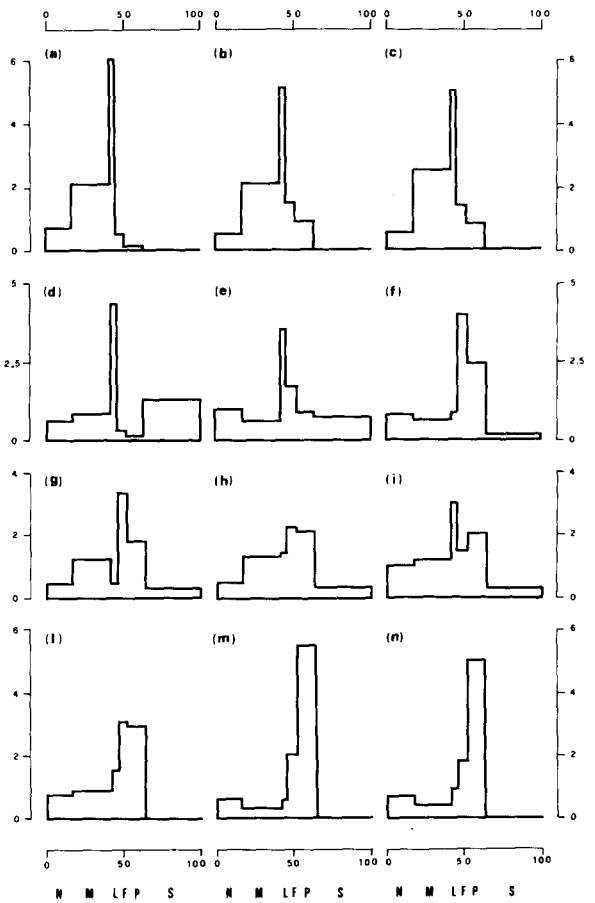

Fig. 1. Distribution patterns of enzymes. Ordinate: mean relative specific activity of fractions (percentage of total recovered activity in the fraction/percentage of total recovered protein in the fraction). Abscissa: relative protein content of fractions, represented in the order in which they are isolated; from left to right: nuclear (N), heavy mitochondrial (M), light mitochondrial (L), fluffy layer (F), microsomal (P) and final supernatant (S). (a) cytochrome oxidase; (b) monoamine oxidase, (substrate tyramine); (c) monoamine oxidase, (substrate benzylamine); (d) catalase; (e) acid phosphatase; (f) alkaline phosphatase; (g) $(Na^+ + K^+)$-ATPase; (h) NADPH-cytochrome *c* reductase; (i) NADH-cytochrome *c* reductase; (l) glucose-6-phosphatase; (m) diamine oxidase, (substrate histamine); (n) diamine oxidase, (substrate cadaverine).

Fig. 1 shows the distribution patterns of enzymes presented as proposed by de Duve et al. [34].

The distribution of protein and marker enzymes closely resembles that obtained both with rat kidney [18] and with rabbit liver [32].

The heavy mitochondrial fraction contains the bulk of mitochondria (50% of the marker enzymes, cytochrome oxidase and monoamine oxidase), but also a large portion (28–30%) of $(Na^+ + K^+)$-ATPase and of NADPH- and NADH-cytochrome-*c* reductase.

Light mitochondrial fraction exhibits the highest specific activities of mitochondrial marker enzymes and of acid phosphatase and catalase (Fig. 1).

The fluffy layer fraction appears to be heterogeneous, both biochemically (Table I and Fig. 1) and morphologically (Fig. 2A): it is contaminated by damaged lysosomes and by mitochondrial outer membranes (10–12% of acid phosphatase and monoamine oxidase, respectively); it contains membranes of the brush border and of the basolateral infoldings (23.5% of both alkaline phosphatase and $(Na^+ + K^+)$-ATPase), which are somewhat concentrated in this fraction, as demonstrated by the relative specific activities of their marker enzymes (Fig. 1); membranes of the endoplasmic reticulum are also present (22% of glu-

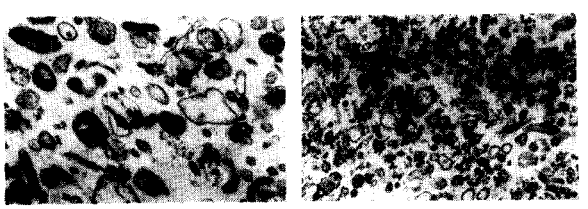

Fig. 2. Electron micrographs of rabbit kidney cortex 'fluffy layer' fraction and microsomal fraction. Both fractions were fixed in suspension as described in Materials and Methods. a: the fluffy layer fraction contains membranes and vesicles of different origin i.e. plasma membranes, brush border, smooth and rough vesicles, tubules and dense vesicles and is contaminated by damaged mitochondria and lysosomes. b: the microsomal fraction is constituted of smooth vesicles predominantly of endoplasmic reticulum origin, some brush borders and numerous free ribosomes. (× 12500).

cose-6-phosphatase), many of which are of the rough type (Fig. 2A).

Diamine oxidase activity, assayed both with histamine and with cadaverine as substrates, is greatly concentrated (> 60%) in the microsomal fraction, which exhibits high glucose-6-phosphatase, NADPH- and NADH-cytochrome *c* reductase activities, but also contains alkaline phosphatase and $(Na^+ + K^+)$-ATPase (31 and 21%, respectively).

The distribution pattern of diamine oxidase relative specific activity (Fig. 1), however, resembles that of the endoplasmic reticulum marker enzymes more closely than that of plasma membranes marker activities, thus suggesting a microsomal localization of diamine oxidase.

Morphologically (Fig. 2B), the microsomal fraction appears constituted mainly by small, closed vesicles predominantly smooth walled, most probably derived from the endoplasmic reticulum; free ribosomes are very numerous; large vesicles of plasma membrane origin and microvilli are also observed.

Since both biochemical and morphological results indicate that the microsomal fraction isolated from kidney cortex has a somewhat heterogeneous composition, we decided to further purify it, according to Jakobsson [19].

Table II shows the chemical and enzymatic composition of total 'cushion' microsomes and the percentage distribution and recovery of protein and enzymes in the subfractions and pellet obtained by gradient centrifugation.

Total 'cushion' microsomes show protein content and enzymatic activities of about one half with respect to the microsomal fraction of Table I. However, the specific activities of membrane marker enzymes are very similar, with only a slight increase in total 'cushion' microsomes.

Thus, these data further confirm the heterogeneous composition of total microsomal fraction from kidney.

The protein content of the microsomal subfractions is very similar to that obtained by Jakobsson [19], excepts that our pellet and subfraction 4 contain twofold and half the protein amount of the corresponding Jakobsson's [19] fractions, respectively. This fact may be due both to the more prolonged centrifugation time and to the different animals employed.

The percentage distribution of marker enzymes of basolateral plasma membranes, brush border and endoplasmic reticulum shows that the pellet, together with subfraction 1, contains more than 50% of $(Na^+ + K^+)$-ATPase and alkaline phosphatase activities, while glucose-6-phosphatase,

TABLE II

PROTEIN AND ENZYMATIC COMPOSITION OF TOTAL 'CUSHION' MICROSOMES OF RABBIT KIDNEY CORTEX AND PERCENTAGE DISTRIBUTION AND RECOVERY IN THE MICROSOMAL SUBFRACTIONS

Total 'cushion' microsomes and microsomal subfractions were prepared according to Jakobsson [19] as described in Materials and Methods. Number of experiments 5. All values are given as mean ± S.D.

	Total microsomes		Microsomal subfractions				Recovery (%)
	Absolute values	Pellet	Percentage values				
			1	2	3	4	
Protein	6.95 ± 1.15	28.64 ± 3.8	11.61 ± 2.0	18.12 ± 2.9	27.04 ± 2.7	13.60 ± 2.6	99.01 ± 8.7
$(Na^+ + K^+)$-ATPase	3.49 ± 0.60	30.03 ± 6.1	24.90 ± 5.4	17.30 ± 0.3	19.45 ± 3.8	12.26 ± 2.3	103.94 ± 9.2
Alkaline phosphatase	2.12 ± 0.50	29.76 ± 3.6	20.62 ± 2.2	24.53 ± 4.4	21.00 ± 2.6	4.26 ± 0.7	100.17 ± 11.7
Glucose-6-phosphatase	0.76 ± 0.06	11.27 ± 1.5	5.48 ± 1.4	24.75 ± 1.8	45.96 ± 4.5	6.56 ± 1.4	94.02 ± 8.7
NADPH cytochrome *c* reductase	0.70 ± 0.06	19.31 ± 2.7	9.17 ± 0.6	14.47 ± 1.5	39.91 ± 5.5	13.88 ± 2.2	96.74 ± 9.2
NADH cytochrome *c* reductase	3.90 ± 0.19	16.25 ± 1.7	6.12 ± 0.6	18.04 ± 1.6	44.65 ± 3.0	8.67 ± 0.9	93.73 ± 9.8
Diamine oxidase (substrate histamine)	11.56 ± 2.40	12.58 ± 2.2	5.02 ± 0.7	18.29 ± 1.1	52.31 ± 9.9	5.23 ± 0.5	93.43 ± 9.1

NADPH- and NADH-cytochrome *c* reductase and diamine oxidase are concentrated (40–50%) in subfraction 3. Diamine oxidase is present for only 17–18% in the pellet and in subfraction 1, while amounting to more than 50% in subfraction 3.

Fig. 3 shows the relative specific activities of enzymes in subfractions and pellet obtained from the gradient: the distribution pattern of diamine oxidase is quite different from that of $(Na^+ + K^+)$-ATPase and alkaline phosphatase, while resembling that of the endoplasmic reticulum markers, strongly suggesting that this enzyme is localized in vesicles originating from these membranes.

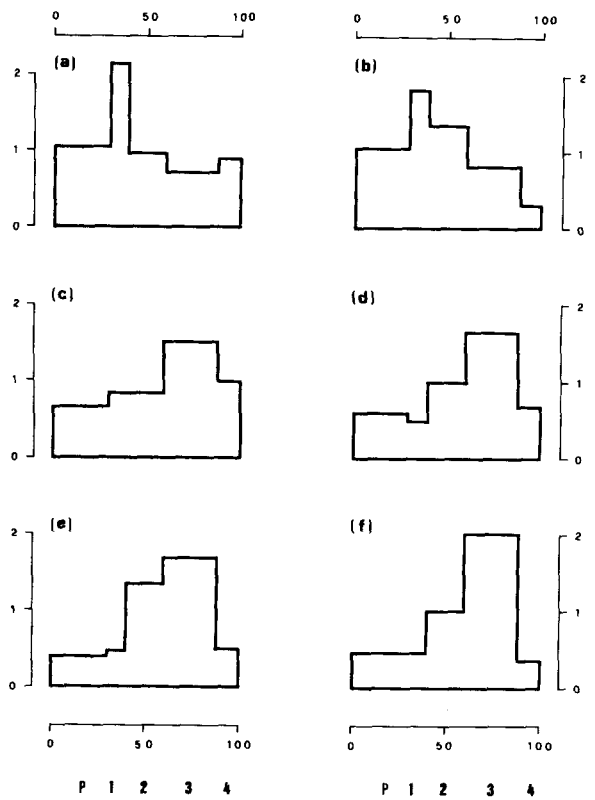

Fig. 3. Distribution patterns of enzymes in rabbit kidney cortex microsomal subfractions. Ordinate: mean relative specific activity of subfractions (% of total recovered activity in the fraction/% of total recovered protein in the fraction). Abscissa: % of total protein (related to the total microsomal fraction). The gradients were prepared, loaded and centrifuged as described in Materials and Methods. A pellet and four subfractions were collected. (a) $(Na^+ + K^+)$-ATPase; (b) alkaline phosphatase; (c) NADPH-cytochrome *c* reductase; (d) NADH-cytochrome *c* reductase; (e) glucose-6-phosphatase; (f) diamine oxidase (substrate histamine).

The morphological composition of the total 'cushion' microsomes, and their subfractions and pellet, examined by electron microscopy, is shown in Fig. 4.

Total 'cushion' microsomes (Fig. 4a) are composed by vesicles of different size, probably deriving from both the endoplasmic reticulum and the plasma membranes, together with brush border microvilli and ribosomes, most of which in the free state.

The pellet (Fig. 4b) contains typical brush

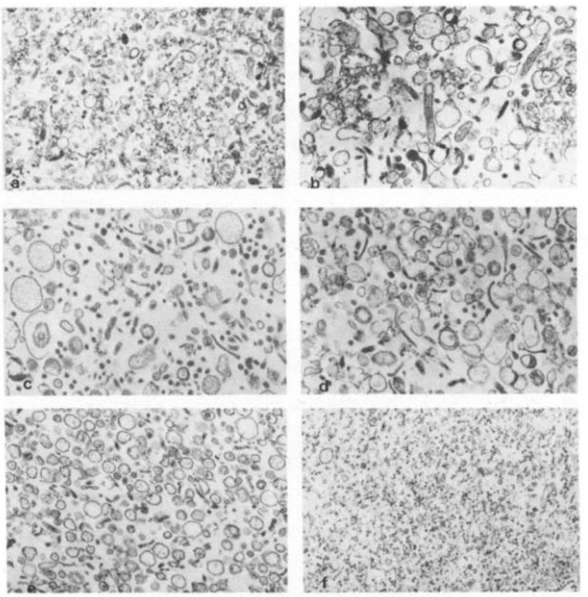

Fig. 4. Electron micrographs of rabbit kidney cortex total microsomes and microsomal subfractions. The subfractions were obtained by rate differential centrifugation of the total 'cushion' microsomes in a sucrose-Ficoll gradient as described in Materials and Methods. Total microsomes, subfractions and pellet from the gradient were fixed in suspension in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, centrifuged and postfixed with 1% OsO_4 in the same buffer. (a) Total 'cushin' microsomes: smooth walled vesicles of endoplasmic reticulum and plasma membrane origin, brush border microvilli and free ribosomes are present. (b) Pellet: closed and open plasma membrane fragments, brush border and very electron-dense tubules and vesicles are visible. (c) Subfraction 1: numerous very dense vesicles and tubules together with large plasma membrane vesicles and sheets are predominantly present. (d) Subfraction 2: small dense vesicles and tubules, plasma membrane profiles, smooth and rough vesicles are visible. (e) Subfraction 3: rounded vesicles, smooth walled, of endoplasmic reticulum origin are predominant. (f) Subfraction 4: free ribosomes and some very small vesicles are present. (× 12500).

borders and smooth profiles probably deriving from the plasma membrane; tubules and small vesicles, filled with a very electron-dense material, probably originating from the tubular invaginations typical of the apical region of the proximal convoluted tubule cells [35], are also observed. Rare ribosome-loaded vesicles are present.

Subfraction 1 (Fig. 4c) is mainly composed of the above mentioned very dense tubular and vesicular profiles, together with large smooth vesicles of plasma membrane origin. Some vesicles of smaller size, probably derived from the endoplasmic reticulum and microvilli from the brush border are also present; rough membranes are rarely seen.

Subfraction 2 (Fig. 4d) appears heterogeneous, as also demonstrated by its biochemical composition (Table II and Fig. 3). It still contains small dense vesicles and tubules, together with large smooth profiles of plasma membrane origin, but the vesicles derived from the endoplasmic reticulum, among which the rough ones are often seen, appear more abundant than in the preceding fractions.

Subfraction 3 (Fig. 4e) is essentially constituted of smooth vesicles ranging from 50 to 200 nm in diameter. Rough vesicles, as well as plasma membranes and brush borders are rarely seen.

Subfraction 4 (Fig. 4f) is almost exclusively constituted by free ribosomes and polisomes; very small, smooth surfaced vesicles, are also present, while large plasma membrane profiles and brush borders are almost completely lacking.

Finally, some kinetic parameters of diamine oxidase activity in the total microsomal fraction were studied. The enzyme was tested in the presence of several concentrations of histamine, cadaverine and putrescine (Fig. 5). Similar V_{max} values were obtained with both cadaverine and histamine at concentration of 50 and 10 mM, respectively; higher concentration of histamine inhibited the enzyme, as reported for rabbit liver diamine oxidase [32] and for pig kidney diamine oxidase [36]. With putrescine as substrate V_{max} was much lower.

Fig. 6 shows the diamine oxidase activity as a function of different pH: the rate of oxidation of the three different substrates showed a maximum in the range of pH 7.5–8.0. Beyond pH 8.5 a second rise in activity could be observed with all

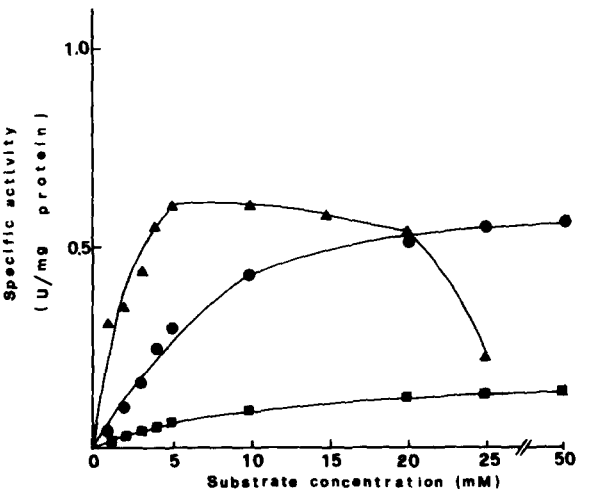

Fig. 5. Diamine oxidase activity of the microsomal fraction from rabbit kidney cortex in the presence of different concentrations of various substrates. ●, cadaverine; ■, putrescine; ▲, histamine. The incubation mixture was composed of: 0.8 ml 0.1 M sodium-potassium phosphate buffer, pH 7.2; 0.05 ml substrate solution; 0.05 ml 0.04% (w/v) horseradish peroxidase; 0.05 ml 0.5% (w/v) *o*-dianisidine hydrochloride; 0.05 ml (0.4–0.5 mg protein) of the microsomal fraction. After 15 min of incubation at 37°C, the enzymatic reaction was stopped by the addition of 0.5 ml of cold 9.8 M HCl. The results are the mean of three experiments.

substrates, particularly with cadaverine. This activity, however, cannot be entirely ascribed to the diamine oxidase because, as shown in Fig. 7, it is not inhibited by the specific diamine oxidase inhibitor aminoguanidine [3]; most probably the

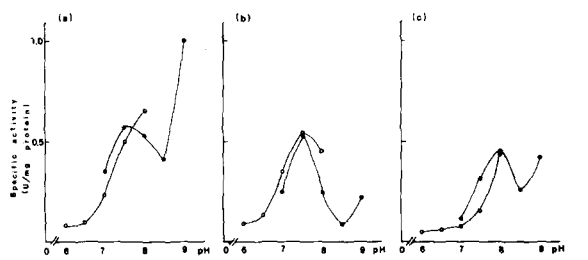

Fig. 6. pH dependency of diamine oxidase activity of the microsomal fraction from rabbit kidney cortex. Microsomes (0.4–0.5 mg protein) were incubated as described in the legend of Fig. 3. Two different 0.1 M buffer solutions were employed: ○, sodium-potassium phosphate buffer; ●, Tris-HCl buffer. (a) 50 mM cadaverine; (b) 10 mM histamine; (c) 50 mM putrescine.

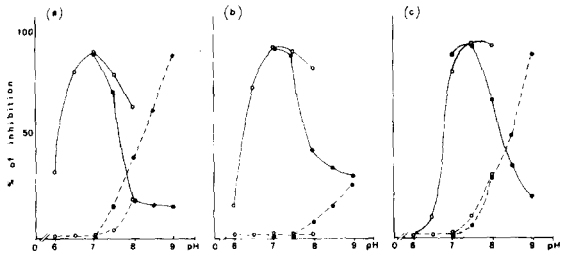

Fig. 7. Inhibition pattern of diamine oxidase activity by aminoguanidine and pargyline at different pH values. The incubation medium (see legend to Fig. 3) contained 10^{-4} M aminoguanidine or 10^{-4} M pargyline. O——O, sodium-potassium phosphate buffer and aminoguanidine; ●——●, Tris-HCl buffer and aminoguanidine; O------O, sodium-potassium phosphate buffer and pargyline; ●------●, Tris-HCl buffer and pargyline. (a) 50 mM cadaverine; (b) 10 mM histamine; (c) 50 mM putrescine.

monoamine oxidase activity, present in mitochondrial outer membranes, contaminating the microsomal fraction, oxidizes diamines at alkaline pH as shown by the inhibition pattern of pargyline, inhibitor of the monoamine oxidase A and B type [37].

This fact may be interpreted by recalling that mitochondrial monoamine oxidase undergoes qualitative modifications on its catalytic activity under the conditions which facilitate partial oxidation of the SH groups and acquires the ability to deaminate diamine oxidase substrates [38].

Discussion

Since it is well known that kidney cortex is mainly composed by proximal convoluted tubules [17], whose cells possess highly specialized membranes, very different both biochemically and morphologically, one can predict that a microsomal crude fraction will be a very heterogeneous one, containing membrane fragments of brush borders, baso lateral infoldings, Golgi elements and vesicles of endoplasmic reticulum [19].

The modification we introduced in the fractionation scheme of Wattiaux-De Coninck et al. [18], i.e. to separate and assay the fluffy layer as an individual fraction instead of sedimenting it together the microsomal fraction, as usual [34,18], appears to be very effective not only because it reduces cross contamination between mitochondrial and microsomal fraction, as already demonstrated for rabbit liver [32], but also because it produces a gross separation between membranes of different origin. In fact, while alkaline phosphatase, marker of brush border, and $(Na^+ + K^+)$-ATPase, marker of basolateral plasma membranes, are more concentrated in the fluffy layer rather in the microsomal fraction, the endoplasmic reticulum marker enzymes are not (Table I).

The distribution pattern of these last marker enzymes deserves a brief discussion. NADPH-cytochrome *c* reductase exhibits its maximum specific activity in the fluffy layer and in the microsomal fractions, but it is also present (31% of its total) in the heavy mitochondrial fraction. This fact might be interpreted as a mitochondrial localization of this activity, as suggested by Masters et al. [39] and by Jakobsson [19] for rabbit and rat kidney, respectively; however the presence of this enzyme in the plasma membranes cannot be excluded.

The heavy mitochondrial fractions, in fact, contains 28% of $(Na^+ + K^+)$-ATPase; moreover NADPH-cytochrome *c* reductase is present only to a little extent in the light mitochondrial fraction, which, on the other hand, exhibits the highest specific activities of mitochondrial marker enzymes.

The reverse is true for NADH-cytochrome *c* reductase, which is concentrated in the light mitochondrial and in the microsomal fractions, thus confirming, for rabbit kidney too, the demonstrated presence of this activity in the mitochondria from rat liver [40].

Finally it must be recalled that the two reductases have also been localized in the Golgi apparatus from rat liver [40,41].

Glucose-6-phosphatase relative specific activity is equally high both in the fluffy layer and in the microsomal fraction; this fact may be interpreted by considering that the enzyme has been suggested to be more abundant [42] or more active [43] in rough than in smooth microsomes from rat liver. Electron microscopy, in fact, shows that rough microsomes are more frequently observed in the first than in the latter fraction (Fig. 2).

The distribution pattern of diamine oxidase activity obtained in the first series of experiments (Table I and Fig. 1) strongly suggests a localiza-

tion of this enzyme in the endoplasmic reticulum: in fact it is almost absent in the heavy and light mitochondrial fractions thus excluding its presence in these organelles; moreover, since heavy mitochondrial fraction is contaminated by plasma membranes, the absence of diamine oxidase from this fraction, seems to exclude a plasma membrane localization of the oxidase; this conclusion is further supported by the low relative specific activity of the diamine oxidase in the fluffy layer fraction, which exhibits the highest specific activities of plasma membrane marker enzymes. On the other hand the resemblance with the marker enzymes of the endoplasmic reticulum and its extremely high relative specific activity in the microsomal fraction strongly suggest a true microsomal localization of the diamine oxidase.

This suggestion is supported by the results obtained in the second series of experiments, carried out by adapting the Jakobsson's [19] continuous sucrose-Ficoll gradient to our material.

Preliminary experiments (data not shown) with gradients of different densities, (ranging from 1.036 to 1.040 at the top and from 1.064 to 1.066 at the bottom, respectively) centrifuged for various time lengths (from 45 to 90 min) showed that the distribution pattern of diamine oxidase activity always paralleled that of endoplasmic reticulum marker enzymes, while differing from that of plasma membrane marker activities. However, the most satisfactory results, in terms of purity of the microsomes, were obtained with the gradient described in Materials and Methods, which had the same density as Jakobsson's [19], but was centrifuged for 75 min. This centrifugation time was critical to achieve a good separation between plasma membranes and endoplasmic reticulum. In fact a shorter centrifugation time did not succeed in separating the different membranes, which were recovered all together in the middle portion of the gradient, while a more prolonged one resulted in a spreading of the endoplasmic reticulum membranes towards the bottom of the tube, thus resulting in a partial overlapping of plasma membrane and endoplasmic reticulum marker activities, also at the highest gradient densities tested.

The distribution pattern of diamine oxidase activity in the microsomal subfractions obtained allows to conclude that this enzyme is present in membranes of the endoplasmic reticulum origin: in fact it is accumulated in subfraction 3, a fraction which, both biochemically and morphologically, appears to be consituted essentially by endoplasmic reticulum membrane fragments. Moreover the almost complete lack of rough elements in subfraction 3 (see Fig. 4e), strongly suggests that diamine oxidase is more active or more abundant in the endoplasmic reticulum smooth compartment.

It is worth mentioning that the same intracellular localization was demonstrated for rabbit liver diamine oxidase [32,44].

This resemblence is further strengthened by the results by the kinetic parameters studied.

In fact both kidney and liver diamine oxidase show similar reaction velocities towards different substrates, similar substrate specificity, with histamine more rapidly oxidized than cadaverine; the same inhibition by high concentrations of histamine is also observed. Optimum pH of liver and kidney diamine oxidase activity are also similar, as are the inhibition patterns of diamine and monoamine oxidase inhibitors at different pH.

Concerning the physiological significance of this localization, only hypotheses can be made. In fact, despite of the increasing number of observations on this enzyme in various organs and tissue in physiological and pathological conditions, its role remains still obscure.

However, the demonstrated involvement of dimaine oxidase in the catabolism of putrescine 'in vivo' [45–47], together with the observation that this activity is enhanced whenever an intracellular raise of di- and polyamines occurs [10,48,49] have suggested that diamine oxidase may represent a mechanism of control of di- and polyamine intracellular levels [8].

The localization of diamine oxidase in the endoplasmic reticulum, a cell compartment involved in 'detoxicating' functions [50] would agree with such a suggested role.

Concerning more specifically kidney diamine oxidase, it has been demonstrated to be present only in proximal convoluted tubule cells [14–16], a nephron segment chiefly involved in readsorptive processes. Since the endplasmic reticulum of these specialized cells has been suggested to be involved in the intracellular transport of solutes [51], the

localization of diamine oxidase in this cell compartment suggests a role for kidney diamine oxidase in the control of diamine concentration in a cell exposed to variable levels of these very active molecules [52].

Acknowledgments

The authors wish to express their grateful thanks to Professors Francesco Autuori and Alessandro Finazzi-Agrò for helpful discussion and criticism. Dr. Stefania Stefanini and Dr. Fiorella Leone kindly prepared the electron micrographs. Many thanks are also due to Vera Autuori for her skilfull technical assistance. This work was in part supported by the Italian C.N.R., special Project 'Chimica fine', contract No. 80.02.107.95.

References

1 Zeller, E.A. (1963) in The Enzymes (Boyer, P.D., Lardy, H. and Myrbäck, K; eds.), Vol. 8, pp. 315–335, Academic Press, New York
2 Bardsley, W.G., Crabbe, M.J.C., Shindler, J.S. and Ashford, J.S. (1972) Biochem. J. 127, 875–879
3 Mondovì, B., Guerrieri, P., Costa, M.T. and Sabatini, S. (1981) in Advances in Polyamine Resarch (Caldarera, C.M., Zappia, V. and Bachrach, U., eds.), Vol. 3, pp. 75–84, Raven Press, New York
4 Blaschko, M. (1963) in The Enzymes (Boyer, P.D., Lardy, H. and Myrbäck, K., eds.), Vol. 8, pp. 337–351, Academic Press, New York
5 Yasunobu, K.T., Ishiraki, H. and Miniamura, N. (1976) Mol. Cell. Biochem. 13, 3–9
6 Best, C.H. (1929) J. Physiol. 67, 256–263
7 Crabbe, J.C. (1981) Agents and Actions 11, 3–8
8 Perin, A., Sessa, A. and Desiderio, M.A. (1981) in Advances in Polyamine Research (Caldarera, C.M., Zappia, V. and Bachrach, U., eds.), Vol. 3, pp. 397–407, Raven Press, New York
9 Sessa, A., Desiderio, M.A., Baizini, M. and Perin, A. (1981) Cancer Res. 41, 1929–1934
10 Desiderio, M.A., Sessa, A. and Perin, A. (1982) Biochim. Biophys. Acta 714, 243–249
11 Cotzias, G.C. and Dole, V.P. (1952) J. Biol. Chem. 196, 235–242
12 Zeller, E.A., Barsky, J., Berman, E.R., Cherckes, S. and Fouts, J.R. (1958) J. Pharamacol. Exp. Therap. 124, 282–288
13 Kopple, J.D., Fai Tam, C., Wang, M. and Swendseid, M.E. (1978) Kidney International 13 (Suppl. 8) S.20–S.25
14 Takano, K., Suzuki, T. and Yasuda, K. (1970) Acta Histochem. Cytochem. 3, 105–113
15 Argento-Cerù, M.P., Oratore, A., Mondovì, B. and Finazzi-Agrò, A. (1981) Cell. Mol. Biol. 27, 359–362
16 Argento-Cerù, M.P., Oratore, A., Beninati, S. and Finazzi-Agrò, A. (1981) Cell. Mol. Biol. 27, 363–367
17 Latta, H., Maunsbach, A.B. and Osvaldo, L. (1967) in Ultrastructure of the kidney (Dalton, A.G. and Haguenau, F. eds.), pp. 1–8, Academic Press, New York
18 Wattiaux-De Coninck, S., Rutgeerts, M.J. and Wattiaux, R. (1965) Biochim. Biophys. Acta 105, 446–459
19 Jakobsson, S.V. (1974) Exp. Cell. Res 84, 319–334
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
21 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
22 Warton, D.C. and Tzagoloff, A. (1967) in Methods in Enzymology (Eastabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 245–250, Academic Press, New York
23 Rieske, J.S. (1967) in Methods in Enzymology (Eastabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 488–493, Academic Press, New York
24 Linhardt, K. and Walter, K. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 779–785, Academic Press, New York
25 Lück, H. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 885–894, Academic Press, New York
26 Shibko, S. and Tappel, A.L. (1965) Biochem. J. 95, 731–741
27 Fitzpatrick, D.F., Davenport, G.R., Forte, L. and Landon, E.J. (1969) J. Biol. Chem. 13, 3561–3569
28 Bonting, S.L. (1970) in Membranes and ion transport (Bittar, E.E., ed.), Vol. 1, pp. 257–363, Wiley-Interscience, London
29 Harper, A.E. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 788–792, Academic Press, New York
30 Dallner, G. (1963) Acta Pathol. Microbiol. Scand. suppl. 166
31 Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570–572
32 Argento-Cerù, M.P., Sartori, C. and Autuori, F. (1973) Eur. J. Biochem. 34, 369–374
33 Bergmeyer, H.U. and Bernt, E. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.) pp. 123–130, Academic Press, New York
34 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J., 60, 604–617
35 Rhodin, J.A.G. (1975) An Atlas of histology, p. 369, Oxford University Press
36 Mondovì, B., Rotilio, G., Finazzi-Agrò, A. and Scioscia-Santoro, A. (1964) Biochem. J. 91, 408–415
37 Ryder, T.A., Mac-Kenzie, M.C., Pryse-Davies, J. and Glover, V. (1979) Histochemistry 62, 93–100
38 Baumanis, E.A., Kalnina, I.E., Kaninka, M.E., Mashkovsky, M.D. and Gorkin, V.Z. (1981) Agents and Actions 11, 689–692
39 Masters, B.S.S., Isacsson, E.L., Prough, R.A. and Mason, J.I. (1973) Abstr. 9th Congr. Biochem. (I.U.B.), p. 341, Stockholm
40 Borgese, N. and Meldolesi, J. (1980) J. Cell. Biol. 85, 501–515

41 Howell, H.E., Ito, A. and Palade, G.E. (1978) J. Cell. Biol., 79, 581–589
42 Remmer, H. and Mercker, H.J. (1965) Ann. N.Y. Acad. Sci. 123, 79–97
43 Stetten, M.R. and Gosh, S.B. (1971) Biochim. Biophys. Acta 233, 163–175
44 Argento-Cerù, M.P., Sartori, C. and Autuori, F. (1973) Eur. J. Biochem., 34, 375–382
45 Seiler, N. and Eichentopf, B. (1975) Biochem. J. 152, 201–210
46 Fogel, W.A., Bieganski, T., Schayer, R.W. and Maslinskì, C. (1981) Agents and Actions 11, 679–684
47 Sourkes, T.L. and Missala, K. (1981) Agents and Actions 11, 20–27
48 Royansky, N., Neufeld, E. and Chayen, R. (1979) Biochim. Biophys. Acta 586, 1–6
49 Baylin, S.B., Abelhoff, M.D., Godwin, G., Carney, D.N. and Gazdar, A.F. (1980) Cancer Res. 40, 1990–1994
50 Estabrook, R.W. and Werungloer, J. (1979) in The induction of drug metabolism (Estabrook, R.W. and Lindenlaub, E., eds.), pp. 187–199, Schattauer Verlag, Stuttgart
51 Rostgaard, J. and Møller, O. (1980) Cell. Tissue Res. 212, 17–28
52 Beninati, S., Piacentini, M., Spinedi, A. and Autuori, F. (1980) Biomedicine 33, 140–143