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Liver Monoamine Oxidase in the Obese–Hyperglycaemic (*ob*/*ob*) Mouse

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1. The specific activity of monoamine oxidase was found to be greater in liver mitochondria from *ob*/*ob* mice than from lean mice. The activities of marker enzymes were similar in both tissues. 2. Experiments with various substrates (5-hydroxytryptamine, benzylamine and tyramine) and inhibitors (clorgyline and deprenyl) indicated that, unlike rat liver mitochondria, mouse liver mitochondria contain a predominance of the B-form of monoamine oxidase. 3. The K_m values for lean and *ob*/*ob* mice were the same for any given substrate and were in the increasing order 5-hydroxytryptamine < tyramine < benzylamine. $V_{max.}$ was approximately 50% greater in obese than in lean mice. 4. Extraction of liver mitochondria with acetone/water or acetone/water/NH_3 to remove lipids decreased the enzyme activity relatively more in obese- than in lean-mice preparations, but residual activity was the same in both preparations.

Monoamine oxidase [monoamine–O_2 oxidoreductase (deaminating), EC 1.4.3.4] is believed to be located on the outer mitochondrial membrane in the liver and other tissues (Schnaitman & Greenawalt, 1968; Colbeau *et al.*, 1971; Sottocasa, 1976). It has been suggested that the activity of the enzyme may be regulated by the lipid environment in the membrane (Houslay & Tipton, 1973; Ekstedt & Oreland, 1976). In this respect, the liver of the obese–hyperglycaemic (*ob*/*ob*) mouse would present an interesting system for studying the effect of lipid environment on the activity of the enzyme. It has been reported that the total liver phospholipid fraction in the *ob*/*ob*-mouse liver contains significantly different proportions of fatty acids from the comparable fraction from the lean control (Winand, 1970).

There is general agreement that mitochondrial monoamine oxidase exists in two forms, A and B, which are distinguishable by their relative activities with selected substrates and by the effect of specific inhibitors. In liver mitochondria, form-A monoamine oxidase preferentially deaminates 5-hydroxytryptamine and noradrenaline, whereas form-B monoamine oxidase preferentially deaminates benzylamine and phenethylamine. Tyramine is considered to be a substrate for both enzymes (Johnston, 1968; White & Glassman, 1977; Ekstedt, 1976). Clorgyline and deprenyl are considered to be specific inhibitors for forms A and B respectively (Egashira *et al.*, 1976). Evidence is also available which suggests that forms A and B are affected differently by the lipid environment (Houslay & Tipton, 1973; Ekstedt & Oreland, 1976). In the present study, the properties of mitochondrial monoamine oxidase in the lean and *ob*/*ob*-mouse liver have been investigated by using different substrates and inhibitors. The effect of lipid depletion by aqueous acetone on the enzyme activity was also examined.

Methods

Animals

Male or female C_{57}Bl/6J *ob*/*ob* mice and their lean controls (+/+) were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A., and used in these experiments at 9–12 weeks of age. The animals were housed four to a cage and given Purina chow and water *ad libitum*. Sprague–Dawley rats used in some of the experiments were obtained from Bio-Breeding, Ottawa, Canada.

Tissue preparation

The animals were killed by decapitation. The livers were removed, rinsed and chilled in ice-cold 0.9% NaCl, weighed and minced. All subsequent operations were carried out at 4°C. Homogenates (10%, w/v) were prepared in 0.25M-sucrose containing 5mM-Tris and 1mM-EDTA adjusted to pH7.5 with 0.1M-HCl. The homogenates were centrifuged for 10min at 600*g* in the SS-34 rotor of the Sorvall RC-2B centrifuge. The pellets were discarded and the supernatants were either used directly for assay of total monoamine oxidase activity or used for the preparation of mitochondrial fractions.

Preparation of mitochondrial fractions

Crude mitochondrial fractions were prepared by centrifuging the 600*g* supernatant at 5000*g* for 10min. The mitochondrial pellet was then resuspended in the homogenization medium and washed

three times. The mitochondrial fractions could be stored at −60°C for 4 weeks with no significant loss of monoamine oxidase activity.

Lipid depletion of mitochondria was done as described by Fleischer & Fleischer (1967) on mitochondrial preparations previously purified on Ficoll gradients (Clark & Nicklas, 1970). Essentially, 3ml of purified mitochondrial fraction was added to 72ml of acetone/water mixture (67.5ml of acetone+4.5ml of water). The mixture was left to stand at 0°C for 10–12min with occasional swirling, after which it was centrifuged for 2min at 2000*g*. After removal of the supernatant, the pellet was washed with 2×75ml of 0.88M-sucrose containing 10mM-Tris adjusted to pH7.5 with 0.1M-HCl and centrifuged at 25000*g* for 10min and finally resuspended in 3ml of the same medium. Lipid-depleted mitochondria were also prepared by extracting the purified mitochondria in the same manner, except that 12μl of aq. NH_3 (sp.gr. 0.880) was included per 100ml of acetone/water mixture.

Extraction and analysis of phospholipids

The preparation of mitochondrial lipid extracts for phospholipid analysis was carried out as described by Kates (1972). The phospholipids from the lipid extract were separated by t.l.c. as described by Skipski & Barclay (1969) with the solvent system chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). The lipid phosphorus content was measured by the method of Ames (1966).

Monoamine oxidase activity

This was measured by using radio-labelled substrates as described by Robinson *et al.* (1968). The complete reaction mixture contained the following components in a final volume of 0.1ml: 10mM-potassium phosphate buffer, pH7.5; tyramine, 5-hydroxytryptamine or benzylamine (1mM, at a specific radioactivity of 0.5μCi/μmol) and enzyme. The amount of enzyme was adjusted such that the reaction was linear with time for at least 40min, and in no case was more than 25% of the substrate consumed. After a 30min incubation in a shaking water bath at 37°C a 50μl portion of the reaction mixture was pipetted on to an Amberlite CG-50 (Na^+ form) column (0.5cm×3.5cm). The reaction products, aldehyde or acid, were eluted with water (2×1ml) and after the addition of 10ml of Aquasol-2, they were counted for radioactivity in a Nuclear–Chicago mark I liquid-scintillation spectrometer. Blanks consisted of buffer, substrate and boiled enzyme. One unit of enzyme activity was taken as the amount of enzyme necessary to catalyse the formation of 1nmol of product/min. Specific activity was then defined as units/mg of protein.

When inhibitors were used, the enzyme was preincubated for 10min at 37°C with the given inhibitor at the concentration stated in the individual Tables and Figures before the addition of the radioactive substrate.

Kynurenine hydroxylase (kynurenine,NADPH–O_2 oxidoreductase, EC 1.14.13.9)

This was assayed in a medium containing 0.1M-Tris buffer adjusted to pH8.1 with 1M-acetic acid, 30mM-KCl, 0.14mM-NADPH, mitochondrial preparation (0.6–1.0mg of protein) and water to a final volume of 3.0ml. After temperature equilibration for 10min at 24°C, 10μl of kynurenine sulphate to give a final concentration of 0.3mM was added to start the reaction. The decrease in A_{340} was measured over a 10min period. One unit of activity was defined as the amount of enzyme necessary to produce a change in A_{340} of 0.001/10min. Specific activity was defined as units/mg of protein.

Cytochrome oxidase (ferricytochrome c–O_2 oxidoreductase, EC 1.9.3.1)

This activity was measured at 550nm in a total volume of 1ml. The assay mixture contained 67mM-sodium phosphate buffer, pH7.0, 33.5μM-cytochrome *c* previously reduced by dialysis overnight against twice the molar concentration of sodium ascorbate and 0.09mM-EDTA. The A_{550} of the mixture was read for 30s and the reaction was started with the addition of 5–10μl of mitochondrial preparation containing 5–10μg of protein. The decrease in A_{550} was recorded for 60s. The cytochrome *c* was then completely oxidized by the addition of 10μl of a saturated solution of potassium ferricyanide. The activity is expressed as rate of oxidation of cytochrome *c* per mg of protein (Smith, 1955).

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Statistics

Student's *t*-test was used to assess the significance of differences between mean values.

Materials

[^{14}C]Tyramine (*p*-hydrophenyl[2-^{14}C]ethylamine hydrochloride) was obtained from the Amersham Corp., Oakville, Ont., Canada; [^{14}C]hydroxytryptamine (hydroxy[2-^{14}C]tryptamine binoxalate) was from New England Nuclear Corp., Montreal, Canada; [^{14}C]benzylamine ([*methylene*-^{14}C]-benzylamine hydrochloride) was from ICN Isotope and Nuclear Division, Montreal, Canada. L-

Kynurenine sulphate, NADPH, cytochrome *c* and benzylamine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Tyramine hydrochloride was obtained from Calbiochem, Downsview, Ont., Canada; 5-hydroxytryptamine binoxalate was from ICN Pharmaceuticals, Montreal, Canada, and Amberlite resin CG-50 (H^+ form; 100–200 mesh) was from BCH Chemicals, Toronto, Canada. Clorgyline [3-(2,4-dichlorophenoxy)propyl-*N*-methylprop-2-ynylamine hydrochloride] was a gift from May and Baker, Dagenham, U.K. Deprenyl (phenylisopropylmethylproinylamine hydrochloride) was kindly provided by Professor J. Knoll, Budapest, Hungary.

Results

Substrate specificity of mouse liver monoamine oxidase

Mitochondrial preparations from livers of lean and obese mice were assayed for monoamine oxidase with three different substrates. There appeared to be a significantly greater activity of the enzyme in preparations from the obese livers as compared with the lean. This higher specific activity was observed with all three substrates, but was greatest with tyramine. The substrate specificity of monoamine oxidase in both lean and obese mice was different from that of rat liver mitochondria. Whereas in rat liver mitochondria activity ranked tyramine > hydroxytryptamine > benzylamine, in both types of mouse liver mitochondria the activity with 5-hydroxytryptamine was the lowest, indicating that mouse liver mitochondria contained a much smaller proportion of the A form of monoamine oxidase (Table 1). The higher monoamine oxidase specific activity of the obese-mouse liver mitochondria was not due to a generalized effect on mitochondrial enzymes, since neither the specific activity of kynurenine hydroxylase, an outer-membrane marker, nor that of cytochrome *c* oxidase, an inner-membrane marker (Colbeau *et al.*, 1971), were significantly different in lean and obese mice (Table 2). In addition, neither the brain nor the heart of the obese mice had monoamine oxidase activities which differed from those of the lean mice.

Effects of inhibitors

To determine the relative proportions of A and B forms of monoamine oxidase and possible differences between the enzymes of obese- and lean-mouse liver, the effects of the inhibitors clorgyline and deprenyl were examined. These inhibitors had similar effects on the enzyme from lean and obese mice. Depending on the substrate used, however, the inhibitory power of the two compounds was quite different (Table 3). Clorgyline had little effect on the enzyme activity when benzylamine was the substrate, whereas deprenyl at the same concentrations inhibited almost completely. Likewise, the activity towards tyramine was almost completely abolished by deprenyl, but much less inhibition was found with clorgyline. On the other hand, the activity with 5-hydroxytryptamine as the substrate was inhibited by both compounds, clorgyline being more inhibitory than equal concen-

Table 1. *Substrate specificity of liver mitochondrial monoamine oxidase in lean mice, obese mice and rats*

All values are expressed as means ± S.E.M. for five animals from each of the three groups assayed in duplicate. *$P < 0.001$ when compared with lean groups.

	Monoamine oxidase activity (nmol/min per mg of protein)		
Animal	Tyramine	5-Hydroxytryptamine	Benzylamine
Lean mouse	5.70 ± 0.30	0.85 ± 0.05	1.55 ± 0.06
Obese mouse	9.89 ± 0.14*	1.30 ± 0.03*	2.51 ± 0.08*
Rat	9.31 ± 0.18	3.19 ± 0.09	1.53 ± 0.05

Table 2. *Mitochondrial enzyme activities in lean and obese mice*

Units of enzyme activity and statistical analysis were defined in the Methods section. Monoamine oxidase was assayed with tyramine as the substrate. Values are means ± S.E.M. for four separate preparations for kynurenine hydroxylase and monoamine oxidase and for two separate preparations for cytochrome oxidase. Abbreviation: n.s., not significant.

	Enzyme activity (units/mg of protein)		
	Kynurenine hydroxylase	Monoamine oxidase	Cytochrome oxidase
Lean	69.7 ± 5.4	6.98 ± 0.20	1.81 ± 0.20
Obese	69.7 ± 5.0	10.78 ± 0.30	2.10 ± 0.25
P	n.s.	< 0.001	n.s.

trations of deprenyl. These data indicated that in mouse liver mitochondria the predominant form of monoamine oxidase was form B.

To confirm the above data, inhibitor dose–response curves with tyramine as the substrate were done. As a control, these experiments were carried out in parallel with the better-known rat liver mitochondrial enzyme (Fig. 1). With both inhibitors, biphasic dose–response curves were obtained for rat liver mitochondria, suggesting that monoamine oxidase activity is attributable to approximately equal portions of the A and B forms. In mouse liver mitochondria, on the other hand, an effect of clorgyline (24% inhibition) was observed only at concentrations 10 times that of deprenyl capable of producing nearly total inhibition of the enzyme activity. These data further supported the predominance of the B form of the enzyme in lean- and obese-mouse liver mitochondria. The data presented in Fig. 1 give the results for obese mice only. Data obtained with lean mice were essentially the same.

Kinetics of monoamine oxidase in mouse liver mitochondria

The kinetic characteristics of monoamine oxidase of lean and obese mice were studied with amine substrates. It was observed that the $V_{max.}$ value, but not the apparent K_m, was different for the two groups of animals. The kinetic data are given for tyramine (Fig. 2). The apparent K_m for tyramine was found to be 0.199mM and the values for 5-hydroxytryptamine and benzylamine were 0.118mM and 35μM respectively and were the same in lean and obese mice.

Effect of lipid environment

It seemed possible that the differences in specific activity of monoamine oxidase in lean and obese mice might be due to differences in the lipid environment. Mitochondria were therefore extracted in order to deplete them of lipids. Treatment with aqueous acetone, which removes up to 80% of neutral phos-

Table 3. *Substrate-selective inhibition of liver mitochondrial monoamine oxidase activity*
Mitochondrial preparations were preincubated at 37°C for 10min in the absence and presence of clorgyline and deprenyl before the addition of the substrate. The enzyme activity was then assayed as described in the Methods section. Values given are the averages of two individual experiments assayed in duplicate. The variation between the experiments was less than 5%.

		Inhibition (%) by			
		Clorgyline		Deprenyl	
Animal	Substrate	0.1μM	1μM	0.1μM	1μM
Lean	Tyramine	4	24	83	92
	5-Hydroxytryptamine	54	65	33	45
	Benzylamine	0	1	68	96
Obese	Tyramine	1	24	86	94
	5-Hydroxytryptamine	50	65	35	47
	Benzylamine	0	3	68	98

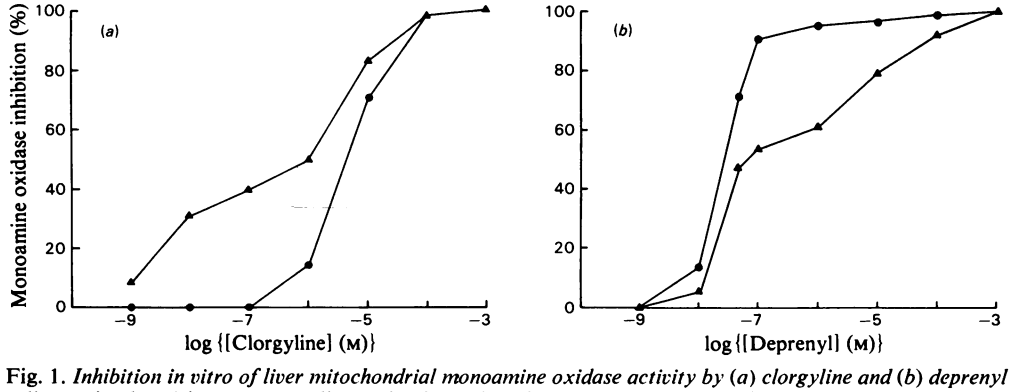

Fig. 1. *Inhibition in vitro of liver mitochondrial monoamine oxidase activity by (a) clorgyline and (b) deprenyl*
▲, Rat liver mitochondria; ●, mouse liver mitochondria. The substrate was tyramine and the assays were done as described in detail in the Methods section.

pholipids (Fleischer & Fleischer, 1967), decreased the activity of the enzyme in both lean-mouse and obese liver mitochondria. Although the specific activity in the untreated obese mitochondria was higher than that in untreated lean mitochondria, residual activity after extraction with aqueous acetone was the same in both cases. Likewise, extraction with aqueous acetone and NH_3, which removes some of the acidic phospholipids as well as the neutral phospholipids, left the same residual activity in both groups (Table 4). The decreases in activity produced by lipid removal was much more profound when 5-hydroxytryptamine was the substrate.

The initial gross phospholipid composition of liver mitochondria did not differ in the lean and obese mice (Table 5).

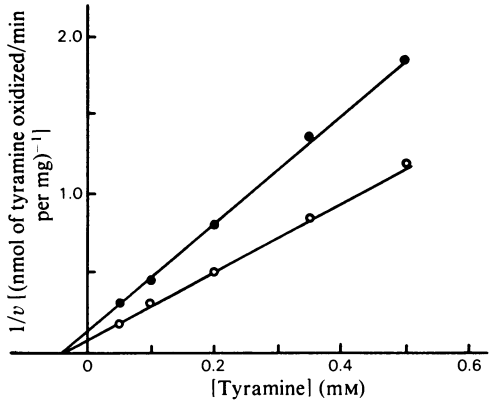

Fig. 2. *Effect of substrate concentration on the oxidative deamination of tyramine by liver mitochondria*
○, Lean mice; ●, obese mice. Velocity (v) is expressed as nmol of tyramine oxidized/min per mg of protein. The assays were done as described in the Methods section.

Discussion

In this paper, we have presented evidence that monoamine oxidase activity was significantly greater in the liver of the obese-hyperglycaemic mouse than in its lean counterpart, whereas there was no difference in the activity of two other mitochondrial enzymes, kynurenine hydroxylase and cytochrome *c* oxidase, between the two groups of animals. These results are different from the recently published data of Feldman & Henderson (1978), who did not find significant differences in the monoamine oxidase activity of crude liver homogenates of lean and obese

Table 4. *Effect of acetone extraction on hepatic mitochondrial monoamine oxidase activity*
I, Mitochondrial fraction; II, mitochondrial fraction extracted with acetone; III, mitochondrial fraction extracted with acetone and aq. NH_3. Details of the extraction procedure are given in the Methods section. Values given are those of two individual experiments assayed in duplicate.

	Monoamine oxidase activity (units/mg of protein)	
Fraction	5-Hydroxytryptamine	Benzylamine
Lean		
I	0.96–1.14	1.79–2.01
II	0.42–0.54	1.27–1.43
III	0.17–0.25	0.70–0.80
Obese		
I	1.59–1.81	2.65–2.95
II	0.44–0.56	1.45–1.65
III	0.20–0.30	0.71–0.79

Table 5. *Phospholipid composition of lean- and obese-mouse liver mitochondria*
The results represent means ± S.E.M. for four experiments each done in triplicate and are expressed as percentage of total phospholipid fraction.

	Composition (%)	
Phospholipid class	Lean	Obese
Sphingomyelin+lysophosphatidylcholine	12.7±3.7	13.5±3.2
Phosphatidylcholine	29.8±1.2	31.8±1.8
Phosphatidylinositol	12.9±1.6	11.4±2.1
Phosphatidylethanolamine	22.1±2.4	21.7±1.8
Phosphatidylglcyerol	7.7±1.1	8.1±1.9
Cardiolipin+phosphatidic acid	13.7±2.1	12.8±2.1

mice. Apart from the difference in the tissue fraction used, their experimental protocol differed from ours in many respects, including the age of the experimental animals, the genotype of the controls and the substrate used for the assays. The studies on substrate specificity, kinetic characteristics and sensitivity toward inhibitors presented here demonstrated that monoamine oxidase from obese-mouse liver mitochondria is similar to the enzyme from lean-mouse liver mitochondria. Therefore it seems unlikely that the greater monoamine oxidase activity in the obese mouse is due to a difference in substrate affinity or in the proportions of the form-A and form-B enzyme. Our finding that liver monoamine oxidase is predominantly form-B in the strain of mouse under investigation is in accord with published results on albino mice (Laverty *et al.*, 1973). The fact that both forms of the enzyme were present in rat liver mitochondria is in harmony with data published by others (Ekstedt, 1976; Egashira *et al.*, 1976; White & Glassman, 1977).

The possibility that an alteration in the liver mitochondrial-membrane composition of the obese mouse leads to a greater monoamine oxidase activity is an attractive hypothesis which needs to be studied further. It has been reported that monoamine oxidase activity may be dependent on the lipid environment of the mitochondrial membrane. Houslay & Tipton (1973) have suggested that multiple forms of rat liver mitochondrial monoamine oxidase were due to differential binding of membrane material to a single enzyme species. Ekstedt & Oreland (1976) have demonstrated that removal of 90% of the phospholipid without liberation of the activity produced marked changes in the relative activities of the A and B forms of the enzyme in rat liver mitochondria. Their results indicated that only a minor portion of the A form remained after lipid extraction, whereas half of the B-form activity remained. With a different extraction procedure, Kandaswami & D'Iorio (1976) found that half of the A-form activity was lost by removal of phospholipids, whereas the B-form activity was actually enhanced. These authors also reported losses in the activity of the enzyme in rats fed on fat-free diets, with a recovery of a major portion of the activity on supplementation with essential fatty acids. The evidence available indicates that differences in lipid composition may indeed be an important factor to explain the differences between preparations from obese and lean animals, since manipulations of the lipid content produced much greater changes in the monoamine oxidase activity of obese than of mitochondria from lean animals. The results described in the present paper indicated that the 'extra' activity of the monoamine oxidase in obese-mouse liver mitochondria was very readily lost on extraction of the mitochondria with aqueous acetone, so that the residual activity was the same in obese- and lean-mouse preparations, with both 5-hydroxytryptamine and benzylamine as substrates. Although we did not find any significant differences in the gross phospholipid composition of the two types of mitochondria (Table 5), it has been reported by Winand (1970) that the fatty acid composition of the total liver phospholipid fraction was markedly different in obese- and lean-mouse livers, particularly the relative proportions of C_{20} polyunsaturated fatty acids. It is not known yet, however, whether there are significant differences in the fatty acid composition of liver mitochondrial phospholipids in these animals.

Alternatively the membrane or the enzyme itself could be altered by glycosylation. It has been proposed that in hyperglycaemia the increased concentration of glucose present in cells where insulin is not required for its transport (e.g. the liver) may lead to non-enzymic glycosylation of proteins and particularly membrane protein (cf. Bunn *et al.*, 1978). This could account for changes in the properties of membrane enzymes such as monoamine oxidase. It should be noted that differences in the carbohydrate component of liver plasma membranes in the *ob/ob* mouse have already been demonstrated and have been proposed as the main factor responsible for the decreased insulin-receptor activity in this tissue (Chang *et al.*, 1975).

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References

Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
Bunn, H. F., Gabbay, K. H. & Gallop, P. M. (1978) *Science* **200**, 21–27
Chang, K. J., Huang, D. & Cuatrecasas, P. (1975) *Biochem. Biophys. Res. Commun.* **64**, 566–573
Clark, J. B. & Nicklas, W. J. (1970) *J. Biol. Chem.* **245**, 4724–4731
Colbeau, A., Nachbaur, J. & Vignais, P. M. (1971) *Biochim. Biophys. Acta* **249**, 462–492
Egashira, T., Ekstedt, B. & Oreland, L. (1976) *Biochem. Pharmacol.* **25**, 2583–2586
Ekstedt, B. (1976) *Biochem. Pharmacol.* **25**, 1133–1138
Ekstedt, B. & Oreland, L. (1976) *Biochem. Pharmacol.* **25**, 119–124
Feldman, J. M. & Henderson, J. H. (1978) *Diabetes* **27**, 389–395
Fleischer, S. & Fleischer, B. (1967) *Methods Enzymol.* **10**, 406–433
Houslay, M. D. & Tipton, K. F. (1973) *Biochem. J.* **135**, 173–186
Johnston, J. P. (1968) *Biochem. Pharmacol.* **17**, 1285–1297
Kandaswami, C. & D'Iorio, A. (1976) *Proc. Can. Fed. Biol. Soc.* **19**, 216
Kates, M. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), p. 351, American Elsevier Publishing Co., New York

Laverty, R., Browne, B. J. & Callingham, B. A. (1973) *J. Pharmacol.* **25**, 1001–1002

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Robinson, D. S., Lovenberg, W., Keiser, H. & Sjoerdsma, A. (1968) *Biochem. Pharmacol.* **17**, 109–119

Schnaitman, C. & Greenawalt, J. W. (1968) *J. Cell Biol.* **38**, 158–175

Skipski, V. P. & Barclay, M. (1969) *Methods Enzymol.* **14**, 530–598

Smith, L. (1955) *Methods Enzymol.* **2**, 732–740

Sottocasa, G. L. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 55–78, Chapman and Hall, London

White, H. L. & Glassman, A. T. (1977) *J. Neurochem.* **29**, 987–997

Winand, J. (ed.) (1970) *Aspects Qualitatifs et Quantitatifs du Métabolisme Lipidique de la Souris Normale et de la Souris Congénitalement Obèse*, p. 200, Arscia, Bruxelles