Distribution of membrane cholesterol of adrenal cortical cells after corticotropin stimulation

Orla M. CONNEELY,* Joyce M. GREENE,† Denis R. HEADON,* Joyce HSIAO‡ and Frank UNGAR‡

*Cell and Molecular Biology Group, Department of Biochemistry, University College, Galway, Ireland,

†Department of Biochemistry, University College, Cork, Ireland, and ‡Department of Biochemistry, Medical School, University of Minnesota, Minneapolis, MN 55455, U.S.A.

(Received 25 April 1983/Accepted 3 May 1983)

Membrane cholesterol in adrenal cortical cells is enriched in the plasma membrane. Stimulation of isolated adrenal cortical cells with corticotropin leads to the production of corticosterone. At high levels of corticotropin, cholesterol for corticosterone synthesis arises by hydrolysis of cellular cholesteryl ester, whereas at lower levels of corticotropin cholesteryl ester levels are unchanged from control values and there is a decrease in plasma-membrane cholesterol levels.

Steroidogenesis in adrenal cortical tissue is regulated by corticotropin ('adrenocorticotrophic hormone'; 'ACTH'). Steroid hormones are derived from cholesterol, which may arise by synthesis *de novo* (Balasubramaniam *et al.*, 1977), from extracellular lipoproteins (Kovanen *et al.*, 1979) and from endogenous cholesteryl esters stored in cytoplasmic lipid droplets (Moses *et al.*, 1971). It has been shown that the metabolism of each of these sources is under the control of corticotropin. In addition, the hormone-dependent cholesteryl esterase, which hydrolyses cytoplasmic cholesteryl esters, is activated by a cyclic AMP-dependent protein kinase (Beckett & Boyd, 1977).

Although it is well established that corticotropin stimulates cyclic AMP production and that cyclic AMP and its analogues are capable of stimulating adrenal steroid hormone formation, some major observations suggest that cyclic AMP is not an obligatory intermediate in the steroidogenic action of corticotropin. Several authors (Grahame-Smith *et al.*, 1967; Beall & Sayers, 1971; Rivkin & Chasin, 1971; Seelig & Sayers, 1972) have found that low doses of corticotropin stimulate corticosterone production without causing detectable changes in the concentration of cyclic AMP. Furthermore, nitrophenyl sulphenyl-corticotropin, an analogue of corticotropin, does not stimulate cyclic AMP production but can stimulate steroidogenesis (Moyle *et al.*, 1973). The observation that a protein kinase was stimulated at low levels of corticotropin has been made (Ling & Marsh, 1977), suggesting that cyclic AMP changes had occurred but below detection levels. It was of interest, therefore, to investigate the subcellular source of cholesterol utilized for corticosterone synthesis at low levels of corticotropin.

Amar-Costesec *et al.* (1974), by using the density perturbant digitonin, have shown that, in liver, cholesterol is concentrated primarily in vesicles of plasma-membrane origin. De Wolf *et al.* (1977) have reported that cholesterol is predominantly located in the plasma membranes of bovine thyroid tissue. Evidence that cholesterol in muscle membranes has a similar distribution to that of liver has been reviewed by Headon *et al.* (1977).

Tissue fractionation of rat adrenal cortex by differential pelleting into four classical fractions and subsequent analysis of marker enzymes gives an overall picture of the subcellular distributions of various constituents (de Duve, 1971). Further fractionation on sucrose density gradients allows the calculation of median densities for these constituents and gives an indication of the distribution of these constituents in the cell. Fractionation of adrenal cortical tissue exposed to various concentrations of corticotropin would be expected to yield information on the source of cholesterol for steroid hormone formation.

Materials and methods

Glucose 6-phosphate dehydrogenase, glucose 6-phosphate, AMP and NADP⁺ were obtained from Boehringer Corporation, Mannheim, Germany. Collagenase (type 11; cat. no. C-6885), bovine serum albumin (fraction V), DNAase, steroids, imidazole and dimethylglutarate were obtained from Sigma Chemical Co., London S.W.6, U.K. Fluon was from ICI, Macclesfield, U.K., silica-gel G (GF 254) was obtained from Merck, Darmstadt, Germany. Corticotropin was obtained from Schering A.G., Berlin Bergkamen, Germany. Radioactive steroids were obtained from The Radiochemical Centre,

Amersham, Bucks., U.K. Antiserum B21-42 was a product of Endocrine Sciences, Tarzana, CA 91356, U.S.A. All solvents and other chemicals were of AnalaR grade and were supplied by BDH, Poole, Dorset, U.K.

Subcellular fractionation of rat adrenal cortical tissue

A 10% (w/v) homogenate of fresh rat adrenal cortical tissue was prepared in ice-cold 0.25 M-sucrose, buffered at pH 7.4 with 3 mM-imidazole hydrochloride (buffered sucrose) using a hand-operated Potter homogenizer. Centrifugations were carried out in a Beckman JA21 centrifuge using the JA20 rotor. Nuclei and cell debris (N fraction) were removed at 400g for 10 min. The supernatant was centrifuged at 4000g for 10 min to prepare the M fraction, rich in mitochondria. The post-4000g supernatant was then centrifuged at 12000g for 15 min to prepare the M+L fraction, enriched in lysosomes and mitochondria. A microsomal fraction (P) was prepared from the resulting supernatant by centrifugation at 105000g for 60 min. This final centrifugation and subsequent density-gradient centrifugations were carried out in a Beckman L5-75 ultracentrifuge using an SW-27 swinging-bucket rotor.

Density-gradient centrifugations were carried out at 5°C for 12 h. Linear sucrose density gradients of 30 ml volume were prepared from 0.5 and 2.0 M buffered sucrose. Samples (2 ml) were applied to the gradients. Data for differential centrifugation are presented according to the method of de Duve *et al.* (1955) and those for density-gradient centrifugation as described by Amar-Costesec *et al.* (1974).

The preparation of isolated intact adrenal cortical cell suspensions and subsequent corticotropin stimulations were carried out as described previously (Ungar *et al.*, 1979). Portions containing approx. 300000 isolated cells of 10 quartered adrenals per ml were incubated with corticotropin. Assays were set up as follows: (a) a control to which no corticotropin was added; (b) a minimal corticotropin assay containing 50 µunits/ml; (c) a maximal corticotropin assay containing 25 units/ml.

The assay tubes were incubated in a shaking water bath for 2 h at 37°C. After removal of the incubation medium, the samples were homogenized in buffered sucrose and microsomal fractions prepared as described above.

Density perturbation of microsomes with digitonin was carried out as described by Amar-Costesec *et al.* (1974).

Analyses

Refractive indices of the fractions were determined by using an Abbe 60 refractometer (Bellingham and Stanley, London, U.K.). The molarity of sucrose was calculated from the refractive index

by using standard tables (Dobrota & Reid, 1971) and the sucrose density, at the temperature of centrifugation, calculated by computer using the data of Barber (1966). Protein concentration was determined by the automated method of Leighton *et al.* (1968) adapted from the manual procedure of Lowry *et al.* (1951) and P_1 was measured by the method of Stewart (1974). Succinate dehydrogenase was estimated by a modification (Porteous & Clark, 1965) of the method of Pennington (1961). Acid phosphatase and 5'-nucleotidase were estimated by the methods of Hinton *et al.* (1970) and steroid 21-hydroxylase by the method of Satre *et al.* (1971).

Samples for lipid analysis were extracted into 10 vol. of chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). Neutral lipid classes were separated on silica-gel G plates using n-hexane/diethyl ether/acetic acid (80:20:1, by vol.) as a solvent system. Neutral-lipid spots were stained with I_2 vapour. Neutral lipids, with the exception of cholesterol, were quantified by the method of Marsh & Weinstein (1966).

The cholesterol content of lipid extracts was quantitatively determined by g.l.c. in a Pye-Unicam (Series 104, model 24) gas chromatograph with dual-flame ionization detectors. The chromatograph was coupled to a Servoscribe IS recorder with disc-integrating facilities (R.E. 542.20; Smiths Industries, London, U.K.). Separations were carried out in a glass column (150 cm × 4 mm internal diameter) packed with 3% SP-2100 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA, U.S.A.). Both the column and the support were pretreated with a Sylon-CT solution. The carrier was N_2 at a flow-rate of 50 ml/min. The flow-rates of H_2 and air were 50 and 600 ml/min respectively. The chromatograph was operated isothermally at 270°C. Samples of volume 5 µl were injected. Peak areas were calculated either from the integrator trace on the recorder or, alternatively, by using the method of Carroll (1961). Corticosterone was estimated by radioimmunoassay using serum B21-42 (Endocrine Sciences).

Results

The distribution patterns of the measured constituents after differential centrifugation are shown in Fig. 1. Recoveries for all constituents measured ranged from 90 to 107%. Approx. 39% of succinate dehydrogenase activity was recovered in the M fraction with 73% recovered in the N and M fractions taken together. A relative specific activity of 3.24 was obtained in the mitochondrial fraction.

Approx. 29% of acid phosphatase activity was recovered in the M+L fraction with 39.9% of the total recovered in the M+L and P fractions together.

Fig. 1. Distribution of measured constituents after fractionation of adrenal cortex by differential centrifugation. Fractions are plotted in the order N, M, M + L, P and S. The ordinate represents the relative specific activity or concentration of constituents, and the abscissa scale represents the percentage protein, cumulatively, in each fraction (Amar-Costesec *et al.*, 1974).

The relative specific activity of acid phosphatase activity was 2.66 in the M + L fraction; 5'-nucleotidase was recovered in the P fraction to the extent of 30.2%; cholesterol and steroid 21-hydroxylase were also recovered primarily in the P fraction to the extent of 36.1% and 31.4% respectively. The relative specific activities or concentrations for these constituents in the microsomal fraction were 3.23, 3.54 and 3.26 respectively as shown in Fig. 1.

Although the overall recovery of measured constituents was in the range 90–107%, the percentage of succinate dehydrogenase recovered in the M fraction was 39%, with 30% of the 5'-nucleotidase being found in the P fraction. Although analytical fractionation data for the adrenal cortex are not available from other groups, these percentage

recovery values might be considered to be low. This reflects the choice of relatively mild homogenization conditions to prevent excessive fragmentation of subcellular organelles such as mitochondria and lysosomes, which would complicate the subsequent fractionation. The yield of plasma-membrane markers in the microsomal fraction was within the range of 30–32% with relative specific activities of 3.12–3.45 for all three conditions, i.e., control, low-corticotropin and high-corticotropin treatments.

The origin of cholesterol present in the P fraction has been discussed and the high cholesterol to protein or phospholipid ratios characteristic of liver, bovine thyroid and muscle plasma membranes have already been mentioned. The distribution of cholesterol after density-gradient centrifugation of

Table 1. *Comparison of cholesterol/protein ratios in microsomes, cholesteryl ester/protein ratios in homogenates and corticosterone production in control and samples treated with corticotropin*

Results for ratios are expressed as a percentage of the control value and the corticosterone content represents excess over the control ($n = 6$).

	Control	Minimal corticotropin	Maximal corticotropin
Cholesterol/protein	100.3 $\pm$ 2.24%	62.0 $\pm$ 2.48%	101.1 $\pm$ 2.63%
Cholesteryl ester/protein	100.1 $\pm$ 2.39%	99.2 $\pm$ 2.52%	56.4 $\pm$ 3.21%
Corticosterone	1.0	5.8	12.9

the microsomal fraction may be expected to indicate the origin of the cholesterol contained in the P fraction isolated from rat adrenal tissue.

Cholesterol content of rat adrenal membranes at different corticotropin concentrations

Control, minimal and maximal corticotropin analyses were set up as described. After incubation, the cell suspensions were homogenized and a microsomal fraction was prepared by differential centrifugation. The homogenates were assayed for corticosterone production, cholesteryl esters and protein to monitor the disappearance of cholesteryl esters on corticotropin stimulation. Cholesterol/protein ratios were determined in the microsomal fraction.

The results shown in Table 1 indicate that under conditions of minimal corticotropin treatment there is a 5.8-fold increase in the quantity of corticosterone produced above that evident in the control. Associated with this increase in steroid output is a drop in the cholesterol/protein ratio of the microsomal fraction to 62% of the control value, whereas the cholesteryl ester level of the homogenate remains constant, indicating that at low levels of corticotropin the cholesterol content of the microsomal fraction is decreased.

In the case of maximal stimulation with corticotropin, the microsomal cholesterol/protein ratio remains at the same level as that in the control, whereas the cholesteryl ester level of the homogenate decreases to 56% of the control value. Under these conditions, it appears that cholesterol for corticosterone production is derived from hydrolysis of endogenous cholesteryl esters.

To confirm these results, and to determine the origin of cholesterol appearing in the microsomal fraction, microsomes from unstimulated and corticotropin-stimulated adrenals were subjected to density-gradient centrifugation on sucrose density gradients using the density perturbant digitonin. Digitonin increases the density of cholesterol-containing membranes by its ability to bind the 3β -hydroxy group of sterols, forming an insoluble equimolar complex. The increase in density depends on the cholesterol content of the membranes. Changes in the extent of digitonin shift would,

Table 2. *Summary of changes in median densities of constituents after digitonin treatment of microsomes isolated from unstimulated adrenals and adrenals stimulated with low (50 μ units/ml) and high (25 units/ml) doses of corticotropin ($n = 6$)*

	10 ⁴ $\times$ Density change (median density of treated — median density of untreated)		
	Control	Low-corticotropin	High-corticotropin
Steroid	76 $\pm$ 2.5	100 $\pm$ 3.2	166 $\pm$ 3.5
21-hydroxylase			
5'-Nucleotidase	405 $\pm$ 13.2	258 $\pm$ 8.6	452 $\pm$ 11.9
Cholesterol	418 $\pm$ 12.6	282 $\pm$ 9.1	416 $\pm$ 11.7

therefore, be expected to reflect changes in membrane cholesterol levels induced by corticotropin.

The median density distributions of cholesterol, 5'-nucleotidase and steroid 21-hydroxylase in untreated and digitonin-treated microsomes are shown in Figs. 2–4. The changes in median densities of the various constituents with digitonin are summarized in Table 2. Examination of the density distributions in the control samples (not treated with digitonin) of cholesterol (Fig. 2a) and the plasma-membrane marker enzyme 5'-nucleotidase (Fig. 3a) indicates a close association between these two constituents with median densities of 1.1002 and 1.1025 g/ml respectively, whereas steroid 21-hydroxylase (Fig. 4a) equilibrates at a higher density of 1.1223 g/ml. In addition, treatment of control microsomes with digitonin further distinguishes cholesterol-containing membranes from the endoplasmic-reticulum marker steroid 21-hydroxylase. The distributions of 5'-nucleotidase and cholesterol show an increase in density after digitonin treatment by 0.04405 and 0.0418 g/ml respectively, whereas the median density of steroid 21-hydroxylase increases by 0.0076 g/ml. These results demonstrate that the cholesterol in the microsomal fraction is located mainly in the plasma membrane.

After stimulation of adrenals with low doses of corticotropin the shifts in cholesterol (Fig. 2b) and 5'-nucleotidase (Fig. 3b) produced by digitonin are reduced to 0.0258 and 0.0282 (g/ml) respectively. The cholesterol shift obtained in these fractions

Fig. 2. Distribution of cholesterol in untreated (—) and digitonin-treated microsomes (----) from control adrenals (a) and adrenals stimulated with low (b) and high (c) doses of corticotropin

Arrows indicate the median densities of cholesterol in digitonin-treated (broken arrow) and untreated (solid arrow) microsomes.

Fig. 3. Distribution of 5'-nucleotidase in untreated (—) and digitonin-treated microsomes (----) from control adrenals (a) and adrenals stimulated with low (b) and high (c) doses of corticotropin

Arrows indicate the median densities of 5'-nucleotidase in digitonin-treated (broken arrow) and untreated (solid arrow) microsomes.

corresponds to 67% of that obtained for control membranes. These results indicate that the decreased cholesterol/protein ratios obtained after stimulation of adrenal cortical cells with low levels of corticotropin result from a mobilization of plasma-membrane cholesterol, presumably for corticosterone formation.

The digitonin shifts in microsomes from adrenals stimulated with high doses of corticotropin show an increase in median density of 5'-nucleotidase (Fig. 3c) and cholesterol (Fig. 2c) of 0.0452 and 0.0416 g/ml respectively. The increase in median density of

these two constituents is similar to the control, indicating that the plasma-membrane cholesterol in these fractions has remained unchanged. In addition, stimulation of adrenals with corticotropin brings about a change in the median density of endoplasmic-reticulum membranes. The changes in median density distribution of steroid 21-hydroxylase after exposure to low and high doses of corticotropin and treatment with digitonin may be accounted for by the ability of digitonin to bind the 3 β -hydroxy intermediates generated during the production of corticosterone.

Fig. 4. Distribution of steroid 21-hydroxylase in untreated (—) and digitonin-treated microsomes (---) from control adrenals (a) and adrenals stimulated with low (b) and high (c) doses of corticotropin

Arrows indicate the median densities of steroid 21-hydroxylase in digitonin-treated (broken arrow) and untreated (solid arrow) microsomes.

Discussion

With regard to membrane cholesterol distribution in rat adrenal cortical cells, the data obtained indicate that in this tissue membrane cholesterol is located predominantly in the plasma membrane. This result is in agreement with data regarding membrane cholesterol distribution in other tissues. The significance of this is relevant when one considers the possible source of cholesterol for steroid hormone synthesis when adrenal cortical cells are treated with corticotropin. Corticotropin is known to bind to plasma-membrane receptors. The experiments carried out in this work might be expected to indicate whether this membranal cholesterol is mobilized under conditions of minimal corticotropin treatment.

With reference to the 'control' assay in these experiments, the ratios of cholesterol and cholesteryl esters is taken as 100%. In the 'minimal' corticotropin assay, i.e. where sufficient corticotropin (50 μ units/ml) is added to result in corticosterone synthesis but not to cause measurable increases in cholesteryl esterase activity and at concentrations where others have shown that no measurable increase in cyclic AMP levels occurs (Sayers *et al.*, 1972; Ling & Marsh, 1977), it can be seen that there is a decrease in the cholesterol/protein ratio to 62% of the control value with a digitonin shift of 67% of the control, and the production of corticosterone is increased by a factor of 5.8 compared with the control. There is no appreciable decrease in the cholesteryl ester/protein ratio (99.4% of control value), indicating that cholesteryl ester levels are not appreciably affected.

In the maximal corticotropin assay, i.e. where sufficient corticotropin is added to cause corticosterone synthesis and readily detectable cyclic AMP accumulation as evidenced by substantial cholesteryl ester breakdown, the cholesterol/protein ratio in the microsomal fraction and the digitonin shift are effectively the same as that in the control, whereas the cholesteryl ester/protein ratio of the homogenates is decreased by 44%, indicating mobilization of cholesterol from lipid stores in the cytoplasm. The production of corticosterone is 12.9 times the control value.

Beaufay *et al.* (1974) examined the distribution of cholesterol in rat liver with respect to 27 other constituents on an analytical basis, and they concluded that the endoplasmic reticulum is essentially free of cholesterol. Treatment of rat liver microsomes with digitonin resulted in a shift to higher density in the distribution patterns of cholesterol and enzymes associated with plasma membrane such as 5'-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase.

Subfractionation of adrenal membranes on sucrose density gradients indicates that the cholesterol in the microsomal fraction is predominantly located in the plasma membrane, whereas the endoplasmic reticulum is essentially free of cholesterol. Treatment of microsomes with digitonin results in a shift of cholesterol and 5'-nucleotidase, an enzyme associated with plasma membranes (Amar-Costesec *et al.*, 1974), to a higher density. In addition, digitonin treatment of microsomes isolated from adrenals stimulated with low doses of corticotropin indicated that the observed decrease in cholesterol/protein

ratio results from a mobilization of plasma-membrane cholesterol pools, presumably for steroid synthesis. Under these conditions, the density shift of 5'-nucleotidase and cholesterol is approx. 67% of that observed in the control. The digitonin shifts of these constituents from adrenals stimulated with high doses of corticotropin are effectively the same as in the control, indicating that the plasma-membrane cholesterol has not been altered.

Digitonin binds to 3β -hydroxysterols of which non-esterified cholesterol is the major such compound in membranes. The extent of the digitonin shift is a function of the accessible free 3β -hydroxysterol content of the membranes. From the work of others and in the present study the highest concentration of membrane cholesterol is in the plasma membrane, with smaller amounts being present in membranes such as the endoplasmic-reticulum membranes. In the present study the content of the 3β -hydroxysterols in the membranes of endoplasmic-reticulum origin is low under control conditions; however, it does show an increase after stimulation with corticotropin. This situation could in part be due to an increase in the pregnenolone content of endoplasmic reticulum in addition to the presence of cholesterol in these membranes.

Whatever factors are involved in stimulation of steroid synthesis at low doses of corticotropin, it is suggested that the plasma membrane may be involved in providing a convenient source of cholesterol for immediate synthesis of corticosterone. Such a mechanism could be postulated to operate by corticotropin binding to its receptor sites on the plasma membrane, bringing about the release of cholesterol for corticosterone synthesis. Balasubramaniam *et al.* (1977) have shown that extracellular lipoproteins are important in the regulation of adrenal cholesterol and up to 90% of the substrate cholesterol for steroid production may arise from uptake of extracellular lipoproteins (Gwynne & Hess, 1978). It is likely, therefore, that the cholesterol removed from the plasma membrane after corticotropin stimulation would be replenished *in vivo* by cholesterol derived from serum lipoproteins.

References

- Amar-Costesec, A., Wibó, M., Thines-Sempoux, D., Beaufay, H. & Berthet, J. (1974) *J. Cell Biol.* **62**, 717-745
 Balasubramaniam, S., Goldstein, J. L., Faust, J. R., Brunschede, G. Y. & Brown, M. S. (1977) *J. Biol. Chem.* **252**, 1771-1779
 Barber, E. J. (1966) *Natl. Cancer Inst. Monogr.* **21**, 219-239

- Beall, R. J. & Sayers, G. (1971) *Arch. Biochem. Biophys.* **148**, 70-76
 Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibó, M., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 213-231
 Beckett, G. J. & Boyd, G. S. (1977) *Eur. J. Biochem.* **72**, 223-233
 Carroll, K. K. (1961) *Nature (London)* **191**, 377-378
 de Duve, C. (1971) *J. Cell Biol.* **50**, 20D-55D
 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604-619
 De Wolf, M. J. S., Hilderson, H. J. J. & Dierick, W. H. S. (1977) in *Methodological Surveys in Biochemistry* (Reid, E., ed.), vol. 6, pp. 251-257, Ellis Horwood, Chichester
 Dobrota, M. & Reid, E. (1971) in *Separation with Zonal Rotors* (Reid, E., ed.), pp. Z-6.1-Z-6.2, Wolfson Bioanalytical Centre, University of Surrey, Guildford
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497-509
 Grahame-Smith, D. G., Butcher, R. W., Ney, R. L. & Sutherland, E. W. (1967) *J. Biol. Chem.* **242**, 5535-5541
 Gwynne, J. T. & Hess, B. (1978) *Metab. Clin. Exp.* **27**, 1593-1600
 Headon, D. R., Barrett, E. J., Joyce, N. M. & O'Flaherty, J. (1977) *Mol. Cell. Biochem.* **17**, 117-123
 Hinton, R. H., Dobrota, M., Fitzsimons, J. T. R. & Reid, E. (1970) *Eur. J. Biochem.* **12**, 349-361
 Kovanen, P. T., Faust, J. R., Brown, M. S. & Goldstein, J. L. (1979) *Endocrinology* **104**, 599-609
 Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482-513
 Ling, W. Y. & Marsh, J. M. (1977) *Endocrinology* **100**, 1571-1578
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
 Marsh, J. B. & Weinstein, D. B. (1966) *J. Lipid Res.* **7**, 574-576
 Moses, H. L., Davis, W. W., Rosenthal, A. S. & Garren, L. D. (1971) *Science* **163**, 1203-1205
 Moyle, W. R., Kong, Y. C. & Ramachandran, J. (1973) *J. Biol. Chem.* **248**, 2409-2417
 Pennington, R. J. (1961) *Biochem. J.* **80**, 649-654
 Porteous, J. W. & Clark, B. J. (1965) *Biochem. J.* **96**, 159-171
 Rivkin, I. & Chasin, M. (1971) *Endocrinology* **88**, 664-670
 Satre, M., Chambaz, E. M., Vignais, P. V. & Idelman, S. (1971) *FEBS Lett.* **12**, 207-212
 Sayers, G., Beall, R. J. & Seelig, S. (1972) *Science* **175**, 1131-1133
 Sayers, G., Beall, R. J. & Seelig, S. (1974) in *Biochemistry Series One* (Rickenberg, H. V., ed.), vol. 8, pp. 25-60, Butterworth and Co., London
 Seelig, S. & Sayers, G. (1972) *Arch. Biochem. Biophys.* **154**, 230-239
 Stewart, D. G. (1974) *Anal. Biochem.* **62**, 349-364
 Ungar, F., Hsiao, J., Greene, J. M. & Headon, D. R. (1979) *FEBS Lett.* **108**, 331-334