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EVALUATION OF THE SPECIFIC DICYCLOHEXYLCARBODIIMIDE BINDING SITES IN BROWN ADIPOSE TISSUE MITOCHONDRIA

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Summary

1. The content of the membrane sector of the ATPase complex (F_0) in brown adipose tissue mitochondria was determined by means of specific [^{14}C]-DCCD binding.

2. The specific DCCD binding to the F_0 protein was distinguished from the nonspecific binding to the other membrane proteins and phospholipids by: (a) Scatchard plot analysis of the equilibrium binding data, (b) SDS-polyacrylamide gel electrophoresis of the ^{14}C-labelled membrane proteins, (c) partial purification of the chloroform-methanol extractable DCCD-binding protein. It was found that the specific DCCD binding was present in three polypeptides of a relative molecular weight of 9000, 16 000 and 32 000. In brown adipose tissue mitochondria the specific binding was 10-times lower than in heart or liver mitochondria. The binding to the other membrane proteins and to phospholipids was quite similar in all mitochondrial preparations studied.

3. The decreased quantity of the specific binding sites in brown adipose tissue mitochondria demonstrated that the reduction of F_0 parallels the reduction of the F_1-ATPase and revealed that in these mitochondrial membranes the ratio between the respiratory chain enzymes and the ATPase complex is 10- to 20-times higher than in heart or liver mitochondria.

Abbreviations: F_1 and F_0 refers to catalytical and membrane-bound moiety of mitochondrial ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulphate; Temed, *N,N,N',N'*-tetramethylethylenediamine.

Introduction

It was found that the low activity of the mitochondrial ATPase in the brown adipose tissue is sufficient to allow only a small fraction of the respiratory chain capacity to be expressed [1,2]. This is due to the extremely low content of the catalytic part (F_1) of the ATPase complex [3,4].

Properties of the isolated F_1-ATPase from brown adipose tissue mitochondria were shown to be identical with other F_1-ATPase [3,4], however, the oligomycin-sensitive ATPase complex has not yet been sufficiently characterised. Thus, a question arises whether the reduction of F_1-ATPase is accompanied by a parallel reduction of the F_0 part of ATPase.

Energy transfer inhibitors DCCD and oligomycin specifically interact with the membrane part of the ATPase complex [5—7]. Until now only the concentrations of these inhibitors required for a 50% inhibition of the ATPase activity have been used to estimate the relative content of F_0 in brown adipose tissue and rat liver mitochondria [8]. It was the aim of the present work to use a more direct approach, the equilibrium binding study with [^{14}C]DCCD in order to quantify the membrane sector part of ATPase complex in brown adipose tissue mitochondria. It could be shown that the quantity of the F_0 part of the ATPase complex evaluated as specific-DCCD-binding sites is reduced in parallel to the reduction of F_1-ATPase.

Materials and Methods

Mitochondria were isolated from brown adipose tissue [9], beef heart [10] and rat liver [11], disrupted by freezing-thawing (3-times) and sedimented by centrifugation at 30 000 × *g* for 15 min [8].

The binding of [^{14}C]DCCD to mitochondrial membranes was measured at different concentrations of [^{14}C]DCCD in the media as previously described [12]. The binding data were analysed according to Scatchard [13]. The nonspecific binding was calculated as suggested by Chamness and McGuire [14] and the specific binding was obtained after subtraction of nonspecific binding from the total binding.

The [^{14}C]DCCD-labelled mitochondria (1.6 nmol per mg protein) were analysed by gradient polyacrylamide gel electrophoresis in the presence of SDS as described by O'Farrell [15]. For autoradiography the slab gels were dried under vacuum and radioactivity was detected with the aid of Kodak X-OMAT R films.

The [^{14}C]DCCD-labelled mitochondria (1.6 nmol per mg protein) were extracted with chloroform/methanol (2 : 1, v/v) according to Folch et al. [16]. The crude chloroform-methanol extract was applied to the thin layer of Silica gel (Merck G). Chromatography was carried out in chloroform/methanol/water (65 : 24 : 4, v/v). The thin-layer chromatography plates were exposed to iodine vapour and then sprayed with ninhydrin to localize the lipids, amino-positive lipids and proteins. The radioactivity was detected either by autoradiography with Kodak X-OMAT R films or directly by liquid scintillation of scrambled spots.

The content of cytochrome aa_3 in mitochondrial membranes was determined

from the absorbance change (reduced minus oxidized) at 605 nm using an extinction coefficient of 24 $mM^{-1} \cdot cm^{-1}$. The experimental conditions were similar to those of Williams [17].

Results

Evaluation of the specific-[^{14}C]DCCD-binding sites in various types of mitochondrion

Incubation of brown adipose tissue mitochondria with increasing concentrations of [^{14}C]DCCD resulted in labelling of the mitochondria directly proportional to the total [^{14}C]DCCD concentrations offered (see Fig. 1B). Hence, the [^{14}C]DCCD binding was not saturable even at concentrations much higher than 0.25 nmol DCCD/mg protein which is required for the maximum inhibition of the ATPase activity [8]. The [^{14}C]DCCD binding to the beef heart mitochondria, measured under identical conditions, was 2—3 times higher and revealed similar nonsaturability (Fig. 1A).

As was shown before, the extraction of the beef heart mitochondria with water/acetone (10 : 90, v/v) removes unspecifically bound DCCD localized in phospholipids from the mitochondrial membrane [12,19]. The [^{14}C]DCCD binding becomes saturable (Fig. 1C) and the specific-DCCD-binding sites can be distinguished [12]. The identical extraction of brown adipose tissue mitochondria suppressed the binding of the [^{14}C]DCCD to very low levels, indicating that a high portion of the ^{14}C-label was present in phospholipids (Fig. 1D).

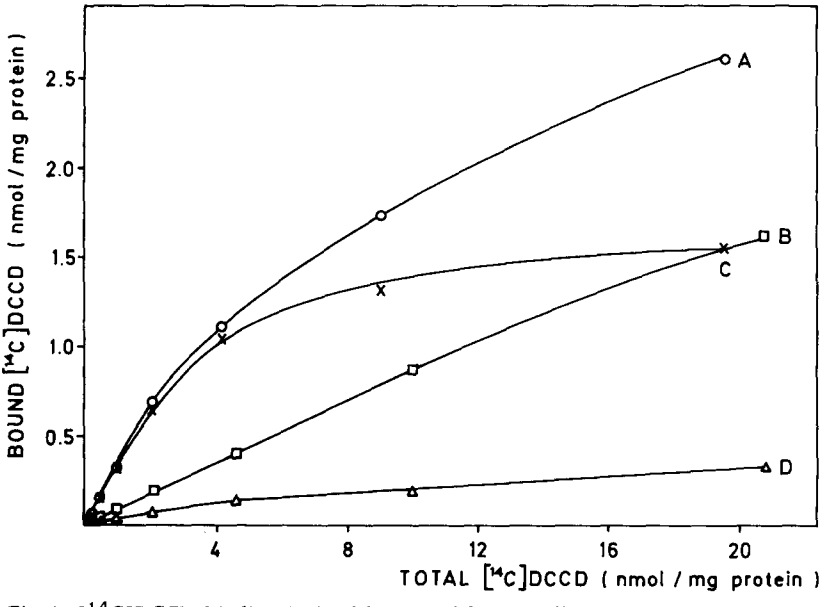

Fig. 1. [^{14}C]DCCD binding to beef heart and brown adipose tissue mitochondria. The mitochondrial membranes were incubated with increasing concentrations of [^{14}C]DCCD and the bound radioactivity was determined as described in Methods. A. Beef heart mitochondria washed with 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. B. Brown-fat mitochondria washed with 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. C. Beef heart mitochondria washed with 10% water in acetone. D. Brown adipose tissue mitochondria washed with 10% water in acetone.

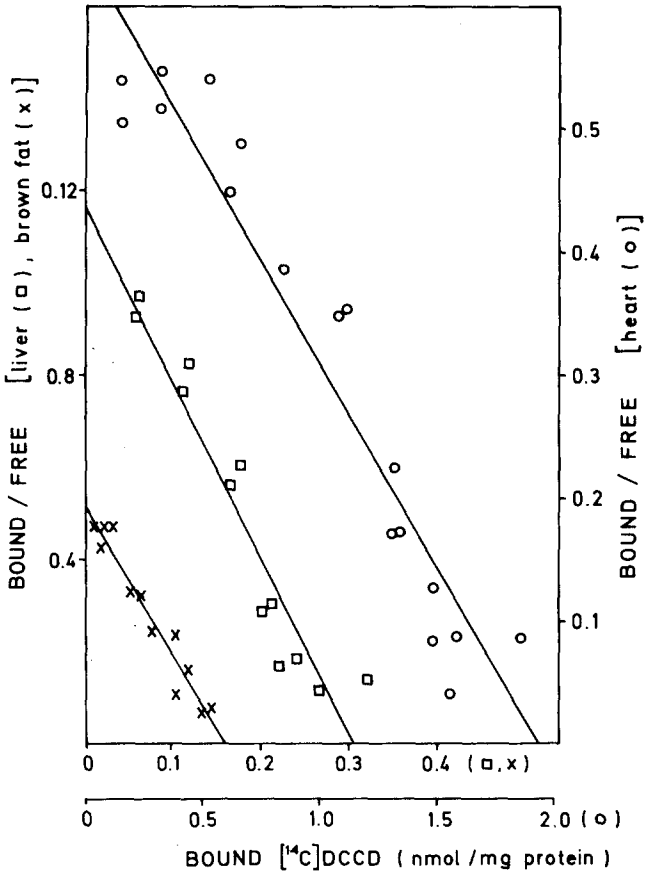

Fig. 2. The Scatchard plot of the specific [^{14}C]DCCD binding to various types of mitochondrion. The DCCD binding was measured as described in Fig. 1. Free radioactivity was calculated as a difference between the bound radioactivity and the total radioactivity to which mitochondria were exposed. The binding data obtained after acetone extraction of the mitochondrial membranes (beef heart, rat liver and brown adipose tissue) were corrected for the residual nonspecific binding as described in methods and analysed according to Scatchard [13].

The ^{14}C-label of the brown adipose tissue was 6- to 8-times lower than in the beef heart mitochondria under these conditions, however, the saturation of the binding sites was not reached. Thus, the maximum binding capacity of specific-binding sites could not be evaluated from the binding curves directly. Therefore, the results were analysed according to Scatchard [13]. To eliminate the residual portion of nonspecific binding remaining after the acetone extraction, the binding data were corrected according to Chamness and McGuire [14].

As shown in Fig. 2, the Scatchard plots of the corrected binding data appeared as parallel straight lines with negative slopes and a correlation coefficient close to one. Intercepts with the abscissa, indicative of B_{max} values, were obtained at 0.14 and 1.9 nmol DCCD per mg of membrane protein for brown fat and beef heart mitochondria, respectively. The B_{max} value of the rat liver mitochondria determined for comparison with our previous results [8] was 0.31 nmol per mg protein.

TABLE I

EVALUATION OF THE SPECIFIC-DCCD-BINDING SITES IN VARIOUS TYPES OF MITOCHONDRION

The maximum binding capacity for DCCD (B_{max}) and the content of cytochrome aa_3 were determined as described in Methods. The molar ratio of the specific-DCCD-binding sites and the respiratory chain components was calculated for each type of mitochondrial membrane.

	Beef heart	Rat liver	Brown adipose tissue
B_{max} (nmol DCCD/mg protein)	1.9	0.29	0.14
Cytochrome aa_3 (nmol/mg protein)	0.72	0.15	0.48
nmol DCCD/nmol cytochrome aa_3	2.6	1.86	0.30

The B_{max} values were further related to the respiratory chain components (cytochrome aa_3) in individual mitochondrial membranes (Table I). It appeared that the B_{max} for DCCD parallels the cytochrome aa_3 content in the beef heart and rat liver mitochondria, whilst it is significantly decreased in the brown fat mitochondria. Therefore, it could be concluded that the DCCD-binding capacity of the brown adipose tissue mitochondria is highly reduced as compared with other types of mitochondrial membrane.

Analysis of the [^{14}C]DCCD-labelled mitochondria by polyacrylamide gel electrophoresis

To distinguish between the binding of [^{14}C]DCCD to different membrane proteins, polyacrylamide gel electrophoresis of the ^{14}C-labelled mitochondria was performed. To ensure relatively specific labelling, the mitochondria were incubated with 1.6 nmol DCCD per mg protein and acetone-extracted and non-extracted samples were analysed (see Methods).

Autoradiograms and their densitometric scans are presented in Fig. 3. Beef heart mitochondria revealed three dominant radioactive bands with relative molecular weights of 32 000, 16 000 and 9500, the low molecular weight band diffusing toward the gel front (see Fig. 3). Approximately 30% of the radioactivity was present in several low intensity bands.

The overall picture of the brown adipose tissue mitochondria revealed the labelling of at least nine proteins. In comparison with beef heart mitochondria, the intensity of only the 9500, 16 000 and 32 000 bands was highly reduced. The reduction was identical in all three bands. Therefore it was assumed that only these bands represent the specific-DCCD-binding sites. When the reduction was evaluated per the cytochrome aa_3 content, the brown adipose tissue mitochondria contained 10.0-times less specific-binding sites than beef heart mitochondria.

Due to the fact that the slab gels were fixed, stained and destained in acidic methanol known to remove phospholipids from the gel [19], there was no difference between acetone-extracted and non-extracted samples.

Separation of the specific DCCD-binding protein by chloroform-methanol extraction and thin-layer chromatography

The hydrophobic nature of the DCCD-binding subunit of the ATPase complex permits its solubility in chloroform/methanol [20]. Experiments were per-

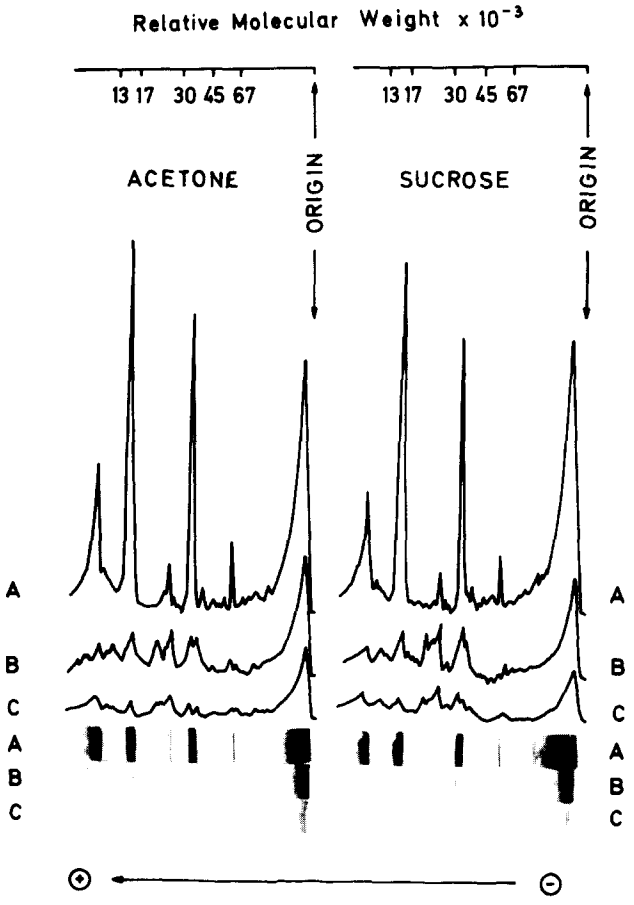

Fig. 3. Autoradiography of [^{14}C]DCCD-labelled mitochondrial proteins after SDS-polyacrylamide slab gel electrophoresis. Mitochondria were labelled with [^{14}C]DCCD (1.6 nmol per mg of protein) and then washed either with 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 or with 10% water in acetone. After electrophoresis, the slab gel was stained, destained and dried. Autoradiography was performed for 21 days and autoradiograms were scanned at 630 nm. 100 μg of beef heart mitochondria were run in slot A, 250 μg of brown adipose tissue mitochondria in slot B and 100 μg of brown adipose tissue mitochondria in slot C. The scale indicates the mobility of the molecular weight standards.

formed with the aim to separate the DCCD-binding protein from other nonspecific contaminants.

Chloroform-methanol extraction according to Folch [16] was followed by separation of the DCCD-binding protein from the bulk of phospholipids by TLC on Silica gel (Merck G).

Equal amounts of brown fat and beef heart mitochondria were labelled with [^{14}C]DCCD and the membrane material was extracted. When the original mitochondria and crude chloroform-methanol extract obtained in the course of the Folch procedure (see Table II) were examined, the radioactive label was only slightly different in the two types of membrane. Also the amount of [^{14}C]-DCCD nonspecifically bound to hydrophilic proteins non-extractable with chloroform/methanol (precipitate) was almost the same in both preparations.

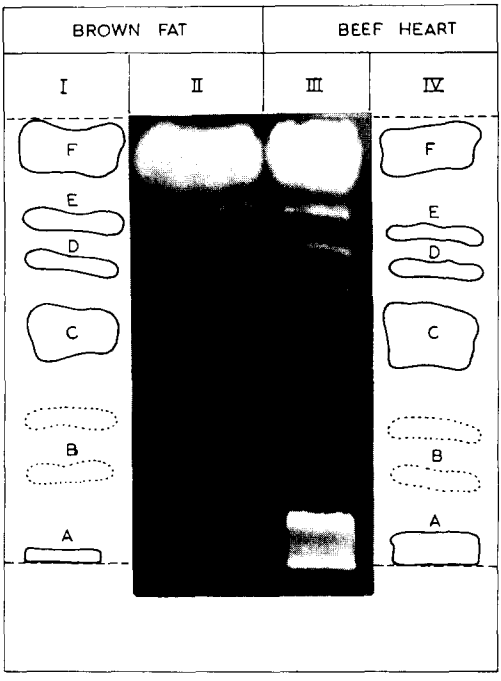

Fig. 4. Fractionation of the chloroform-methanol extract of the [^{14}C]DCCD-labelled mitochondria by thin-layer chromatography on Silica gel G. Final chloroform-methanol extract prepared according to Folch [16] was examined by TLC as described in Methods. I, IV, Schematic picture of the chromatograph after staining with ninhydrin and iodine vapour. II, III, Autoradiographic detection of the ^{14}C-labelled components after chromatographic separation. A, DCCD-binding protein; B, minority phospholipids; C, phosphatidylethanolamine; D, phosphatidylcholine; E, cardiolipin; F, yellow pigment, probably flavins.

TABLE II

PARTIAL PURIFICATION OF THE DCCD-BINDING PROTEIN

The same amount of the beef heart and brown adipose tissue mitochondria (100 mg protein) was labelled with [^{14}C]DCCD. Mitochondria were extracted with chloroform/methanol and the crude extract was separated into phospholipid and protein fractions by TLC. Data represent total ^{14}C-label in various fractions obtained during isolation procedure (see Methods).

	nmol [^{14}C]DCCD		
	Brown adipose tissue (A)	Beef heart (B)	B/A
Mitochondria	18.7	34.0	1.8
Precipitate	2.5	3.0	1.2
Interphase	0.4	0.9	2.2
Crude extract	13.2	26.3	2.0
Phospholipids	10.8	11.8	1.1
DCCD-binding proteins	0.3	7.7	25.6

The difference in the specific binding capacity between the two membrane preparations became apparent after the fractionation of the crude chloroform-methanol extract by TLC. As shown in Fig. 4, the hydrophobic proteins remained at the start (spot A) and were separated from minority phospholipids (spot B), phosphatidylcholine (spot C), phosphatidylethanolamine (spot D) and cardiolipin (spot E). A yellow substance, most probably flavins, migrated with the front. Radioactivity detected in the hydrophobic DCCD-binding proteins was 25-times lower in the brown adipose tissue mitochondria than in the beef heart mitochondria when expressed per mg protein and 16.9-times lower when evaluated per cytochrome *a* content. The rest of the radioactivity recovered from the thin-layer chromatography plates and belonging to the phospholipid-associated DCCD was comparable in both types of mitochondrion (see Table II). This is in agreement with the similar content of phospholipids in these membranes [21].

Discussion

The previous results from this laboratory [8] indicated that in the brown adipose tissue mitochondria as compared with rat liver mitochondria, 8-times less aurovertin and 2- to 3-times less DCCD is required for an equal inhibition of the ATPase activity. Such a difference in the titre of individual inhibitors would suggest a lack of stoicheiometry between the F_1 and F_0 parts of the ATPase complex in brown adipose tissue mitochondria. However, the total concentration of the inhibitor represents only an indirect estimate of the specific binding capacity as DCCD is known to interact not only with the F_0 part of the ATPase complex [5—7], but also with other membrane proteins and with phospholipids [19,22—24]. In order to evaluate the specific binding sites for DCCD localised in the membrane sector of the ATPase complex of brown adipose tissue mitochondria, the binding of [^{14}C]DCCD by isolated mitochondria was measured.

Identification and quantification of these binding sites was more complicated than e.g. in heart mitochondria [12], because the percentage of specific binding sites was considerably decreased. The evaluation of specific binding sites was based on the assumption of their higher affinity to the ligand, and on the finding that saturation of these binding sites at low ligand concentrations is accompanied by inhibition of the ATPase activity. Three lines of experimental evidence were used in these studies: (a) Scatchard plot analysis of the equilibrium binding data, (b) polyacrylamide gel electrophoresis combined with autoradiography of ^{14}C-labelled membrane proteins, (c) extraction and purification of the DCCD-binding protein.

Contrary to the beef heart mitochondria, in brown adipose tissue mitochondria, the Scatchard plot analysis of the equilibrium binding data results in a non-linear concave plot (not shown). Only after correction for nonspecific binding [14] was a linear plot obtained, and could the maximum binding capacity of the specific-DCCD-binding sites be calculated. The results showed that B_{max} values in brown adipose tissue mitochondria related to the content of cytochrome aa_3 were 9-times lower than in heart and 6-times lower than in liver mitochondria.

This result was further verified by SDS-polyacrylamide gel electrophoresis of the [^{14}C]DCCD-labelled mitochondrial polypeptides. In the beef heart mitochondria 70% of the total ^{14}C-label was associated with polypeptide bands of relative molecular weights of 9500, 16 000 and 32 000. In parallel samples of the brown adipose tissue, all these three bands were 10-times reduced.

As opposed to the findings with the specific sites, the number of nonspecific-DCCD-binding sites was found to be almost identical in beef heart and brown fat mitochondria by both methods. They could be further distinguished as nonspecific sites on phospholipids and on proteins. In Scatchard plots the latter type appeared as a nonspecific binding in the acetone extracted material and was present on autoradiograms as the bands of the same intensity in both types of mitochondrion.

To support further the above conclusion, the DCCD-binding protein was extracted from heart and brown adipose tissue mitochondria, and its radioactivity was determined. This procedure made it also possible to determine the nonspecific binding of DCCD to individual phospholipids. The data also showed that the ^{14}C-label could be detected in all phospholipid fractions, and in agreement with previous data the radioactivity detected in the DCCD binding protein fraction was 15-times lower in brown adipose tissue mitochondria than in the heart mitochondria.

Assuming that the same stoicheiometry of the DCCD units in the membrane sector of the ATPase complex exists in various mitochondrial membranes [25], it may be concluded that the membrane (F_0) and catalytic (F_1) parts of the ATPase complex in brown adipose tissue mitochondria are reduced simultaneously. Accordingly, the synthesis of all protein subunits of the ATPase complex should be equally depressed, irrespective of their cytoplasmic or mitochondrial origin.

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References

1 Nicholls, D.G. (1979) Biochim. Biophys. Acta 549, 1—29
2 Nicholls, D.G. (1976) Eur. J. Biochem. 62, 223—228
3 Cannon, B. and Vogel, G. (1977) FEBS Lett. 76, 284—289
4 Houštěk, J. and Drahota, Z. (1977) Biochim. Biophys. Acta 484, 127—139
5 Beechey, R.B. (1974) in Membrane Adenosine Triphosphatases and Transport Processes (Bronk, J.R., ed.), pp. 41—62, The Biochemical Society, London
6 Cattell, K.J., Lindop, C.R., Knight, I.E. and Beechey, R.B. (1971) Biochem. J. 125, 169—177
7 Norling, B., Glaser, E. and Ernster, L. (1978) in Frontiers of Biological Energetics: From Electrons to Tissues (Dutton, L.P., Leigh, J.S. and Scarpa, A., eds.), pp. 504—515, Academic Press, New York
8 Houštěk, J., Kopecký, J. and Drahota, Z. (1978) Comp. Biochem. Physiol. 60B, 209—214
9 Hittelman, K.J., Lindberg, O. and Cannon, B. (1968) Eur. J. Biochem. 11, 183—192
10 Smith, A.L. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 81—86, Academic Press, New York
11 Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem. 183, 1123—1128
12 Svoboda, P., Kopecký, J., Houštěk, J. and Drahota, Z. (1979) Biochim. Biophys. Res. Commun. 89, 981—987

13 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660—671
14 Chamness, G.C. and McGuire, W.L. (1975) Steroids 26, 538—542
15 O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007—4021
16 Folch, J., Lees, M., Stanley, S. and Stanley, G.H. (1957) J. Biol. Chem. 226, 497—553
17 Williams, J.M. (1964) Arch. Biochem. Biophys. 107, 537—543
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Dianoux, A.-C., Bof, M. and Vignais, P.V. (1978) Eur. J. Biochem. 88, 68—77
20 Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630—6637
21 Lišková, Z., Strunecká, A. and Drahota, Z. (1974) Physiol. Bohemoslov. 23, 229—234
22 Stekhoven, E.S., Waikus, R.F. and Moerkerk, H.T.B. (1973) Biochemistry 11, 1144—1150
23 Holloway, C.T., Roberton, A.M., Knight, I.G. and Beechey, R.B. (1966) Biochem. J. 100, 79P
24 Casey, R.P., Thelen, M. and Azzi, A. (1979) Biochem. Biophys. Res. Commun. 87, 1044—1051
25 Sebald, W., Graf, T. and Lukins, H.B. (1979) Eur. J. Biochem. 93, 587—599