Biochimica et Biophysica Acta, 291 (1973) 557–563

BBA 76166

THE EFFLUX OF BETAINE FROM RAT-LIVER MITOCHONDRIA, A POSSIBLE REGULATING STEP IN CHOLINE OXIDATION

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(Received August 2nd, 1972)

SUMMARY

1. Rat-liver mitochondria accumulate betaine during coupled "State 3" oxidation of choline.

2. At low salt concentrations, betaine efflux from mitochondria occurs, when also adenine nucleotides leak out of the mitochondria.

3. At high salt concentrations betaine efflux is slow.

4. It is concluded that the overall oxidation of choline by rat-liver mitochondria may be limited by the efflux of betaine.

INTRODUCTION

Isolated rat-liver mitochondria are able to oxidize choline to betaine *via* betaine aldehyde[1–5]. The reactions involved take place inside the mitochondrial matrix[3–8] and therefore, the substrate choline and the product betaine have to pass the inner membrane for complete reaction.

It was suggested some years ago that the entry of choline is a limiting factor in its overall oxidation especially under conditions where high concentrations of monovalent cations are present[16]. An effect of adenine nucleotides on choline movement across the mitochondrial membrane was also postulated[4].

However, it seemed equally possible to us that the efflux of products of choline oxidation is a limiting step. Evidence favouring this possibility came forward when we found that under some conditions high concentrations of betaine accumulate in the mitochondrial matrix[8]. When choline is oxidized under "State 3" conditions the rate of oxygen consumption is only 10% of that in the presence of an uncoupler. Under these conditions the final product of choline oxidation, betaine, is retained almost completely within the mitochondria. Betaine aldehyde is not present in appreciable concentrations either inside or outside the mitochondria[8]. These conditions then, provided us the possibility to study the conditions under which the accumulated

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; S-13, 5-chloro, 3-*tert*-butyl, 2′-chloro, 4′-nitro-salicylanilide; MOPS, morpholinopropane sulphonic acid.

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betaine can leave the matrix space. We concluded from these studies, that the movement of betaine across the mitochondrial inner membrane is one of the limiting steps in overall choline oxidation.

RESULTS AND DISCUSSION

When rat-liver mitochondria are allowed to oxidize choline in the presence of ADP and phosphate or glucose and hexokinase most oxidation products remain within the matrix (Fig. 1). Evidently, there is a barrier preventing the final oxidation product,

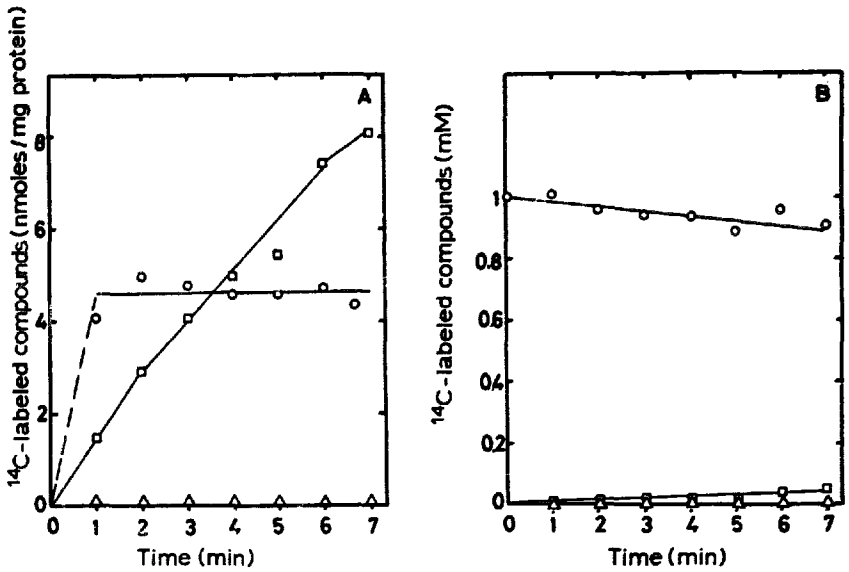

Fig. 1. Distribution of choline and its products during "State 3" choline oxidation. Rat-liver mitochondria, 4.2 mg protein/ml, were incubated in a medium containing 175 mM sucrose, 10 mM glucose, 5 mM Tris–morpholinopropane sulphonic acid (MOPS) buffer, 5 mM potassium phosphate buffer, 5 mM ADP, 0.2 mM $MgCl_2$, 4 I.U. hexokinase, 1 mM choline, 7 μCi [^{14}C]choline, final volume, 7 ml; pH 6.9; temperature, 25 °C. At the times indicated in the figure, samples were taken, centrifuged and analysed as described under Materials and Methods. ○—○, choline; □—□, betaine; △—△, betaine aldehyde; A, ^{14}C-labeled compounds in the matrix; B, ^{14}C-labeled compounds in the medium.

betaine, from leaving the mitochondrion. This results in a severely inhibited choline oxidation compared with uncoupler stimulated oxidation, when most products are found outside the mitochondria[8]. The betaine is firmly enclosed within the mitochondrial inner membrane, because the suspension can be diluted with cold sucrose and washed without appreciable loss of betaine, whereas by the same treatment most choline is removed. In subsequent experiments betaine-loaded mitochondria were prepared by incubation under conditions of Fig. 1, followed by dilution with cold sucrose and reisolation. It was verified by paper chromatography in each case that more than 90 % of the intramitochondrial ^{14}C label was betaine.

In Fig. 2A the effect of several additions on the efflux of betaine from betaine-loaded mitochondria is shown. There is a marked increase in efflux in the presence of 2,4-dinitrophenol in a concentration that also stimulates the overall oxidation of choline. The effect of 2,4-dinitrophenol is duplicated by other uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 5-chloro,3-*tert*-butyl, 2'-chloro,4'-nitro-salicylanilide (S-13) and lauric acid. In each case excessive concentrations of the uncoupler (although these concentrations did not exceed usual uncoupling concentrations for oxidation of substrates) resulted in diminished efflux, up to

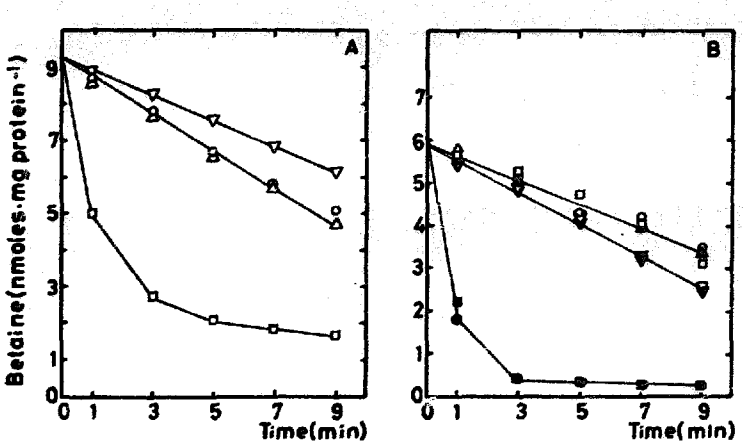

Fig. 2. Efflux of betaine from mitochondria at different "energy states". Betaine-loaded mitochondria (1.5 mg protein/ml and 2.1 mg protein/ml, in Expts 2A and 2B, respectively) were incubated in a medium containing 175 mM sucrose, 10 mM glucose, 1 mM $MgCl_2$, 5 mM potassium phosphate buffer (Expt 2A) or 185 mM sucrose and 5 mM potassium phosphate buffer (Expt 2B). Final volume, 5 ml; temperature, 25 °C; pH 7.0. At different times samples were taken and analysed for betaine as described under Materials and Methods. Additions: A. ▽—▽, 1 mM ATP, ○—○, no addition; △—△, 5 I.U. hexokinase; □—□, 20 μM 2,4-dinitrophenol. B. ▼—▼; no additions; ○—○, 1 mM ATP and 0.85 μg oligomycin per mg protein; ▲—▲, 1 mM ATP, 0.85 μg oligomycin per mg protein and 20 μM 2,4-dinitrophenol; ▽—▽, 0.85 μg oligomycin per mg protein; ■—■, 20 μM 2,4-dinitrophenol; ●—●, 0.85 μg oligomycin per mg protein and 20 μM 2,4-dinitrophenol. Oligomycin, if added, was present 2 min before starting the experiment.

50 % at the highest concentrations tested. On the other hand, ATP slightly inhibits the slow efflux of betaine. Combination of these results suggest an effect of the energetic condition of the mitochondria on the rate of betaine efflux, *i.e.* a rapid efflux under low energy conditions. However, this is contradicted by the fact that in the presence of hexokinase – which also should lower the mitochondrial energy content – there is no increase in betaine efflux, as compared to the control. In Fig. 2B it is shown even more markedly that it is not the energetic condition that regulates the betaine efflux. When ATP, oligomycin and 2,4-dinitrophenol are added the efflux of betaine is slow although this is certainly a low energy condition.

The enhancement of betaine efflux by 2,4-dinitrophenol is dependent on the presence of phosphate (Fig. 3A). The effect of phosphate is partly duplicated by mersalyl, whereas the combined addition of phosphate and mersalyl leads to an intermediate rate of betaine efflux (Fig. 3B). These effects correspond nicely with results found when choline oxidation was measured (Ridder, J. J. M. de, unpublished): optimal oxidation required the presence of both an uncoupler and phosphate[9]. The probable reason for the effect of mersalyl is that this compound inhibits phosphate movement across the mitochondrial membrane and thereby under these conditions maintains a high intramitochondrial level of phosphate[10].

At this stage of the investigation it became apparent that conditions leading to betaine efflux in some respects were reminiscent of those leading to efflux of adenine nucleotides from mitochondria as reported by Meisner and Klingenberg[11]. To test this idea further we measured the betaine efflux in the presence of bongkrekic acid, a specific inhibitor of both exchange[12,13] and leakage of adenine nucleotides from mitochondria[14]. Indeed, bongkrekic acid inhibits the betaine efflux completely (Fig. 4A), even in the presence of phosphate and an uncoupler. Also the addition of extramitochondrial adenine nucleotides markedly inhibits the uncoupler-stimulated betaine efflux (Fig. 4B).

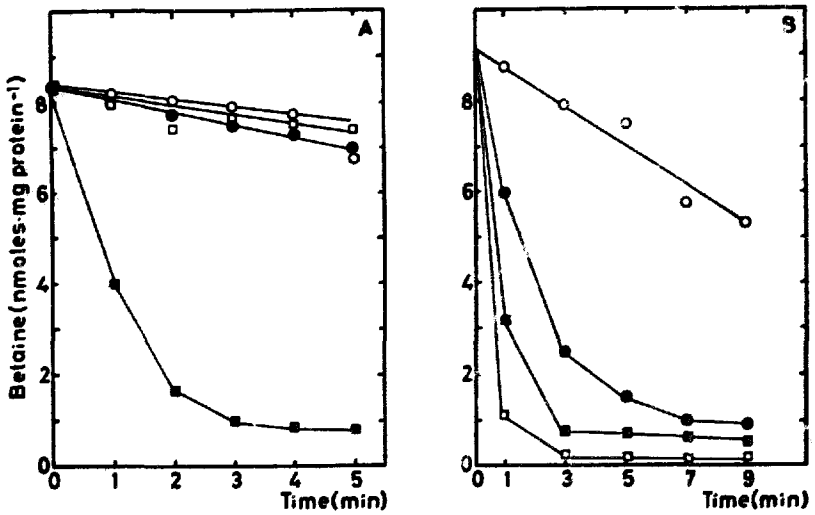

Fig. 3. Influence of phosphate on betaine efflux. Betaine-loaded mitochondria (3.2 mg protein/ml in Expt 3A and 3.9 mg protein/ml in 3B) were incubated in a medium containing 185 mM sucrose and 5 mM Tris–MOPS buffer at a temperature of 25 °C and a pH of 7.0. Final volume, 5 ml. Samples were taken as described under Materials and Methods. Additions: A. ○—○, 1 mM ATP; ●—●, 20 μM 2,4-dinitrophenol; □—□, 1 mM ATP and 5 mM phosphate; ■—■, 20 μM 2,4-dinitrophenol and 5 mM phosphate; B. ○—○, 20 μM 2,4-dinitrophenol; ●—●, 20 μM 2,4-dinitrophenol *plus* 28 nmoles mersalyl per mg protein; □—□, 20 μM 2,4-dinitrophenol, 28 nmoles mersalyl per mg protein and 5 mM phosphate; ■—■, 20 μM 2,4-dinitrophenol *plus* 5 mM phosphate. Mersalyl, if added, was present 2 min before starting the experiment.

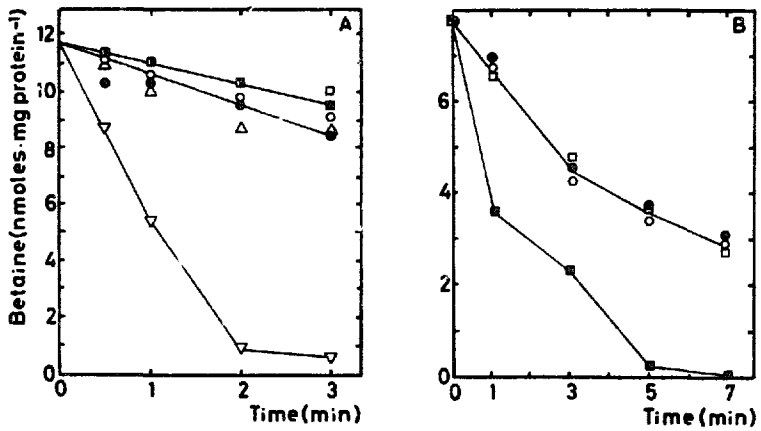

Fig. 4. The effects of bongkrekic acid and adenine nucleotides on betaine efflux from mitochondria. Betaine-loaded mitochondria (4.1 mg protein/ml in Expt 4A and 2.4 mg protein/ml in Expt 4B) were incubated in a medium containing 185 mM sucrose and 5 mM potassium phosphate buffer, at a pH of 7.0 and a temperature of 25 °C. Final volume, 4 ml. Samples were taken as described under Materials and Methods. Additions: A. □—□, 1 mM ATP + 10 μM bongkrekic acid; ■—■, 1 mM ATP + 10 μM bongkrekic acid + 20 μM 2,4-dinitrophenol; ○—○, no addition; ●—●, 10 μM bongkrekic acid; △—△, 10 μM bongkrekic acid + 20 μM 2,4-dinitrophenol; ▽—▽ 20 μM 2,4-dinitrophenol; B. ■—■, 20 μM 2,4-dinitrophenol; ○—○, 20 μM 2,4-dinitrophenol + 1 mM AMP + 1.6 μg oligomycin/mg protein; ●—●, 20 μM 2,4-dinitrophenol + 1 mM ADP -- 1.6 μg oligomycin/mg protein; □—□, 20 μM 2,4-dinitrophenol + 1 mM ATP + 1.6 μg oligomycin/mg protein. Bongkrekic acid and oligomycin, if added, were present 2 min before starting the experiments.

In Table I the adenine nucleotide efflux under our experimental conditions is given. Comparison with the results given in Figs 2–4 shows good correlation between adenine nucleotide efflux and betaine efflux. Evidently the mitochondrial membrane becomes permeable toward betaine only under conditions when adenine nucleotides

TABLE I

EFFLUX OF ADENINE NUCLEOTIDES FROM RAT-LIVER MITOCHONDRIA

Betaine-loaded mitochondria (9.1 mg/ml) were incubated in a medium comparable to that normally used for measuring betaine efflux, containing 185 mM sucrose. Additions as indicated in the table. pH 7.0; temperature, 25 °C; incubation time, 3 min; final volume of each incubation, 1 ml. Samples were taken and adenine nucleotides were measured as described in Materials and Methods. In Incubation 6 160 mM sucrose was replaced by 80 mM KCl. Bongkrekic acid or mersalyl, if added, was present 2 min before starting the experiments. Results are expressed as nmoles/mg protein.

Addition	*ATP*	*ADP*	*AMP*	*Total*
1. None	4.3	5.0	6.0	15.3
2. 2,4-Dinitrophenol (20 μM)	0.8	4.9	5.3	11.0
3. 2,4-Dinitrophenol + P_i (5 mM)	0.9	0.7	3.1	4.7
4. 2,4-Dinitrophenol + mersalyl (32 nmoles/mg protein)	0.6	2.0	3.0	5.6
5. 2,4-Dinitrophenol + P_i + bongkrekic acid (10 μM)	0.6	6.4	6.4	13.4
6. 2,4-Dinitrophenol + P_i + KCl (80 mM)	0.7	0.6	3.1	4.3

also leak out. We wish to point out, however, that this is not a generalized non-specific leakage, because it is inhibited for instance by bongkrekic acid.

To gain more understanding of the actual mechanism by which betaine is transported out of the mitochondria, we tested whether its metabolic precursors could stimulate its efflux. As can be seen in Fig. 5 neither betaine aldehyde, nor choline can increase the rate of betaine efflux under the conditions tested. Even betaine itself cannot exchange with intramitochondrial betaine so as to produce an increased rate of loss of label. Thus, the mechanism of betaine movement must be very different from that of the better known anion translocators in the mitochondrial inner membrane, that act as exchange-diffusion carriers[15].

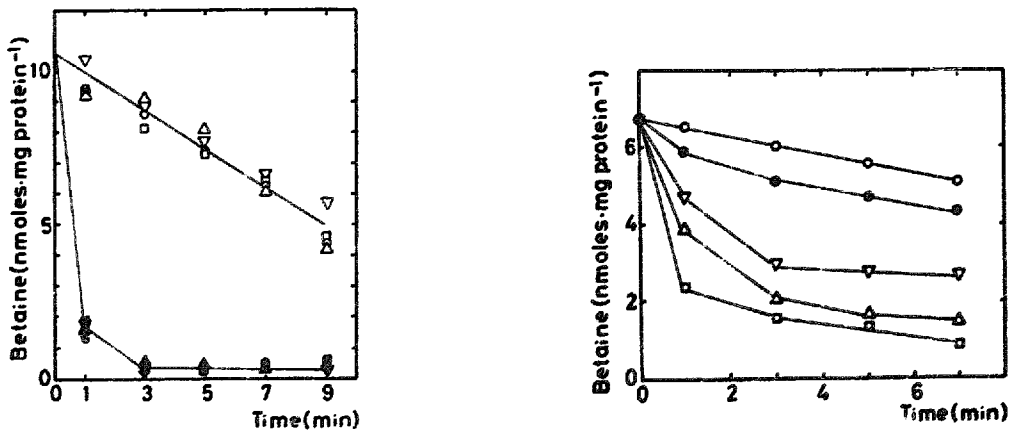

Fig. 5. Effect of added choline, betaine aldehyde and betaine on betaine efflux. Betaine-loaded mitochondria (2.0 mg protein/ml) were incubated in a medium containing 185 mM sucrose, 5 mM potassium phosphate buffer; pH 7.0, temperature, 25 °C. Final volume, 5 ml. Samples were taken as described under Materials and Methods. Open symbols: *plus* 1 mM ATP; closed symbols: *plus* 20 μM 2,4-dinitrophenol. ○—○ and ●—●, no addition; □—□ and ■—■, 10 mM choline; △—△ and ▲—▲, 10 mM betaine aldehyde; ▽—▽ and ▼—▼, 10 mM betaine.

Fig. 6. Inhibition of betaine efflux by K^+. Betaine-loaded mitochondria (1.9 mg protein/ml) were incubated in a medium containing 2.5 mM potassium phosphate buffer, 20 μM 2,4-dinitrophenol and sucrose to adjust the osmolarity to 185 mosM. Samples were taken as described under Materials and Methods. Additions: ○—○, 75 mM KCl; ●—●, 40 mM KCl; ▽—▽, 15 mM KCl; △—△, 5 mM KCl; □—□, no addition.

Since there is a marked effect of cations on uncoupler-stimulated choline oxidation by rat-liver mitochondria[16] (Ridder, J. J. M. de, unpublished), it was of interest to test the effect of the cationic composition of the medium on betaine efflux. In Fig. 6 an experiment is shown in which sucrose was replaced by KCl. From other experiments we know that it is K^+ and not Cl^- that is effective in inhibiting choline oxidation (Ridder, J. J. M. de, unpublished). A number of other monovalent cations (choline$^+$, Na^+ and $Tris^+$ in concentrations of 80 mM) were tested. They all inhibited betaine efflux tc the same extent as they inhibited choline oxidation. The inhibitory effect of these cations was partly overcome by high concentrations of phosphate, also in agreement with findings in metabolic experiments.

Mg^{2+} also inhibits betaine efflux and so does La^{3+}. The concentrations at which the uncoupler-stimulated betaine efflux was inhibited completely were 20 and 1 mM, respectively. However, at a Mg^{2+} concentration of 5 mM, which inhibits choline oxidation at least 95 %, no or nearly no inhibition of betaine efflux was found.

The effect of temperature on the uncoupler-induced betaine efflux is very marked. At 0 °C hardly any efflux was observed. The Q_{10} value of the process is approximately 3. Since adenine nucleotide efflux also has a very high temperature coefficient[17], it is hard to say at present which step is primarily responsible for the high Q_{10} value of betaine efflux: adenine nucleotide efflux or betaine efflux itself.

Two main factors appear to govern the rate of betaine efflux from mitochondria. First, the betaine efflux is inhibited strongly by the presence of high concentrations of monovalent cations in the surrounding medium. Secondly, only conditions leading to an efflux of adenine nucleotides from mitochondria show an increased rate of betaine efflux. These results point to betaine efflux from the mitochondrial matrix as a limiting step in choline oxidation.

This means that in the cell, which contains a high concentration of K^+ and also appreciable concentrations of adenine nucleotides, leakage of betaine from the mitochondria will be practically impossible. Choline oxidation therefore will be slow also and wasteful breakdown of choline — required as a building block for phospholipids — is prevented.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated according to the method of Hogeboom[18], as described by Myers and Slater[19].

Protein was determined according to Cleland and Slater[20].

Separation of mitochondria from incubation mixtures by centrifugation–filtration was performed according to the method of Werkheiser and Bartley[21] as modified by Harris and van Dam[22].

ATP, ADP and AMP were determined according to the method described by Williamson and Corkey[23].

^{14}C-labeled choline, betaine aldehyde and betaine were separated by paper chromatography as described by Wilken[6].

^{14}C-labeled compounds were measured by liquid scintillation counting in a mixture of toluene and ethanol (3:1, v/v) containing 50 mg POPOP and 4 g PPO per l.

[U-^{14}C]Choline was obtained from New England Nuclear. Choline and betaine were obtained from British Drug Houses. Betaine aldehyde was a gift from Drs F. M.

Kaspersen. Bongkrekic acid was a gift from Prof. Dr G. W. M. Lijmbach. Oligomycin was obtained from Sigma Chemical Company.

ACKNOWLEDGEMENTS

We wish to thank Mrs C. Verdouw and Mrs D. voor in 't Holt for co-operation in some of the experiments. This investigation was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 Jellinek, M., Strength, D. R. and Thayer, S. A. (1959) *J. Biol. Chem.* 234, 1171–1173
2 Bianchi, G. and Azzone, G. F. (1964) *J. Biol. Chem.* 239, 3947–3955
3 Kagawa, T., Wilken, D. R. and Lardy, H. A. (1965) *J. Biol. Chem.* 240, 1836–1842
4 Wilken, D. R., Kagawa, T. and Lardy, H. A. (1965) *J. Biol. Chem.* 240, 1843–1846
5 Speed, D. and Richardson, M. (1968) *J. Chromatogr.* 35, 497–505
6 Wilken, D. R. (1970) *Anal. Biochem.* 36, 323–331
7 Wilken, D. R., McMacken, M. L. and Rodriquez, A. (1970) *Biochim. Biophys. Acta* 216, 305–317
8 Ridder, J. J. M. de, *8th Meet. Fed. Eur. Biochem. Soc., Amsterdam, 1972*, Abstr. 1136
9 Guha, S. and Wegmann, R. (1963) *Nature* 200, 1218–1219
10 Tyler, D. D. (1968) *Biochem. J.* 107, 121–123
11 Meisner, H. and Klingenberg, M. (1968) *J. Biol. Chem.* 243, 3631–3639
12 Henderson, P. J. F. and Lardy, H. A. (1970) *J. Biol. Chem.* 245, 1319–1326
13 Klingenberg, M., Grebe, K. and Heidt, H. W. (1970) *Biochem. Biophys. Res. Commun.* 39, 344–351
14 Out, T. A., Kemp Jr, A. and Souverijn, J. M. (1971) *Biochim. Biophys. Acta* 245, 299–304
15 Dam, K. van, and Meijer, A. J. (1971) *Annu. Rev. Biochem.* 40, 115–160
16 Williams, G. R. (1950) *J. Biol. Chem.* 235, 1192–1195
17 Kemp Jr, A., Groot, G. S. P. and Reitsma, H. J. (1969) *Biochim. Biophys. Acta* 180, 28–34
18 Hogeboom, G. H. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 16–19, Academic Press, New York
19 Myers, D. K. and Slater, E. C. (1957) *Biochem. J.* 67, 558–572
20 Cleland, K. W. and Slater, E. C. (1953) *Biochem. J.* 53, 547–556
21 Werkheiser, W. C. and Bartley, W. (1957) *Biochem. J.* 66, 79–91
22 Harris, E. J. and Dam, K. van (1968) *Biochem. J.* 106, 759–766
23 Williamson, J. R. and Corkey, B. E. (1969) *Methods in Enzymology* (Lowenstein, J. M., ed.), Vol. 13, pp. 434–513, Academic Press, New York.