Biochimica et Biophysica Acta, 975 (1989) 330–335
Elsevier

BBABIO 43028

Chelating agents and rat liver mitochondria

Patricia Lund and David Wiggins

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford (U.K.)

(Received 3 March 1989)

Key words: Mitochondria; EDTA; EGTA; Adenine nucleotide; P_i-induced swelling; (Rat liver)

The chelating agents (EGTA and EDTA) and inorganic phosphate (P_i) are the most variable components of experiments involving isolated liver mitochondria. In the absence of EGTA or EDTA, swelling induced by P_i leads to rapid loss of endogenous adenine nucleotides to adenosine. Chelating agents prevent swelling and loss of adenine nucleotides. Concentrations below about 0.1 mM are ineffective. The protective effects depend on the continuous presence of the chelating agent; they are lost on washing EGTA-containing suspensions with chelating-agent-free medium. We question the accepted view that chelating agents stabilize mitochondria by binding Ca^{2+} to prevent activation of phospholipase.

Introduction

Isolated liver mitochondria are commonly used to study enzymic reactions in situ, with little or no consideration of the morphological changes that can occur to affect both the permeability of the membranes and the enzyme activities. This is not surprising in view of the tremendous variety of media for isolation and incubation that a beginner encounters in published papers. Each laboratory has its own cocktail, and no one anymore sets out the reasons for inclusion of this or that concentration of buffer, inhibitor or chelating agents. We began to realise that apparently insignificant alterations in the composition of the isolation or incubation medium could have very pronounced effects, as measured by liver glutaminase activity. Perhaps our experience may be of some use to others.

This paper is concerned with effects of EGTA, EDTA and P_i, the most variable components of experiments involving liver mitochondria. P_i is almost universally added to incubations. Chelating agents may be included in the isolation and/or incubation medium; they are reported to stabilize the preparation by binding Ca^{2+}, thus inhibiting activity of the Ca^{2+}-dependent phospholipases [1]. This implies that Ca^{2+} is removed from the mitochondrial membranes by the chelating agent. In fact, our experiments show that the stabilizing effects of EGTA are totally reversible. The continuous presence of the chelating agent (more than 0.1 mM) in the incubation medium is required to prevent P_i-induced mitochondrial swelling and the biochemical changes associated with it.

Materials and Methods

Fed male rats were killed by cervical dislocation and their livers were removed into ice-cold 0.3 M mannitol containing 5 mM Hepes adjusted to pH 7.4 with KOH. Mitochondria were isolated in the mannitol/Hepes medium by the rapid method described by Kun et al. [2]. Unless stated otherwise incubation was in the same medium with aqueous additions contributing no more than 10% of the final volume. In some experiments mitochondria were isolated and incubated in mannitol/Hepes containing 1 mM EGTA. In others, homogenization and the centrifugations to the stage of the first mitochondrial pellet were in mannitol/Hepes/EGTA with the two washes of the pellet in EGTA-free medium.

Mitoplasts were prepared in mannitol/Hepes medium from freshly isolated mitochondria as described by Greenawalt [3] in the absence or presence of 1 mM EGTA.

Incubations were in 25 ml stoppered conical flasks, gas phase O_2, with shaking in a water bath at 37°C. Components of the incubation were equilibrated to 37°C before addition of mitochondrial suspension. Reactions were stopped with 0.1 volume 20% $HClO_4$. Acid supernatants were neutralized with KOH before mea-

Abbreviations: P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Correspondence: P. Lund, Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

surements of metabolites. When lipid components were examined, a volume of suspension was centrifuged briefly in an Eppendorf microfuge, the supernatant poured off, and the pellet resuspended in 0.5 ml H_2O before being transferred to 10 ml $CHCl_3$/methanol (2 : 1) for extraction. Further treatment and preparation of samples for TLC on Silica gel G plates (Analtech, Newark, USA) was by conventional methods [4]. Chromatography in hexane/ether/glacial acetic acid (70 : 30 : 2) was followed by treatment with iodine vapour to visualize the lipid components.

Interference by mannitol in protein determination

Protein was determined in suspensions by the biuret method after solubilizing the protein with deoxycholate [5]. We noticed that the concentration of mitochondrial protein appeared to decrease over the range of sample size (0.02–0.05 ml) taken for assay. This was traced to a non-stoichiometric reaction of mannitol with the biuret reagent. After correction of the readings at 520 nm for this interference, the mitochondrial protein values became linearly related to sample size. It is important to appreciate that this source of error can make a difference of more than 50% to the apparent protein concentration of a suspension containing approx. 30 mg per ml if a small sample size (0.01 ml) is assayed. Neither Hepes (the other component of the mannitol medium) nor sucrose interferes in the biuret assay.

Measurements of [^{14}C]EDTA distribution space

In a series of incubations the matrix and extramatrix spaces were measured using [^{14}C]sucrose or [^{14}C]mannitol and ^3H_2O, and to a duplicate series [^{14}C]EDTA was added. For these experiments the flasks contained approx. 10 mg mitochondrial protein in 2 ml, with other additions as shown. For measurement of matrix and total H_2O spaces, the marker ^3H_2O/[^{14}C]mannitol or ^3H_2O/[^{14}C]sucrose containing 5 mM sucrose (0.05 ml containing approx. $1 \cdot 10^6$ d.p.m. and $1 \cdot 10^5$ d.p.m. for ^3H and ^{14}C, respectively) was added at 8 min, whereas in the duplicate flasks the [^{14}C]EDTA (0.02 ml containing approx. $2.5 \cdot 10^5$ d.p.m.) was added to the carrier EDTA at the beginning of the incubation. At 10 min, 2×0.8 ml samples were taken and pipetted onto 0.5 ml silicone fluid (Dow Corning 550/Dow Corning 200-1cs; 55 : 1) in 1.5 ml Eppendorf tubes and centrifuged in an Eppendorf microfuge. Full details of the subsequent procedure are given in an earlier publication [6]. The only difference was that the tissue was included in the sample taken for scintillation counting because of the possibility of mitochondrial binding of EDTA.

Metabolite assays

ATP [7], ADP and AMP [8], and adenosine [9] were determined enzymically, using either a Zeiss or an Aminco DW-2 double-beam spectrophotometer.

Light scattering

Mitochondria, suspended in mannitol/Hepes or mannitol/Hepes/EGTA, were diluted in the same medium for measurement of absorption at 520 nm [10].

Chemicals

Ethylenediaminetetra [2-^{14}C]acetic acid, D-[1-^{14}C] mannitol, [U-^{14}C]sucrose and ^3H_2O were obtained from Amersham International. Enzymes, co-enzymes and adenine nucleotides were from Boehringer (London).

Results

P_i causes loss of adenine nucleotides from mitochondria

It is well known that P_i causes the so-called large amplitude swelling of liver mitochondria [10]. This type of swelling is easily visible during incubation as a decrease in turbidity of the suspension. Loss of endogenous adenine nucleotides is associated with swelling; the time course of the loss at 37 °C is given in Table I. Endogenous adenine nucleotides of freshly isolated mitochondria are present largely as ADP. In the absence of P_i or added oxidizable substrate the distribution of ATP, ADP and AMP remains constant and there is no loss. In the presence of 10 mM P_i, loss of total activity begins after 2 min, ATP virtually disappears and only about 25% of the total remains at 10 min. The lost adenine nucleotides appear as adenosine (see below).

Protection of endogenous adenine nucleotides by EGTA or EDTA

Chelating agents prevent P_i-induced swelling and protect mitochondria from the loss of adenine nucleotides that occurs as a result of swelling. However, the protective effects are dependent on both the concentration of chelating agent and the amount of mitochondrial protein (Table II). At 1 mM and 0.1 mM EGTA and 2.8 mg protein/ml there was no obvious swelling

TABLE I

Rate of loss of adenine nucleotides from mitochondria

Mitochondria were isolated and incubated in mannitol/Hepes (pH 7.4). Final volume was 2 ml, containing 14.8 mg protein.

Condition and time (min)	nmol/flask			
	ATP	ADP	AMP	Total
No P_i				
0	48	132	11	191
2	25	144	18	187
6	28	136	16	180
10	31	132	14	177
+10 mM P_i				
2	74	96	18	188
6	5	34	65	104
10	2	22	30	54

and no loss of adenine nucleotides. The transition occurred below 0.1 mM. Doubling the protein concentration (5.6 mg/ml) altered the distribution of adenine nucleotides at 0.1 mM EGTA in favour of AMP, which appears to be the first stage in their loss. The lost adenine nucleotides are virtually accounted for as adenosine.

Similar effects are observed when EDTA replaces EGTA.

The approach of preparing mitochondria in the absence of a chelating agent and then adding EGTA or EDTA to the subsequent incubation is quite common. However, the reverse is also very common. We have noticed that the chelating agent is more effective in protecting against swelling and loss of adenine nucleotide when a suspension containing 1 mM EGTA or EDTA is subsequently diluted in chelating agent-free incubation medium than when the same low concentration is incubated with chelating agent-free suspension (as in the experiment of Table II).

Effects of chelating agents are reversible

The finding that the protective effects of chelating agents on mitochondrial morphology are lost on dilution led us to suspect that their stabilizing effects required the presence of the chelating agent and could not be explained simply as binding and removal of Ca^{2+}. This was confirmed by experiments in which homogenization of the liver and the steps are far as the isolation of the first mitochondrial pellet included 1 mM EGTA. The two subsequent washes of the pellet were in EGTA-free mannitol/Hepes.

The evidence that the effects of EGTA are reversible is as follows. Firstly, the washed EGTA mitochondria appear less opaque on incubation with P_i than the EGTA-containing suspension and are obviously therefore swollen. Secondly, the washed suspension (C) loses adenine nucleotides on incubation with P_i, unlike the suspension (B) prepared and incubated with EGTA (Table III). The loss is somewhat less than the 90% that disappeared to adenosine in the control (A) after 10 min, but there is the usual shift of adenine nucleotides to AMP in those conditions where losses occur. Thirdly, as in the control (A), phospholipid breakdown occurs on incubation with P_i after EGTA is washed from the mitochondrial suspension (C). The evidence for this, observed after TLC, is the increase in long-chain fatty acid under these conditions (not shown). Production of long-chain fatty acid (U-factor [10]), an indicator of mitochondrial swelling [10] and of phospholipid degradation, occurs in both suspensions A and C in the presence of P_i. On the other hand, there is no indication of phospholipid breakdown when EGTA is also present (suspension B). This information was obtained from the mitochondrial pellet extracted after incubation. It is interesting that there is no detectable release of long-chain fatty acid into the incubation medium, which may explain the failure of bovine serum albumin to prevent P_i-induced swelling while inhibiting the swelling brought about by thyroxine, Ca^{2+} or phloridzin [10]. The swelling agents were divided into two classes [10] depending on whether their effects were inhibited by albumin, which was assumed to prevent uncoupling by binding the long-chain fatty acid produced from membrane phospholipid during swelling.

TABLE II

Prevention of loss of adenine nucleotides by EGTA

Mitochondria were incubated with 10 mM P_i and EGTA at the concentrations shown. Further incubation was for 10 min. Final volume was 4 ml. Total adenine nucleotide contents of mitochondria before incubation were 166 and 332 nmol per flask, respectively.

Protein (mg/ml)	EGTA (mM)	nmol/flask			Total	Adenosine
		ATP	ADP	AMP		
2.8	0	< 5	36	20	56	85
2.8	1	112	48	12	172	< 5
2.8	0.1	131	30	12	173	< 5
2.8	0.05	< 5	22	37	59	74
5.6	0	< 5	39	30	69	190
5.6	1	220	85	20	325	< 5
5.6	0.1	< 5	112	217	329	19
5.6	0.05	< 5	53	37	90	183

TABLE III

Reversibility of protective effects of EGTA

Mitochondria from the same liver isolated in mannitol/Hepes (A); mannitol/Hepes/1 mM EGTA (B); mannitol/Hepes/1 mM EGTA to the stage of the first mitochondrial pellet. Two subsequent washes of pellet with mannitol/Hepes (C). Suspensions were incubated subsequently in mannitol/Hepes, mannitol/Hepes/EGTA, mannitol/Hepes for 10 min at 37 °C. Final volume was 4 ml.

Mitochondria and condition	Protein (mg)	nmol/flask			Total
		ATP	ADP	AMP	
A					
Fresh suspension	18.8	42.4	188	15.2	246
Incubated, no P_i	18.8	18.2	169	44.1	231
Incubated, 10 mM P_i	18.8	3.0	6.1	15.2	24.3
B					
Fresh suspension	15.6	57.5	133	36.5	227
Incubated, no P_i	15.6	53.0	142	31.9	227
Incubated, 10 mM P_i	15.6	150	81.7	10.6	242
C					
Fresh suspension	15.3	22.7	160	22.8	206
Incubated, no P_i	15.3	21.2	166	21.3	209
Incubated, 10 mM P_i	15.3	3.0	3	59.3	62.3

Adenine nucleotide content of freshly isolated mitochondria

The previous section implies that mitochondria isolated in the presence of EGTA or EDTA may have a

higher adenine nucleotide content than when the chelating agents are omitted. This is not the case. In our experiments the values were 15.2 ± 0.59 and 15.3 ± 1.3 nmol/mg protein, respectively, for total adenine nucleotides for mitochondria prepared from the same five livers in the absence and presence of 1 mM EGTA (means S.E.M.). The light-scattering properties at 520 nm of the fresh suspensions with and without 1 mM EGTA [10] are also identical. It is the subsequent treatment of the mitochondria that determines whether they swell and lose their adenine nucleotide content. On storage for 24 h at 4°C a suspension containing 1 mM EGTA retained 70% of total adenine nucleotide whereas the suspension in mannitol/Hepes alone retained 62%. However, light scattering measurements at 520 nm showed that mitochondria swell during storage without chelating agent, with little further swelling on addition of 10 mM P_i. Suspensions stored with 1 mM EGTA, on the other hand, are stable as measured by light scattering. Extraction and TLC of lipid components after storage show that long-chain fatty acid, the indicator of mitochondrial swelling [10], accumulates only in the absence of chelating agent.

Mitoplasts do not lose adenine nucleotides on incubation

Mitoplasts, isolated in the absence of chelating agent, are totally permeable to [^{14}C]sucrose after 10 min incubation irrespective of the presence or absence of P_i. There is little or no loss of the adenine nucleotides (12 nmol per mg protein) present in freshly prepared mitoplasts. Mitoplasts isolated in the presence of 1 mM EGTA are condensed, with the ^3H_2O space (as defined in Materials and Methods) approximately 30% of that found without chelating agent. However, 1 mM EGTA does not completely inhibit P_i-induced swelling of mitoplasts; the ^3H_2O space doubled on incubation with 10 mM P_i. Again, little or no loss of adenine nucleotides occurs.

From these experiments we conclude that the phosphatase or 5′-nucleotidase that forms adenosine when mitochondria swell and lose their adenine nucleotides to the medium is associated with the outer membrane, intermembrane space, lysosomes or other subcellular contaminant.

Added adenine nucleotides are not lost to adenosine

The experiments consistently showed that endogenous adenine nucleotides that disappear on swelling of mitochondria by P_i are virtually quantitatively recovered as adenosine. However, when 1–2 mM ATP, ADP or AMP were added to the incubations, there appeared to be no measurable losses. To allow more accurate determination of adenosine, mitochondria were incubated with a low concentration (0.2 mM) of adenine nucleotide. After 10 min at 37°C the added ATP, ADP or AMP led to only small increments of adenosine (Table IV) in the presence of 10 mM P_i. In all conditions the added adenine nucleotide accumulated as AMP.

TABLE IV

Fate of added adenine nucleotides in the presence of P_i

Mitochondria were isolated in mannitol/Hepes. Suspension was incubated 10 min at 37°C in mannitol/Hepes. Final volume was 2 ml, containing 6 mg protein. Additions were as shown.

Condition and additions	nmol/ml			Adenosine	Total
	ATP	ADP	AMP		
Fresh suspension					
–	12	30	2	< 5	44
0.2 mM ATP	221	30	2	< 5	253
0.2 mM ADP	12	193	2	< 5	207
0.2 mM AMP	12	30	215	< 5	257
Incubated					
10 mM P_i	3	6	7	36	52
10 mM P_i 0.2 mM ATP	5	20	172	63	250
10 mM P_i 0.2 mM ADP	2	8	143	69	222
10 mM P_i 0.2 mM AMP	2	6	158	73	239

EDTA is not bound to mitochondria

The reversibility of the effects of EGTA (see Table III) suggests that chelating agents are not tightly bound to mitochondria. Lehninger [10] had reported tight binding of EDTA, whereas others found evidence to the contrary (see Ref. 11). Non-binding was confirmed in experiments with [2-^{14}C]EDTA (Table V) in that the EDTA space corresponded to the sucrose space when mitochondria were incubated with 10 mM P_i in the presence of 1 mM EDTA, 0.1 mM EDTA or 1 mM EDTA plus 1 mM $CaCl_2$. These data are included in detail because they illustrate several points: (i) in the absence of P_i, or in the presence of P_i plus 1 mM EDTA the matrix spaces are identical, i.e., the mitochondria are not swollen; (ii) 0.1 mM EDTA, or 1 mM EDTA plus 1 mM $CaCl_2$, gives too low a concentration of free

TABLE V

Distribution space of EDTA in liver mitochondria

Total ^3H_2O permeable space and [^{14}C]sucrose and [^{14}C]EDTA impermeable spaces were determined after 10 min incubatiion as described in Materials and Methods. Values are means of two determinations.

Condition	Distribution volume (μl per mg protein)			
	H_2O	Sucrose	EDTA	Matrix
No substrate	3.86	2.59		1.27
10 mM P_i	12.3	12.3		
10 mM P_i + 1 mM EDTA	3.48	2.39	2.27	1.08
10 mM P_i + 0.1 mM EDTA	12.2	12.2	12.7	
10 mM P_i + 1 mM EDTA + 1 mM $CaCl_2$	11.6	11.5	12.7	

chelating agent to prevent the P_i-induced swelling and the matrix space is totally penetrated by sucrose and EDTA; (iii) in the conditions associated with extensive swelling the total H_2O carried through the silicone, which gives some indication of the mitochondrial volume, increases 3-fold.

When [^{14}C]mannitol was used in place of [^{14}C]sucrose virtually identical results were obtained, except for variations in the calculated matrix spaces. However, a more careful control of protein concentration is necessary if this space is to be accurately determined [12].

Discussion

The information in this paper may be helpful to those who intend to use isolated mitochondria to examine individual enzymic reactions in situ as opposed to the integrated process of oxidative phosphorylation. The methodology for mitochondria was developed with the aim of producing coupled mitochondria for study of the mechanism of oxidative phosphorylation and few of us take the trouble to read the hundreds of papers that led to the acceptance of a particular component for the isolation or incubation medium. There is no standard method; a quick scan through recent papers yields a bewildering variety. The presence and amount of chelating agent in the isolation and/or incubation medium is probably the most random variable. For many purposes this may not be important. But it is important to be aware of how EGTA or EDTA may affect the results. Our experiences convince us that many discrepant results can be attributed to small, apparently insignificant, differences in technique. In some papers the composition of the medium used for the isolation of the mitochondria is not even mentioned. In others there is a totally random inclusion of chelating agent. In many instances, respiratory control ratios are measured to prove the adequacy of the preparation but the experiments are carried out under conditions in which morphological integrity must be rapidly lost. Obviously the full implicaitons of the metabolic effects of chelating agents, particularly on the swelling induced by P_i, are not appreciated, even though P_i is universally present in mitochondrial incubations.

It is often stated that EDTA causes mitochondrial swelling (for example, Refs. 11 and 13). In fact, the original papers of Azzi and Azzone [14] show that EDTA brings about the so-called low-amplitude swelling, a different phenomenon from P_i-induced swelling that is readily visible to the naked eye. EDTA clearly prevents this and measurements of mitochondrial volume prove it. It is also often stated that Ca^{2+} causes mitochondrial swelling. Again, it is important to distinguish between the swelling brought about by the uptake of Ca^{2+} [15] and the chelation of EDTA or EGTA by Ca^{2+} that effectively lowers the concentration of chelating agent to the point where its protective effects against P_i-induced swelling are eliminated. This is relevant to the use of calcium-EGTA or -EDTA buffers to control [Ca^{2+}] in that the concentration of free chelating agent is a variable that should also be considered. Matrix volume is crucially important in the determination of ion and metabolite gradients, for calculations of the membrane potential and in assessing the mechanism of action of hormones at the mitochondrial level; all variables that can influence the matrix volume have to be taken into account. In the specific instance of hormonal effects on mitochondrial processes (for references, see the review of Brand and Martin [16]) a good deal of attention has been given to the influence of the osmotic support used for isolation of liver mitochondria, but little or no consideration has been given to the presence or absence or concentration of chelating agent. Another factor that is critically important in the calculation of matrix volume is the protein concentration. Our observation that mannitol, but not sucrose, interferes in a non-stoichiometric manner in the biuret method of protein estimation must mean that some published work may contain major errors. We are not aware of any previous comment on this reaction.

Our finding that the protection against swelling by EGTA is reversible argues against the widely held belief [1] that chelation of Ca^{2+} to prevent Ca-dependent phospholipase activity is the primary effect. Mörikofer-Zwez et al. [17] found no decrease in calcium content of mitochondria isolated in the presence of 0.1 mM EGTA [17]. The question then is how do the chelating agents protect against mitochondrial swelling. We can only suggest a freely reversible association with positively charged groups on the inner membrane which hinders the morphological changes. To this limited extent the effects of EDTA and EGTA may be similar. Their different, more specific, effects are not considered in this paper.

We have made no attempt to identify the adenosine-forming enzyme. Clearly the enzyme has a very high affinity for the substrate, presumably AMP, since the concentration available from endogenous adenine nucleotide must be extremely low. Experiments not mentioned in Results show that an enzyme-forming adenosine from AMP at pH 7.4 is released into the incubation medium, is not inhibited by EDTA or EGTA, but is inhibited by P_i. This points to a phosphatase, probably lysosomal in origin. The conversion by rat liver mitochondria of added adenine nucleotides to adenosine was reported in 1956 [8], possibly because mitochondria preparations were more heavily contaminated with other subcellular fractions in those days.

The data in this paper concern only P_i as a swelling agent because it is almost invariably included in mitochondrial incubations. It is important to emphasize that the data were obtained under incubation conditions

without added respiratory substrate, phosphate acceptor (ADP) or Mg^{2+}, all of which would provide a different picture. Our aim is to illustrate phenomena that can occur when liver mitochondria are used as a tool to study enzymic reactions in situ.

References

1 Nedergaard, J. and Cannon, B. (1979) Methods Enzymol. 55, 3–28.
2 Kun, E., Kirsten, E. and Piper, W.N. (1979) Methods Enzymol. 55, 115–118.
3 Greenawalt, J.W. (1974) Methods Enzymol. 31, 310–323.
4 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
5 Jacobs, E.E., Jacob, M., Sanadi, D.R. and Bradley, L.B. (1956) J. Biol. Chem. 223, 147–156.
6 Lund, P. and Wiggins, D. (1987) Biosci. Rep. 7, 59–66.
7 Lamprecht, W. and Trautschold, I. (1974) Methods Enzymol. Anal. 4, 2101–2110.
8 Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974) Methods Enzymol. Anal. 4, 2127–2131.
9 Möllering, H. and Bergmeyer, H.U. (1974) Methods Enzymol. Anal. 4, 1919–1922.
10 Lehninger, A. (1962) Physiol. Rev. 42, 467–517.
11 Joseph, S.K., McGivan, J.D. and Meijer, A.J. (1980) Biochem. J. 194, 35–41.
12 Cohen, N.S., Cheung, C.W. and Raijman, L. (1987) Biochem. J. 245, 375–379.
13 Whipps, D.E. and Halestrap, A.P. (1984) Biochem. J. 221, 147–152.
14 Azzi, A. and Azzone, G.F. (1965) Biochim. Biophys. Acta 105, 265–278.
15 Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 378–386.
16 Brand, M.D. and Murphy, M.P. (1987) Biol. Rev. 62, 141–193.
17 Mörikofer-Zwez, S., Kunin, A.S. and Walter, P. (1983) J. Biol. Chem. 248, 7588–7594.
18 Fonnesu, A. and Davies, R.E. (1956) Biochem. J. 64, 769–777.