4. As a result of the findings, the suggestion is put forward that the levels of glutathione and ascorbic acid found in dietetic liver necrosis could be the result of changes occurring in a dead or dying liver left in the living body, and were not specific consequences of the dietetic lesion.

Our thanks are due to Sir Harold Himsworth for his valuable advice and encouragement in this work. We are indebted to Miss S. Botha for her technical assistance. One of us (O.L.) was in receipt of a Research Grant from the Medical Research Council.

REFERENCES

Abell, M. R. & Beveridge, J. M. R. (1950). *Arch. Path.* **50**, 23.
Binet, L., Rouvillois, C. & Bargeton, D. (1939). *Pr. méd.* **47**, 1157.
Binet, L., Weller, G. & Goudard, H. (1937). *C.R. Soc. Biol., Paris*, **124**, 1141.
Dent, C. E. (1947). *Biochem. J.* **41**, 314.
Drochmans, P. (1950). *Arch. Biol. (Liège)*, **61**, 475.
Himsworth, H. P. (1950). *Lectures on the Liver and its Diseases*, 2nd ed. Oxford: Blackwell.
Himsworth, H. P. & Glynn, L. E. (1945). *Biochem. J.* **39**, 267.
Leaf, G. & Neuberger, A. (1947). *Biochem. J.* **41**, 280.
Lindan, O. & Work, E. (1951*a*). *Liver Disease*, p. 47. Ciba Foundation Symp. London: Churchill.
Lindan, O. & Work, E. (1951*b*). *Biochem. J.* **48**, xxxi.
Lindan, O. & Work, E. (1953). *Biochem. J.* **55**, 554.
Neubeck, C. E. & Smythe, C. V. (1944). *Arch. Biochem.* **4**, 443.

The Effect of Calcium on the Respiratory and Phosphorylative Activities of Heart-muscle Sarcosomes

By E. C. SLATER and K. W. CLELAND
Molteno Institute, University of Cambridge

(*Received* 3 *March* 1953)

A long recognized difficulty in the study of the enzyme systems which bring about oxidative phosphorylation is their great instability at temperatures above about 0°. The instability is particularly noticeable if the enzyme preparation is incubated before it is added to the reaction mixture which is used to demonstrate oxidative phosphorylation.

In studies of the oxidative phosphorylation systems in heart-muscle sarcosomes, the respiratory granules of heart muscle corresponding to mitochondria in liver and kidney (Cleland & Slater, 1953*a*, *b*), this instability was particularly troublesome, especially when rat-heart sarcosomes were used. Paul, Fuld & Sperling (1952) have also observed this instability in heart-muscle 'cyclophorase' preparations.

The inactivation of the respiratory enzyme systems is accompanied by marked morphological changes, which have been described in another paper (Cleland & Slater, 1953*a*). In that paper, it is shown that the rate of development of these morphological changes is greatly decreased by the addition of the chelating agent, ethylenediaminetetraacetic acid (Versene). Further, as has already been reported in a preliminary communication (Slater & Cleland, 1952), the instability of the α-ketoglutaric oxidase system of the sarcosomes is due to calcium contained in the sarcosomal preparation, and the addition of ethylenediaminetetraacetic acid greatly stabilizes the system. The present paper describes these experiments in greater detail, and also deals with the mode of action of the calcium. A study was also made of the components of the reaction mixture used in oxidative phosphorylation experiments, which, in the absence of ethylenediaminetetraacetic acid, confer limited stability on the enzyme systems.

Calcium is a normal constituent of blood serum, and since the demonstration of Ringer (1883) that it is required for the beating of frog heart, it has often been included in the medium in studies of the respiration of tissue slices. It has frequently been observed, however, that the addition of calcium depresses the respiration of minces and dispersions made with 'homogenizers'. An exception is the oxidation of succinate by 'homogenates', which was found by Axelrod, Swingle & Elvehjem (1941) to be stimulated by calcium. This was shown by Swingle, Axelrod & Elvehjem (1942) to be due to the activating effect of calcium on the enzyme hydrolysing diphosphopyridine nucleotide (DPN). In the presence of DPN, fumarate, produced from succinate, is oxidized to oxaloacetate, and this is a strong inhibitor of succinic dehydrogenase (Das, 1937; Keilin & Hartree, 1940; Pardee & Potter, 1948). The destruction of DPN, accelerated by calcium, prevents the formation of oxaloacetate

and therefore causes increased oxygen uptake. Keilin & Hartree (1949) have described another type of calcium activation of the succinic oxidase system of heart-muscle preparations in phosphate buffer, using preparations which did not oxidize fumarate. In this case, the activation is caused by the formation of calcium phosphate gel which, like other similar precipitates, activates a dilute heart-muscle preparation by some physical action.

Added calcium has also been recognized as an inhibitor of oxidative phosphorylation. This was first shown by Potter (1947) with rat-kidney 'homogenates' and confirmed by Lehninger (1949), using rat-liver mitochondria. Lehninger thought that this might be due to the activation of adenosine triphosphatase (ATPase), but Potter (1947) had evidence that this could not be the only reason for the inhibition of the esterification of inorganic phosphate. Keltch & Clowes (1951) found that 0·001 M-calcium chloride completely inactivated the α-ketoglutaric oxidase system of liver particles, and that this inactivation could be overcome by the addition of citrate. Our work is, however, the first to show that the known instability of the α-ketoglutaric oxidase system and oxidative phosphorylation, in heart preparations at least, is due to calcium already present in the preparations. All the calcium in the heart is found in the sarcosomal preparations.

METHODS

Sarcosomal preparations. These were made as previously described (Cleland & Slater, 1953*b*). Different types of preparations are designated according to the components of the isolation medium, as described in that paper.

Enzymic activities. The activities of the α-ketoglutaric oxidase system and the accompanying phosphorylation (P:O ratio) and of myokinase and ATPase were measured as in previous papers (Slater, 1953*a*; Slater & Cleland, 1953). Except where stated otherwise, the 'isotonic reaction mixture' (Slater & Cleland, 1953), containing fluoride, was used for the α-ketoglutaric oxidase system. The pH was 7·05–7·4. When other oxidase systems were studied, fluoride and malonate were omitted from this mixture and the appropriate substrate (0·02 M-DL-malate, 0·005 M-oxaloacetate, 0·023–0·027 M-succinate) replaced α-ketoglutarate. In some experiments, fluoride was included in the reaction mixture when succinate was the substrate. The concentration of sarcosomal protein was usually somewhat less and the amount of hexokinase/mg. sarcosomal protein more when succinate was substrate. The oxaloacetic acid used was kindly supplied by Dr I. Harary.

Determination of the degree of inactivation. The sarcosomes were incubated, either alone or in the presence of some substance whose stabilizing effect was being tested, in the side bulb of a Warburg manometer flask. Unless stated otherwise, the sarcosomes were suspended in isotonic medium. After incubation for a specified period, the sarcosomes were tipped into the main compartment of the Warburg flask containing the reaction mixture and measurements of the O_2 uptake commenced. The degree of inactivation was calculated by comparison of the O_2 uptake with that given by a sample of the sarcosomal preparation added to the reaction mixture without any incubation. Since the ethylenediaminetetraacetic acid in the reaction mixture prevented any further inactivation, the inactivation measured was due wholly to the preliminary incubation.

Determination of calcium. Calcium was determined on a solution of the ashed preparation, or on clear extracts without ashing, by the method of Debney (1952). The ash of one or two rat hearts or of the sarcosomes prepared from one or two hearts was dissolved in 0·5 ml. HCl (1 part conc. HCl + 4 parts water), heated on a water bath for 20 min. to hydrolyse pyrophosphate and made up to 10 ml. with glass-distilled water. A sample of this solution (2–5 ml.) was diluted to 5 ml. with glass-distilled water and neutralized with N-KOH (litmus paper); 1 ml. N-KOH and 0·05 ml. of a saturated solution of ammonium purpurate were added and the mixture immediately titrated with 10^{-3} M-ethylenediaminetetraacetic acid until the colour matched that of a control prepared simultaneously with glass-distilled water in place of the solution to be analysed. The solution of ethylenediaminetetraacetic acid was standardized by titration against a standard solution of $CaCl_2$, prepared by dissolving Iceland spar in HCl. The method was tested by ashing two samples (2 ml.) of saline-ethylenediaminetetraacetic acid medium (see Cleland & Slater, 1953*b*), one of which contained 1·19 μmoles $CaCl_2$. No calcium was detected in the medium, while 1·23 μmoles were found in the sample with added calcium (recovery 103%).

RESULTS

Effect of incubation of heart-muscle sarcosomes on respiratory enzyme systems and on oxidative phosphorylation

Table 1 describes an experiment on the effects of incubation of the sarcosomes for 15 min. at 25° on the ability of the preparation to oxidize α-ketoglutarate, succinate, malate and oxaloacetate, and to couple the oxidation with phosphorylation. It can be seen that the incubation inactivated by nearly 90% the oxidation of α-ketoglutarate and malate and markedly inactivated the phosphorylating systems associated with the oxidation of malate and oxaloacetate. The effect on the P:O ratio with α-ketoglutarate as substrate was relatively minor, while with succinate as substrate in this experiment the inactivation of oxidative phosphorylation was considerable but much less than with malate. In other experiments (see Table 7), the effect on the P:O ratio with succinate was greater. The relatively slow rate of oxidation of oxaloacetate was only slightly affected by the incubation.

The increase of the rate of oxidation of succinate after incubation is very likely associated with a decreased concentration of oxaloacetate. Since the unincubated sarcosomes oxidize malate more rapidly than oxaloacetate, some oxaloacetate would accumulate and inhibit succinic dehydrogenase. As the effect of the incubation was to inactivate the

Table 1. *Effect of incubation of heart-muscle sarcosomes on respiratory enzyme systems and on oxidative phosphorylation*

(Saline preparation from rat heart. Malonate and NaF present in reaction mixture only when α-ketoglutarate was substrate. Q_{O_2} (μl. O_2/mg. sarcosomal protein/hr.) calculated from initial rates; P:O calculated from actual O_2 uptakes. Incubated: sarcosomes incubated 15 min. at 25°.)

Substrate	α-Ketoglutarate	Succinate	Malate	Oxaloacetate
Control				
Q_{O_2}	90	180	38	17
P:O	2·63	1·00	1·97	4·20
Incubated				
Q_{O_2}	10	286	5	14
P:O	2·13	0·60	0·33	0·94
Inactivation (−) or activation (+) (%)				
Of Q_{O_2}	−88	+78	−87	−16
Of P:O	−19	−40	−83	−77

oxidation of malate with little effect on that of oxaloacetate, less of the latter would accumulate after incubation and hence the succinic dehydrogenase would be less inhibited.

The high P:O ratio obtained with oxaloacetate as substrate is probably due to an anaerobic side reaction, since the disappearance of oxaloacetate greatly exceeded the oxygen uptake (oxaloacetate disappearance (μmoles): oxygen uptake (μatoms) = 2·8 with unincubated, 2·7 with incubated sarcosomes).

Table 1 shows that the effect of incubation can be conveniently studied by following the effect on the α-ketoglutaric oxidase system.

Inactivation of α-ketoglutaric oxidase system

Fig. 1 shows that, at neutral pH, the α-ketoglutaric oxidase system was rapidly inactivated at 15·5°, 10 min. being sufficient for 50 % inactivation. The inactivation proceeded steadily, without any lag period. This shows that traces of endogenous substrates, which would be rapidly oxidized, do not give any protection.

The inactivation was very greatly affected by temperature, as is shown in Table 2. The preparations were relatively stable at 0°; in fact the activity slightly increased after standing at 0° for a short time. Even at 15°, the inactivation was very rapid. There was no appreciable difference between the stability of the α-ketoglutaric oxidase system in isotonic saline or in sucrose preparations.

Table 2 shows a marked species difference in the rate of inactivation, rat preparations being considerably less stable than those from the cat or pig. The rate of inactivation was only slightly affected by varying the pH between 6·8 and 8·0, or the tonicity of the suspending medium between 0·08- and 0·65-osmolar.

Substances promoting stability of the α-ketoglutaric oxidase system

Reaction mixture. All the above experiments were carried out with the enzyme preparation suspended in 'saline', 'phosphate-saline' or isotonic sucrose

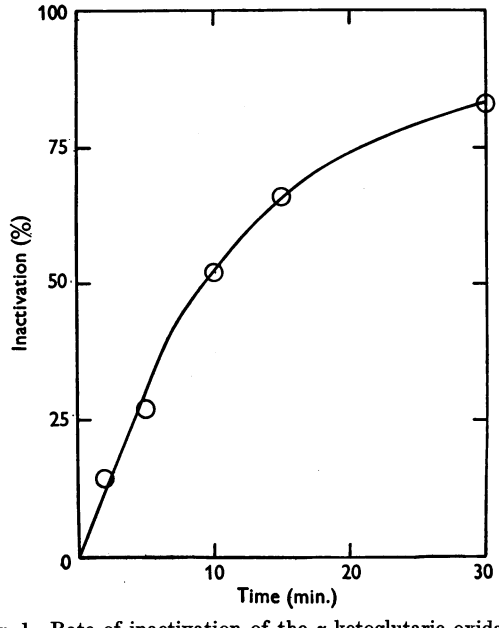

Fig. 1. Rate of inactivation of the α-ketoglutaric oxidase system of rat-heart sarcosomes at 15·5°. Saline preparation.

media (see Cleland & Slater, 1953*b*) without further addition. The α-ketoglutaric oxidase system is much more stable in the presence of α-ketoglutarate and those additional components required for optimum oxidation (adenine nucleotides, magnesium, cyto-

Table 2. *Inactivation of α-ketoglutaric oxidase system of heart-muscle sarcosomes by incubation*

(Phosphate-saline, saline or sucrose preparations.)

	Incubation conditions		Inactivation (−) or activation (+) (%)		
Animal	Temp. (°)	Time (min.)	No. of expts.	Mean	Range
Rat	0	30	1	+18	—
	14	10	1	−26	—
	15·5	10*	2	−46	40–52
	25	10	6	−86	75–94
		15	5	−91	88–94
Cat	0	60†	1	+4	—
		1200†	1	−84	—
	25	10	1	−39	—
		15‡	1	−71	—
		30‡	1	−91	—
Pig	0	180	1	+9	—
	25	5	1	−16	—

* See Fig. 1 for other times.
† Same preparation.
‡ Same preparation.

Table 3. *Effect of omitting different components on protection afforded by reaction mixture to α-ketoglutaric oxidase system*

(See text for description of experiment. Saline preparations from rat.)

Substance omitted from otherwise complete mixture	Inactivation (%)	
	Expt. 1	Expt. 2
None	13	34
α-Ketoglutarate	13	—
AMP	25	35
ADP	40	—
Phosphate	48	48
NaF	29	—
$MgCl_2$	38	—
Malonate	—	31

chrome *c*). For example, the inactivation of rat heart-muscle sarcosomes after 15 min. at 25°, under these conditions, was only about 20 %, compared with about 90 % inactivation when the enzyme was incubated alone. Cat and pig heart preparations are usually stable for about 30 min. at 25°, in the presence of the reaction mixture (see Slater, 1950).

Two types of experiments were carried out, in order to determine which components of the reaction mixture conferred stability on the α-ketoglutaric oxidase system of the sarcosomes. In the first, summarized in Table 3, the sarcosomal preparation, diluted to a concentration of about 1 mg. protein/ml., was incubated in the main compartment of a Warburg flask for 10 min. at 25° either with the complete reaction mixture or with the same mixture minus one of its components. The missing component was then added from the side bulb together with ethylenediaminetetraacetic acid to prevent any further inactivation, and the oxygen uptake measured. A control with the complete reaction mixture with the addition of ethylenediaminetetraacetic acid was included. The experiment showed that the components of the reaction mixture which can be omitted without affecting the degree of protection obtained are α-ketoglutarate, malonate and probably adenosine monophosphate (AMP). The omission of adenosine diphosphate (ADP), phosphate, magnesium and, to a lesser extent, fluoride all caused increased inactivation.

In the second type of experiment, a more concentrated sarcosomal preparation (4–7 mg. protein/ml.) was incubated in the side bulb with each component of the reaction mixture in turn and then added to the main compartment of the flask containing all the other components of the mixture and ethylenediaminetetraacetic acid. Experiments along these lines are shown in Table 4, which also includes the effect of adenosine triphosphate (ATP). The percentages inactivation in Table 4 are based on the maximum rates of oxygen uptake. This was usually uniform, but with the preparations incubated with the higher concentrations of ADP, there was a considerable delay in reaching the maximum rate. This suggests that, although ADP largely protects the system from the irreversible inactivation which usually occurs, even in the presence of ADP there is considerable reversible inactivation.

Table 4. *Effect of various components of the reaction mixture on the stability of the α-ketoglutaric oxidase system*

(Saline preparations from rat incubated in side bulb of Warburg flask, with additions as shown, for 10 min. at 25°.)

Addition during incubation	Inactivation (%)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
None	75	81	90	94
Phosphate	79	79	—	—
Malonate	81	—	—	—
NaF	82	73	—	—
AMP	77	—	—	—
ADP, $0{\cdot}55 \times 10^{-3}$ M	66	—	—	—
ADP, $1{\cdot}7 \times 10^{-3}$ M	—	—	23	—
ADP, $3{\cdot}4 \times 10^{-3}$ M	—	—	29	—
ATP, $0{\cdot}6 \times 10^{-3}$ M	51	—	—	—
ATP, $1{\cdot}2 \times 10^{-3}$ M	—	—	—	44
$MgCl_2$	63	72	—	—
$MgCl_2$ + phosphate	—	73	—	—
$MgCl_2$ + NaF	—	60	—	82
NaF + phosphate	—	76	—	—
NaF + $MgCl_2$ + phosphate	—	—	—	59

Table 4 shows that phosphate, malonate and AMP have little protective effect, while fluoride had no effect in one experiment, but was slightly protective in another. Phosphate and fluoride together

were ineffective. Magnesium had some slight protective action (confirmed in two additional experiments not shown in Table 4), which was increased by the simultaneous presence of fluoride and further increased by the addition of phosphate together with magnesium and fluoride. The component of the mixture which was most protective was ADP, in relatively high concentrations; ATP was also protective, but not AMP. High concentrations of ADP or ATP are required, because in the absence of inhibitors the high concentration of sarcosomes employed rapidly hydrolysed ADP and ATP. For example, analysis showed that all the ADP used in Expt. 1 of Table 4 had been hydrolysed at the end of the incubation period. Since neither AMP nor inorganic phosphate had any appreciable protective effect on the α-ketoglutaric oxidase system, it is probable that it is the energy-rich phosphate groups of ADP and ATP (~P) which are responsible. Owing to the presence of myokinase and ATPase in the sarcosomes, ADP and ATP are interconvertible to a considerable degree.

It is concluded from these experiments that there are two protective mechanisms operating in the reaction mixture: (i), ADP; (ii), magnesium, fluoride and phosphate.

The experiments shown in Tables 3 and 4 were carried out with preparations isolated with saline. ADP gave no protection to a preparation isolated with isotonic sucrose. The reason for this difference is not known; possibly ADP can penetrate more readily to a sensitive site in the sarcosomes after the latter has been isolated with saline than with sucrose.

The protective effect of ATP against the inactivation of various oxidase systems caused by incubation was first shown by Pardee & Potter (1949). Kielley & Kielley (1951) found that ADP and AMP as well as ATP protected the α-ketoglutaric oxidase system of liver mitochondria. The protection by AMP is in contrast to our results, but may be related to the higher myokinase activity of liver mitochondria. Also, unlike our findings, Kielley & Kielley obtained the protection with sucrose preparations.

Ethylenediaminetetraacetic acid. Fig. 2 shows that ethylenediaminetetraacetic acid, in concentrations between 10^{-4} M and 2×10^{-3} M, completely prevented the loss of activity which occurred with rat-heart preparations even in the presence of the reaction mixture.

Table 5 describes the effect of adding ethylenediaminetetraacetic acid to the reaction mixture, using the more stable cat-heart sarcosomes. Over the short time interval (25 min.) used in this experiment, inactivation of the oxidase system was only slight even in the absence of ethylenediaminetetraacetic acid. The latter did, however, prevent this inactivation and appreciably raised the P:O ratio with both α-ketoglutarate and succinate as substrate. The ATPase and myokinase activities were little affected by the ethylenediaminetetraacetic acid, the concentration of which (0·002 M) was considerably less than that of magnesium (0·005 M) which is necessary for optimum activity of these enzymes. Under these conditions, the activity of the added hexokinase was also little affected (we are indebted to Mr F. A. Holton for testing this point).

It should be noted that, in all the experiments described in this paper, except those shown in Fig. 2 and Table 5, ethylenediaminetetraacetic acid

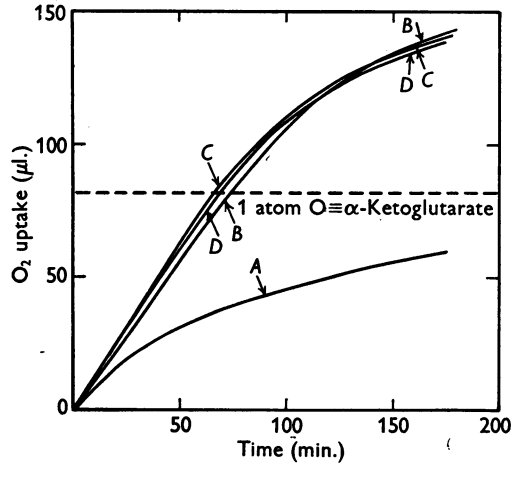

Fig. 2. Effect of added ethylenediaminetetraacetic acid on the oxidation of α-ketoglutarate by rat-heart sarcosomes (saline preparation, 1·4 mg. sarcosomal protein). The sarcosomes were added at zero time without previous incubation. The concentrations of ethylenediaminetetraacetic acid were: *A*, none; *B*, 10^{-4} M; *C*, 10^{-3} M; *D*, 2×10^{-3} M.

Table 5. *Effect of including ethylenediaminetetraacetic acid in reaction mixture*

(Cat-heart sarcosomes, saline preparation. Reaction time, 25 min.)

	Concentration of ethylenediaminetetraacetic acid	
	0	0·002 M
α-Ketoglutaric oxidase system		
Q_{O_2}	45	50
P:O	2·27	2·93
Succinic oxidase system		
Q_{O_2}	53	55
P:O	0·74	0·87
ATPase		
Q_P	182	166
Myokinase		
Q_P	5·8	7·2

Table 6. *Effect of ethylenediaminetetraacetic acid on the stability of the α-ketoglutaric oxidase system and accompanying phosphorylation*

(Preparation incubated at 25°. Sal. = saline preparation. Sal.-E = saline-ethylenediaminetetraacetic acid preparation. Suc. = isotonic sucrose preparation. Suc.-E = isotonic sucrose-ethylenediaminetetraacetic acid preparation.)

Expt.	Animal	Prep.	Incubation time (min.)	Ethylenediaminetetraacetic acid* (M)	Ethylenediaminetetraacetic acid* (μmoles/mg. sarcosomal protein)	Q_{O_2}	P:O
1	Cat	Sal.	0	—	—	50	2·90
			30	0	0	4·6	1·67
			30	0·01	0·88	54	2·67
2	Rat	Sal.	0	—	—	56	—
			15	0	0	3·2	—
			15	0·00002	0·002	4·9	—
			15	0·0001	0·01	6·2	—
			15	0·001	0·12	9·4	—
			15	0·01	1·2	61	—
3	Rat	Sal.	0	—	—	14	2·52
			15	0	0	1·3	6·0†
			15	0·001	0·24	9·3	2·56
4	Rat	Sal.	0	—	—	64	—
			15	0	0	5·4	—
			15	0·005	1·5	71	—
5	Rat	Sal.-E	0	—	—	56	2·56
			17	0·013	2·1	46	2·06
6	Rat	Suc.	0	—	—	59	2·54
			10	0	0	4·1	1·73
			10	0·009	1·2	42	2·51
7	Rat	Suc.-E	0	—	—	55	2·96
			15	0·013	2·1	48	2·78
8	Rat	Suc.-E	0	—	—	73	—
			10	0·01	1·1	70	—

* During incubation. † See text.

(0·001–0·002 M final concentration) was present in the reaction mixture, whether or not it was added to the enzyme preparation during previous incubation. The addition of 0·002 M-ethylenediaminetetraacetic acid to the reaction mixture produced a slight lowering of pH which was counteracted by the simultaneous addition of the same concentration of potassium hydroxide. The lowering of pH is due to the production of protons when ethylenediaminetetraacetic acid combines with the magnesium (Schwarzenbach & Ackermann, 1947).

The effect of ethylenediaminetetraacetic acid is even more striking if it is added to the heart-muscle preparation during a preliminary incubation period before the preparation is added to the reaction mixture. A number of experiments, using both cat and rat preparations, are summarized in Table 6. Expt. 1, with cat-heart preparation, shows that 0·01 M-ethylenediaminetetraacetic acid completely prevented any inactivation of the α-ketoglutaric oxidase system during a 30 min. incubation, compared with 91 % inactivation in its absence. The yield of oxidative phosphorylation (P:O ratio) was also hardly affected by this incubation in the presence of ethylenediaminetetraacetic acid. Expt. 2 shows that a high concentration (0·01 M) is necessary for the optimum effect. In Expt. 3, 0·001 M gave considerable protection to the oxidase system and complete protection of the oxidative phosphorylation. The high P:O ratio obtained when the preparation was incubated in the absence of ethylenediaminetetraacetic acid is undoubtedly due to some anaerobic phosphorylation of the type discussed by Slater & Holton (1952). This anaerobic phosphorylation appears to be relatively resistant to incubation of the preparation.

With saline preparations of sarcosomes, 0·01 M-ethylenediaminetetraacetic acid usually completely prevented inactivation during an incubation of 15 min. at 25°. However, Expt. 5 illustrates that occasionally some inactivation does occur under these conditions. This happens often enough to make it undesirable to adopt this procedure as a routine for temperature equilibration in manometric experiments. If, however, unincubated sarcosomes are added to a reaction mixture containing 0·002 M-ethylenediaminetetraacetic acid, there is only a very slight inactivation of the α-ketoglutaric oxidase system, with no effect on the P:O ratio over 45–60 min. at 25° (Slater & Holton, 1953*a*).

Table 7. *Effect of ethylenediaminetetraacetic acid and ADP on stability of oxidative phosphorylation associated with oxidation of succinate*

(Saline preparations from rat. Incubation at 25°. NaF used in reaction mixture for succinate in Expt. 2, but not in Expt. 1. Q_{O_2}'s are calculated from maximum rates of O_2 uptake, P:O from actual O_2 uptakes.)

	Incubation		Succinate		α-Ketoglutarate	
Expt.	Time (min.)	Addition	Q_{O_2}	P:O	Q_{O_2}	P:O
1	0	—	278	1·01	95	2·50
	15	None	465	0·73	28	2·00
	15	E*	278	1·11	112	2·46
	15	ADP†	273§	0·88	74§	2·31
2	0	—	63	0·64	—	—
	15	None	45	0·10	—	—
	15	E‡	47	0·47	—	—

* 0·01 M-ethylenediaminetetraacetic acid, 1·7 μmoles/mg. sarcosomal protein.
† 0·0028 M-ADP.
‡ 0·001 M-ethylenediaminetetraacetic acid, 0·19 μmoles/mg. sarcosomal protein.
§ Corrected for slight inhibition (measured in a control expt.) caused by the extra amount of ADP in the reaction mixture.

Expt. 6, Table 6, illustrates that sucrose preparations are less well protected by the addition of ethylenediaminetetraacetic acid (note that, although the oxygen uptake has been inactivated by 36%, the P:O ratio was not affected). If, however, the ethylenediaminetetraacetic acid is added to the sucrose used for isolating the sarcosomes, the preparation can be incubated without appreciable inactivation (Expts. 7 and 8, Table 6).

Table 7 shows the effect of ethylenediaminetetraacetic acid on the stability of the phosphorylation associated with the oxidation of succinate. Also included in this table is a study of the protective action of ADP against the effects of incubation on both the succinic oxidase and α-ketoglutaric oxidase systems. In Expt. 1 the effects on the succinic oxidase system of incubation of the sarcosomes alone are similar to those shown in Table 1, namely an activation of the rate of oxidation with a decline of the P:O ratio. Ethylenediaminetetraacetic acid (0·01 M) completely prevented both effects. ADP also gave marked protection to the phosphorylation and prevented the activation of the rate of oxidation. It should be noted that this preparation was unusually stable, the inactivation of the α-ketoglutaric oxidase system being only 71% instead of about 90% (Table 2). Ethylenediaminetetraacetic acid gave complete protection, in fact it caused some activation of the system (this is also apparent in Expts. 1, 2 and 4 in Table 6). The partial protection by ADP shown in Table 4 was also found in this experiment, which adds the additional information that the phosphorylation is also protected. In Expt. 2, of Table 7, the succinic oxidase system was not activated by the incubation; on the contrary there was a slight inactivation which was not protected by ethylenediaminetetraacetic acid. The inactivation of the phosphorylation, which was greater than in other experiments, was largely prevented by a low concentration of ethylenediaminetetraacetic acid.

Effect of added calcium on α-ketoglutaric oxidase system

Since the most striking property of ethylenediaminetetraacetic acid is its ability to chelate with metals, it seemed most likely that it protected the enzyme systems by removing a metal which promoted the inactivation. Table 8 shows that added calcium considerably increased the rate of inactivation. This effect of added calcium was greater if the sarcosomes were prepared by a method aiming at the removal of metals present in the original material. The heart-muscle mince was ground in saline-ethylenediaminetetraacetic acid medium (see below), and the chelating agent then removed by sedimentation of the granules in the centrifuge and washing twice with 100 vol. saline; they were finally suspended in saline solution. This type of preparation (called 'ethylenediaminetetraacetic-acid-washed preparation') is much more stable than the saline preparation. Thus, incubation for 15 min. at 25° caused only 18–27% inactivation (Table 8) compared with about 90% with a normal preparation. The addition of as little as $6·7 \times 10^{-5}$ M-calcium caused a considerably increased inactivation, while 0·01 M inactivated by 84%.

It appeared likely, then, that normal rat-heart preparations contain calcium which, in some way, promotes the inactivation of the α-ketoglutaric oxidase system. The preparation is stabilized if the calcium is bound by ethylenediaminetetraacetic acid, which is an exceptionally powerful chelating agent for this metal. Even if the chelating agent is removed from the preparation, the latter retains its stability to a large degree presumably because the

Table 8. *Effect of added calcium on the inactivation of α-ketoglutaric oxidase system*

(Rat-heart sarcosomes; see text for description of ethylenediaminetetraacetic acid-washed prep.)

Prep.	Incubation conditions: Time (min.)	Incubation conditions: Temp. (°)	Concentration of $CaCl_2$ (*M*)	Inactivation (%)
Saline	5	15	0	18
			0·001	37
			0·01	44
Ethylenediaminetetraacetic acid-washed	5	17	0	11
			0·01	56
	15	25	0	27
			0·001	72
			0·01	84
	15	25	0	18
			0·00007	38
			0·0002	33
			0·00067	58
			0·0067	79

calcium is removed with the ethylenediaminetetraacetic acid. Such a preparation is now particularly susceptible to the addition of calcium. An alternative explanation of the experiments described in Table 8, namely that the calcium was acting by removing the chelating agent adsorbed on the granules, is eliminated by the following considerations: (i) calcium increases the rate of inactivation with a normal preparation; (ii) the amount of calcium required for appreciable inactivation is much greater than the likely concentration of residual ethylenediaminetetraacetic acid; (iii) Fe^{2+}, which binds more strongly than calcium, did not cause inactivation of the ethylenediaminetetraacetic acid-washed preparation.

The relative stability of the ethylenediaminetetraacetic-acid-washed preparation excludes the possibility that the inactivation is caused by metal ions introduced with the reagents.

Sarcosomes isolated in media containing ethylenediaminetetraacetic acid

Since the activity of the α-ketoglutaric oxidase system of rat-heart preparations is decreased by 50% after only 10 min. at 15°, it might be expected that very considerable inactivation would occur during the preparation of the granules, which extends over about 1 hr. That the inactivation is in fact not appreciable if the preparation is carried out near 0°, is due to the very high temperature coefficient of the inactivation. Nevertheless, it is a common experience that oxidative phosphorylation is markedly affected if the temperature is allowed to rise a few degrees during the preparation. This inactivation can now be avoided by including 0·01 M ethylenediaminetetraacetic acid in the media in which the granules are isolated, washed and finally suspended. The properties of a preparation obtained in this way ('saline-ethylenediaminetetraacetic acid preparation') are compared with a saline preparation made from the same rat heart in Table 9. The fresh preparations did not differ markedly, the most noticeable differences being an appreciably higher activity of the α-ketoglutaric oxidase system and lower activities of the ATPase and myokinase in the ethylenediaminetetraacetic-acid preparation. None of these differences was found consistently. A comparison of a large series of preparations made with saline with and without the chelating agent did not reveal any significant differences between the activities of the α-ketoglutaric oxidase system (Cleland & Slater, 1953*b*) or myokinase (Slater, 1953*a*). This indicates that, even in the absence of ethylenediaminetetraacetic acid, our normal procedure using saline does not cause any appreciable inactivation of the α-ketoglutaric oxidase system. However, sarcosomes isolated with isotonic sucrose containing 0·01 M-ethylenediaminetetraacetic acid were considerably more active than those obtained with sucrose alone. An additional experiment in which saline and saline-ethylenediaminetetraacetic acid preparations were made from the same rat heart did not confirm the differences of ATPase activity shown in Table 9.

As would be expected from the experiments already described, the preparations shown in Table 9 markedly differed in their stability to storage at 2°. After 1 day, the activity of the α-ketoglutaric oxidase system and the P:O ratio with the preparation made with ethylenediaminetetraacetic acid had not changed, whereas the α-ketoglutaric oxidase system in the saline preparation had been inactivated by 84% (note again the relatively minor effect on the P:O ratio). Even after 6 days, the saline-ethylenediaminetetraacetic acid preparation retained appreciable activity.

Table 9. *Comparison of activities of preparations made with and without ethylenediaminetetraacetic acid*

(Cat heart; preparations kept at 2°. Fluoride in reaction mixture for both substrates. Q_P = μl. P/mg. sarcosomal protein/hr.)

	Preparation				
	Saline		Saline-E		
Age of prep. (days)	0	1	0	1	6
α-Ketoglutaric oxidase system					
Q_{O_2}	50	7·8	67	70	20
P:O	2·93	2·42	2·73	2·76	1·30
Succinic oxidase system					
Q_{O_2}	55	95	58	39	24
P:O	0·87	0·23	0·89	0·73	0·43
ATPase					
Q_P	166	—	61	—	—
Myokinase					
Q_P	7·2	—	4·9	—	—

The increase in activity of the succinic oxidase system in the saline preparation after 1 day is similar to that shown in Table 1 and undoubtedly has the same explanation. On the other hand, the activity of the succinic oxidase system in the ethylenediaminetetraacetic acid preparation declined on storage. This may be because inactivation of the enzyme systems responsible for the disappearance of oxaloacetate is not dependent upon the presence of calcium and so proceeds at the same rate in saline-ethylenediaminetetraacetic acid and saline preparations whereas the enzyme system bringing about formation of oxaloacetate is stabilized by the chelating agent.

It is shown below that probably much of the calcium in sarcosomal preparations is derived from other components of the heart during isolation. Thus, the inclusion of ethylenediaminetetraacetic acid in the isolation medium will largely prevent the calcium ever becoming bound to the sarcosomes. This is preferable to the subsequent addition of the chelating agent to remove already bound calcium, especially in sucrose preparations (cf. Expts. 6–8, Table 6).

Effect of incubation on the ATPase activity of sarcosomes

Table 10 shows that the ATPase activity of sarcosomes isolated in saline increased by 24% after incubation for 15 min. at 25°, compared with 58% activation by the addition of 10^{-4} M-2:4-dinitrophenol. The latter reagent has been shown by Hunter (1951) and Potter & Recknagel (1951) to increase the ATPase activity of liver mitochondria. The addition of ethylenediaminetetraacetic acid (0·01 M) during the incubation partially prevented the increase of activity, but the differences are too close to the experimental error of the analytical procedure to be significant.

Table 10. *Effect of incubation and of 2:4-dinitrophenol on ATPase activity of sarcosomes*

(Saline preparation from rat heart. Incubated 15 min. at 25°. 2:4-Dinitrophenol (10^{-4} M) added to reaction mixture where shown (DNP). Reaction time, 10 min., 25°. Reaction mixture contained 2×10^{-3} M-ethylenediaminetetraacetic acid and other additions as Slater (1953*a*). Q_P = μl. P/mg. protein/hr.)

	Q_P
Not incubated	238
Not incubated; DNP in reaction mixture	377
Incubated alone	295
Incubated with 0·01 M-ethylenediaminetetraacetic acid	269

Calcium content of heart-muscle and of sarcosomal preparations

The preparations used for calcium analyses were made by grinding with Pyrex glass beads (Ballotini, Chance Grade no. 9) instead of acid-washed sand in order to avoid the introduction of calcium into the ash from fragments of sand. The enzymic activity and the stability of sarcosomes prepared with glass beads were similar to those made with sand.

The results of these analyses are summarized in Table 11. The values for whole heart are similar to those given by Steenbock, Kletzien & Halpin (1932). Sarcosomal preparations made with ethylenediaminetetraacetic acid, whether or not it was subsequently removed, contained only about 15% of the calcium of saline and sucrose preparations. There was no significant difference between the latter two types.

In Table 11, the proportion of the total calcium of the heart found in the sarcosomes is compared with the yield of sarcosomes, calculated from the data of a previous paper (Cleland & Slater, 1953*b*). Several of the preparations were made with very light grinding; hence the yield of sarcosomes was

Table 11. *Calcium content of rat-heart muscle and of sarcosomal preparations*

(Sarcosomal preparations. The yield of sarcosomes was calculated from the total sarcosomal content of the heart estimated from the data of Table 1 of Cleland & Slater (1953*b*) as follows: mg. of sarcosomal protein/g. whole heart $= 0{\cdot}23 \times \frac{226}{1{\cdot}6} = 32{\cdot}4$, where 23% is the estimated proportion of sarcosomes (protein basis) and 226 mg. protein is the total protein in 1·6 g. whole heart. E = ethylenediaminetetraacetic acid.)

Whole heart

1·57, 1·05, 1·15, 1·34: mean 1·28 μmoles calcium/g. heart.

Sarcosomes

Prep.	Wt. of heart* (g.) (*a*)	Sarcosomal protein: Total in intact heart (mg.) (32·4*a*) (*b*)	Sarcosomal protein: Isolated (mg.) (*c*)	Sarcosomal protein: Yield (%) (*c*/*b* × 100)	Calcium: (μmoles/mg. protein) (*d*)	Calcium: Total in isolated sarcosomes (μmoles) (*c* × *d*) (*e*)	Calcium: Yield (%) $\frac{100e}{1{\cdot}28a}$
Sucrose	0·95 (1)	30·8	10·7	35	0·112	1·20	99
	0·85 (1)	27·6	7·9	29	0·110	0·87	80
	0·76 (1)	24·6	6·5	26	0·069	0·45	46
	0·85 (1)	27·6	4·6	17	0·089	0·41	38
	1·00 (1)	32·4	4·9	15	0·102	0·50	39
	1·55 (2)	50·4	11·2	22	0·112	1·25	63
Mean					**0·099**		
Saline	0·84† (1)	27·2	9·4	34	0·126	1·19	111
	1·68† (2)	54·4	18·7	34	0·088	1·64	76
	0·84† (1)	27·2	14·0	51	0·083	1·16	108
Mean					**0·099**		
Saline-E	1·68† (2)	54·4	26·0	48	0·014	0·36	17
Mean					**0·014**		
E-washed	1·68† (2)	54·4	36·0	66	0·016	0·58	27
	1·68† (2)	54·4	23·4	43	0·015	0·36	17
Mean					**0·015**		

* No. of hearts in brackets.

† The weights of these hearts were not determined directly. These values are taken from the mean weight of 12 hearts (range 0·75–1·00 g.).

usually less than the 50% obtained by Cleland & Slater (1953*b*). It can be seen that, in every case, the yield of calcium in sarcosomes prepared with sucrose or saline greatly exceeded the yield of sarcosomes and, when more than about 30% of the sarcosomes were isolated, virtually all the calcium in the heart was obtained in the sarcosomes. This suggests that the calcium might not be in the sarcosomes in the intact heart but becomes incorporated during the isolation. Judging by the absence of the colour of myoglobin in the residue, it is likely that a grinding sufficient to liberate 30% of the sarcosomes liberated all the sarcoplasm. Thus, if all the calcium is in the sarcoplasm, and sarcosomes have a high affinity for calcium, it would be conceivable that all the calcium of the heart would become bound to the isolated sarcosomes. It is also possible that the calcium is derived from extracellular fluid.

Table 12 shows that isolated sarcosomes do in fact take up large amounts of calcium from a solution. The ethylenediaminetetraacetic acid-washed preparation took up all the calcium from a solution of 10^{-4} M-calcium chloride in the usual saline medium, the calcium content of the sarcosomes after treatment with calcium approaching the value for saline preparations. A normal saline preparation took up approximately half the calcium supplied to it in a 10^{-4} M solution, thereby increasing the concentration of calcium in the sarcosomes by about 50%. In this experiment, the calcium was supplied in 0·16 M-potassium chloride without the phosphate present in the 'saline' medium used in the isolation. This shows that inorganic phosphate in the medium is not necessary for the uptake of calcium. The recovery of calcium in the supernatants when the sarcosomes were not added ((*b*) in Table 12) was 86% with the 'saline' medium containing phosphate, and 106% with the phosphate-free potassium chloride. This is probably 100% within experimental error in both cases, since the end point of the titration was more difficult to see when phosphate

was in the medium. In both experiments shown in Table 12, the loss of calcium from the solution agreed, within experimental error, with the gain of calcium by the sarcosomes. The ratio of supernatant to sarcosomes was such as to cause negligible errors due to retention of supernatant in the sarcosomal fraction.

Table 12. *Incorporation of calcium in suspending medium into sarcosomes*

(Rat preparations. *Ethylenediaminetetraacetic acid-washed preparation.* (*a*) 1 ml. prep. containing 9·1 mg. protein + 5 ml. saline (0·02 M-phosphate, 0·135 M-KCl, pH 7·4) + 0·595 μmole $CaCl_2$; (*b*) 6 ml. saline + 0·595 μmole $CaCl_2$. Both kept at 0° for 30 min. and then centrifuged in the cold and Ca determined directly on the supernatant and in the case of (*a*) in the residue (after ashing). Sample of untreated prep. also analysed. *Saline preparation.* (*a*) 1 ml. prep. containing 5·5 mg. protein + 5 ml. 0·16 M-KCl + 0·595 μmole $CaCl_2$; (*b*) 6 ml. 0·16 M-KCl + 0·595 μmole $CaCl_2$. Both treated in same manner as washed ethylenediaminetetraacetic-acid prep.)

	Calcium (μmoles)	
	Ethylenediaminetetraacetic acid-washed prep.	Saline prep.
In sarcosomes		
Before treatment		
Ca/mg. protein	0·015	0·083
Total Ca	0·15	0·49
After treatment		
Ca/mg. protein	0·068	0·127
Total Ca	0·66	0·76
ΔCa	**0·51**	**0·27**
In supernatant (total Ca)		
(*b*)	0·51	0·63
(*a*)	0	0·30
ΔCa	**−0·51**	**−0·33**

DISCUSSION

Instability of oxidative phosphorylation

Most studies of oxidative phosphorylation refer to the great instability of the enzyme systems to even brief treatments at temperatures above 0°. It was early found (Ochoa, 1943) that the system was more stable in the presence of substrates and the co-factors necessary for measuring the oxidative phosphorylation. It was recognized, however, that even under these conditions inactivation during the course of the experiment could be considerable. This has led to the usual practice of using short reaction times, and prompted several investigators to carry out their experiments at low temperatures (Belitzer & Tsibakova, 1939; Hunter & Hixon, 1949; Judah, 1951). The great increase of stability conferred on the oxidative phosphorylation enzymes in heart-muscle sarcosomes by the addition of ethylenediaminetetraacetic acid has proved of considerable technical value in the study of oxidative phosphorylation in these granules. As is shown in another paper (Slater & Holton, 1953*a*), the P:O ratio remains constant for at least 45 min. at 25°, in the presence of ethylenediaminetetraacetic acid, even in the case of the rat-heart sarcosomes which are so unstable in its absence. Preliminary experiments show that it also stabilized liver mitochondria. van Bekkum, Jongepier, Nieuwekerk & Cohen (1953) have found our procedure effective with spleen mitochondria, and Lindberg, Ljunggren, Ernster & Révész (1953) have used it to isolate mitochondria from Ehrlich ascites tumour.

Mechanism of action of ethylenediaminetetraacetic acid

The fact that sarcosomes isolated with ethylenediaminetetraacetic acid and then washed free of the chelating agent are much more stable than saline preparations shows that the ethylenediaminetetraacetic acid is not acting directly in the inactivation process, but must be acting indirectly by removing some substance which is present in the sarcosomes and which promotes the inactivation. Since the characteristic property of ethylenediaminetetraacetic acid is its ability to chelate with metal ions, it is very probable that this substance is a metal ion. Added calcium was found to increase the rate of inactivation both with saline and ethylenediaminetetraacetic acid-washed preparations. Owing to the well-known inhibitory action of heavy metals on the α-ketoglutaric oxidase system, it was not possible to test the specificity of this effect of calcium. However, the high concentration of calcium in the sarcosomes, and their high affinity for added calcium, as measured by direct chemical estimation, is strong support for the view that calcium is the metal responsible for the instability of sarcosomes.

The amount of ethylenediaminetetraacetic acid required for complete protection in the presence of reaction mixture was about 0·07 μmole/mg. sarcosomal protein (Fig. 2), which is of the same order of magnitude as the calcium content of the sarcosomes. However, considerably more was usually required in the experiments in which the sarcosomes were incubated in the absence of reaction mixture (Table 6), although in Expt. 3 of Table 6 and Expt. 2 of Table 7 a high protection was given by an amount of ethylenediaminetetraacetic acid equal to about twice the normal calcium content. Either the calcium is very firmly bound to sarcosomes or it becomes bound to some part of the sarcosome not readily accessible to the chelating agent. It is possible that the different behaviour of saline and sucrose preparations can be explained on this basis.

The protective action of ethylenediaminetetraacetic acid described in the present paper is quite different from its activation of the succinic oxidase

system in the Keilin & Hartree heart-muscle preparation recently found by Bonner (1953). In the latter case, the activation is lost if the chelating agent is removed by sedimentation of the particles and washing in the centrifuge. In Bonner's experiments, it seems clear that ethylenediaminetetraacetic acid has some direct action of its own and is not removing inactivating metals. It also serves the additional function of removing traces of inhibitory heavy metals if these are present in the reagents, as for example in Altmann & Crook's (1953) experiments.

Ethylenediaminetetraacetic acid is ideally suited for binding calcium in experiments of the type described in this paper. Unlike citrate, which is an oxidizable substrate, it is stable in the presence of enzyme preparations. It is remarkably free from inhibitory action on many enzyme systems, even those which require Mg^{2+} (Lehninger, 1951; Slater & Holton, 1953*b*).

Mode of action of calcium

While it appears certain that ethylenediaminetetraacetic acid is able to stabilize the enzyme systems by removing calcium from the preparation, the mechanism whereby calcium exerts its inactivating effect is not established by the present study. Some possibilities will be discussed. First it should be noted that the inactivation requires not only the presence of calcium in the preparation, but also an incubation. It is clear, then, that in our experiments the calcium must act not by directly entering the phosphorylation reaction, as suggested by Lehninger (1951), but by promoting an irreversible destruction of the oxidase system.

As discussed elsewhere (Cleland, 1952; Cleland & Slater, 1953*a*; Cleland, 1953), the sarcosomes have a semi-permeable membrane, which is impermeable to potassium chloride and many other salts. The possibility should therefore be considered that calcium ions are concentrated in the intrasarcosomal fluid by some specific mechanism. If the intrasarcosomal water content is 3·5 times the protein (assuming that the sarcosome contains 20% protein, 10% phospholipid and 70% water), the concentration of intrasarcosomal calcium after treatment with calcium in the two experiments shown in Table 12 would be 0·02 M and 0·04 M compared with 0 and 5×10^{-5} M respectively in the extrasarcosomal fluid. If this intrasarcosomal calcium were in the form of free Ca^{2+} ions, there would be a very high ratio of internal to external $[Ca^{2+}]$. This could conceivably be brought about either by (i) coupling with an energy-yielding mechanism such as respiration (Bartley & Davies, 1952) or (ii) by a Donnan equilibrium. The first possibility is unlikely since the sarcosomes were not respiring or carrying out any other obvious metabolic activity. The second possibility is also made very unlikely by the fact that sarcosomes isolated with and suspended in sucrose contained the same amount of calcium as saline preparations. It is concluded that the calcium in the sarcosomes is not in solution in the intrasarcosomal fluid, but is firmly bound.

The only chemical grouping which seems likely to be present in sufficient concentration is a phosphate group, either free or bound. The total phosphorus content of the heart sarcosomes is 0·5 μg. atoms/mg. of sarcosomal protein (Cleland, 1953), which is more than sufficient to combine with the amounts of calcium found. Most of this phosphorus is in the form of phospholipid which is probably situated in the membrane (Cleland & Slater, 1953*a*). It seems probable, then, that much of this phospholipid as well as other phosphorus compounds in the sarcosome will be combined with calcium. One of these compounds is ADP, present to the extent of about 0·02 μmole/mg. protein (Slater & Holton, 1953*a*).

Before considering the possible mechanism of the action of calcium described in the present paper, it is necessary to consider changes in the properties of sarcosomes or mitochondria which accompany incubation and to see which are known to be affected by calcium. At the same time, the marked protection found by Pardee & Potter (1949), Kielley & Kielley (1951) and ourselves to be given by ADP or ATP (these will henceforth be referred to simply as ~P) in the absence of ethylenediaminetetraacetic acid must also be taken into account. The available information is assembled in Table 13.

Table 13. *Effect of incubation on various properties of liver mitochondria and heart sarcosomes*

Property	Effect of incubation	Accelerated by Ca^{2+}	Inhibited by ~P
Morphology	'Spontaneous transformation'*	+*	+*
α-Ketoglutaric oxidase system	Inactivation*†	+*	+*†
ATPase	Activation*†	?	+†
DPN (endogenous)	Destruction§	+‡	?
~P (endogenous)	Destruction†§	?	?

* Cleland & Slater (1953*a*) or present paper, heart sarcosomes.
† Kielley & Kielley (1951), liver mitochondria.
‡ Axelrod *et al.* (1941); Swingle *et al.* (1942), heart muscle and liver homogenates.
§ Green *et al.* (1949); Huennekens & Green (1950), liver and kidney cyclophorase.

With so many effects of incubation, most of which have been directly related to the presence of calcium, it is difficult to determine which is the primary effect of calcium and which are secondary. Possibly most of the above effects are, in fact, secondary to an unknown primary effect. Some possible causal relationships will be considered.

(1) First it is necessary to state that the loss of oxidative phosphorylation is not caused directly by an increased ATPase activity. The increase of ATPase activity caused by incubating a saline preparation was relatively slight. In any case, the effect of incubation is not only on the P:O ratio but on the activity of various oxidase systems. The effect of ethylenediaminetetraacetic acid on the activation of ATPase by incubation, and the lower ATPase activities sometimes found with preparations made with it, suggest that calcium is concerned in the activation of the ATPase by incubation, but more impressive evidence is required. Heart sarcosomes are not very suitable material for this study, since incubation only slightly increases the ATPase activity. Potter & Simonson's (1952) finding that calcium activates fresh liver mitochondria but not incubated mitochondria is also suggestive that calcium might be concerned in the activation by incubation.

(2) The protective effects of ethylenediaminetetraacetic acid and of ~P were first revealed in the present study by their ability to prevent the morphological changes which occur when isolated sarcosomes are kept at room temperature (the 'spontaneous transformation' of Cleland & Slater, 1953*a*). Consequently, serious consideration has been given to the possibility that the inactivation of the α-ketoglutaric oxidase system is a result of the morphological changes. However, a close examination of the evidence does not support this view. In the first place, the gross morphological changes occur some time after the inactivation of the enzyme systems. Secondly, some factors which have been found to affect the morphological stability (for example, sucrose preparations are morphologically more stable than saline preparations; hypotonicity increases the rate of morphological changes) do not affect the stability of the α-ketoglutaric oxidase system. These considerations suggest that the inactivation of the enzyme system precedes the morphological changes.

(3) Since DPN is a component of the malic oxidase system, the destruction of this coenzyme may be a sufficient explanation of the inactivation of this system. It is possible, but not yet certain (see Slater, 1953*b*), that DPN is also a component of both the α-ketoglutaric and pyruvic oxidase systems. One of these systems (the α-ketoglutaric oxidase system) was inactivated by the incubations used in this paper. Since the oxidative step in the oxidation of oxaloacetate is presumably the oxidation of pyruvate formed by the decarboxylation of oxaloacetate, it can be concluded that the pyruvic oxidase system was not inactivated. The destruction of DPN does not provide an adequate explanation of the inactivation of the phosphorylation associated with the oxidation of succinate or oxaloacetate. It is also noteworthy that in no case where it has been tested does calcium activate soluble enzymes which decompose DPN (McIlwain, 1950; Kornberg & Pricer, 1950; Kaplan, Colowick & Nason, 1951). This strongly suggests that calcium does not act directly on the DPNase in dispersions but promotes some change which makes the DPN accessible to the DPNase. It could do this either by bringing about the morphological disintegration of the granules, or, more specifically, by causing the dissociation of firmly bound DPN-protein complexes (Huennekens & Green, 1950). That DPN is not readily accessible to DPN-degrading enzymes *in vivo* is indicated by the work of Gore, Ibbott & McIlwain (1950).

These considerations make it appear unlikely that the effect of calcium on the DPN content of the preparation is its primary action.

(4) Kielley & Kielley (1951) suggested a relationship between the phosphorylating activity and the loss of ~P from mitochondria (see also Green, Atchley, Nordmann & Teply, 1949). Amongst all the possible explanations of the primary action of calcium, a stimulation of the loss of endogenous ~P is perhaps the most promising, since it would provide a ready explanation of the protective effect of added ~P. The protective effect of added ~P on the morphology of the sarcosomes suggests that a source of energy might be necessary to maintain the structure of the sarcosomal membrane (see also Harman & Feigelson (1952) and Cleland & Slater (1953*a*)).

Two possible ways whereby calcium might affect the endogenous ~P of the sarcosomes may be mentioned. (i) The ~P compounds may actually be bound by the high concentration of calcium entering the sarcosome and thereby be prevented from having their normal effect. (ii) Calcium might displace some metal prosthetic group from an enzyme which operates in conjunction with ~P to maintain the morphological and enzymic structure of the sarcosome. Pressman & Lardy (1952) have obtained evidence that potassium is firmly bound to liver mitochondria, and that this bound potassium is intimately linked with the function of mitochondria. Cleland (1953) has found that the sarcosomal contents contain large amounts of magnesium, which is known as a component of many phosphorylating enzymes.

The slight protective effect of magnesium, fluoride and phosphate might be due to inhibition of phosphatases, which would cause loss of ~P, apart from the possible mechanisms suggested above.

For the reasons given on p. 575, it is probable that the high calcium content and the instability of isolated sarcosomes is an artifact, not representing the state of affairs in the intact fibre. In order to isolate sarcosomes resembling as closely as possible those in the intact cell, it is necessary to prevent the calcium becoming bound to the sarcosome, by including ethylenediaminetetraacetic acid in the isolation medium. Sarcosomes prepared in this way are most suitable for studies of the enzyme systems bringing about the complete aerobic oxidation of intermediary metabolites and oxidative phosphorylation. Studies of the respiratory chain, present in the Keilin & Hartree heart-muscle preparation (Keilin & Hartree, 1949), and of many individual enzymes which are probably localized in the sarcosomes *in vivo* but are relatively easily liberated into solution, e.g. Ochoa's condensing enzyme (Ochoa, Stern & Schneider, 1951), fumarase (Massey, 1952), α-ketoglutaric dehydrogenase (Kaufman, 1951; Sanadi & Littlefield, 1951), require the disruption of the sarcosome, a process which is greatly helped by calcium, either originally present in the heart or introduced from tap water.

SUMMARY

1. Incubation of heart-muscle sarcosomes for 15 min. at 25° largely inactivated the α-ketoglutaric oxidase and malic oxidase systems, activated the succinic oxidase system and had little effect on the oxidation of oxaloacetate. The P:O ratio was greatly decreased with malate or oxaloacetate as substrate, was less affected with succinate, and only slightly decreased with α-ketoglutarate.

2. The inactivation of the α-ketoglutaric oxidase system was studied in greater detail. The inactivation had a high temperature coefficient, proceeded at a steady rate without any lag period and was not markedly affected by the pH or tonicity of the suspending medium.

3. The inactivation of the α-ketoglutaric oxidase system was much less in the presence of the reaction mixture used in oxidative phosphorylation experiments. The component mainly responsible was adenosine diphosphate; adenosine triphosphate was also effective but not adenosine monophosphate. The combined addition of magnesium, fluoride and phosphate also gave some protection.

4. Ethylenediaminetetraacetic acid (0·01 M) usually completely protected the α-ketoglutaric oxidase system against incubation of the sarcosomes for 15 min. at 25°, even in the absence of the reaction mixture. In the presence of ethylenediaminetetraacetic acid and the reaction mixture, sarcosomes oxidized α-ketoglutarate at practically a uniform rate for an hour or more at 25°.

5. Both adenosine diphosphate and ethylenediaminetetraacetic acid prevented the activation of the succinic oxidase system (which is probably due to a decrease of the steady-state concentration of oxaloacetate) and the inactivation of the accompanying phosphorylation caused by the incubation.

6. Sarcosomes isolated in ethylenediaminetetraacetic acid and then freed from this substance were much more stable than those isolated in saline alone, but they were very susceptible to added calcium. Added calcium also increased the rate of inactivation with normal sarcosomes.

7. The most satisfactory preparations of sarcosomes are obtained by including ethylenediaminetetraacetic acid in the isolation medium.

8. By direct analysis it was found that isolated sarcosomes contain all the calcium in the heart muscle, even when only about 30% of the total sarcosomes were isolated. Preparations isolated with ethylenediaminetetraacetic acid contained much less calcium. Both types of sarcosomes took up large amounts of calcium from solutions. The localization of the calcium in the isolated sarcosomes does not, therefore, necessarily reflect the position in the intact heart.

9. It is very probable that the stabilizing effect of ethylenediaminetetraacetic acid is due to removal of calcium from the sarcosomes. The mechanism of action of the calcium was not established, but different possibilities are discussed.

This work was carried out on behalf of the Agricultural Research Council. We wish to thank Prof. D. Keilin, F.R.S., for his interest and advice. One of us (K.W.C.) thanks the Nuffield Foundation for a Dominions Fellowship (while on leave from the Department of Histology and Embryology, University of Sydney) and the Agricultural Research Council for a personal grant during different stages of this work. We also wish to thank the Bersworth Chemical Company for the gift of some ethylenediaminetetraacetic acid (Versene).

REFERENCES

Altmann, S. M. & Crook, E. M. (1953). *Nature, Lond.*, **171**, 76.
Axelrod, A. E., Swingle, K. F. & Elvehjem, C. A. (1941). *J. biol. Chem.* **140**, 931.
Bartley, W. & Davies, R. E. (1952). *Biochem. J.* **52**, xx.
Bekkum, van D. W., Jongepier, H. J., Nieuwekerk, H. T. M. & Cohen, J. A. (1953). *Trans. Faraday Soc.* **49**, 329.
Belitzer, V. A. & Tsibakova, E. T. (1939). *Biochemistry, Leningr.* **4**, 516.
Bonner, W. D. (1953). *Biochem. J.* (in the Press).
Cleland, K. W. (1952). *Nature, Lond.*, **170**, 497.
Cleland, K. W. (1953). In preparation.
Cleland, K. W. & Slater, E. C. (1953*a*). *Quart. J. micr. Sci.* (In the Press).
Cleland, K. W. & Slater, E. C. (1953*b*). *Biochem. J.* **53**, 547.
Das, N. B. (1937). *Biochem. J.* **31**, 1124.

Debney, E. W. (1952). *Nature, Lond.*, **169**, 1104.
Green, D. E., Atchley, W. A., Nordmann, J. & Tepley, L. J. (1949). *Arch. Biochem.* **24**, 359.
Gore, M., Ibbott, F. & McIlwain, H. (1950). *Biochem. J.* **47**, 121.
Harman, J. W. & Feigelson, M. (1952). *Exp. Cell Res.* **3**, 509.
Huennekens, F. M. & Green, D. E. (1950). *Arch. Biochem.* **27**, 418, 428.
Hunter, F. E. (1951). In *Phosphorus Metabolism*, vol. 1, p. 297, ed. by W. D. McElroy & Bentley Glass. Baltimore: The Johns Hopkins Press.
Hunter, F. E. & Hixon, W. S. (1949). *J. biol. Chem.* **181**, 73.
Judah, J. D. (1951). *Biochem. J.* **49**, 271.
Kaplan, N. O., Colowick, S. P. & Nason, A. (1951). *J. biol. Chem.* **191**, 473.
Kaufman, S. (1951). In *Phosphorus Metabolism*, vol. 1, p. 370, ed. by W. D. McElroy & Bentley Glass. Baltimore: The Johns Hopkins Press.
Keilin, D. & Hartree, E. F. (1940). *Proc. Roy. Soc.* B, **129**, 277.
Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.
Keltch, A. K. & Clowes, G. H. A. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 831.
Kielley, W. W. & Kielley, R. K. (1951). *J. biol. Chem.* **191**, 485.
Kornberg, A. & Pricer, W. E. (1950). *J. biol. Chem.* **182**, 763.
Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
Lehninger, A. L. (1951). In *Enzymes and Enzymes Systems*, p. 1, ed. by J. T. Edsall. Cambridge, Mass: Harvard University Press.
Lindberg, O., Ljunggren, M., Ernster, L. & Révész, L. (1953). *Exp. Cell Res.* **4**, 243.
McIlwain, H. (1950). *Biochem. J.* **46**, 612.
Massey, V. (1952). *Biochem. J.* **51**, 490.
Ochoa, S. (1943). *J. biol. Chem.* **151**, 493.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Pardee, A. B. & Potter, V. R. (1948). *J. biol. Chem.* **176**, 1085.
Pardee, A. B. & Potter, V. R. (1949). *J. biol. Chem.* **181**, 739.
Paul, M. H., Fuld, M. & Sperling, E. (1952). *Proc. Soc. exp. Biol., N.Y.*, **79**, 349.
Potter, V. R. (1947). *J. biol. Chem.* **169**, 17.
Potter, V. R. & Recknagel, R. O. (1951). In *Phosphorus Metabolism*, vol. 1, p. 377, ed. by W. D. McElroy & Bentley Glass. Baltimore: The Johns Hopkins Press.
Potter, V. R. & Simonson, H. C. (1952). *Fed. Proc.* **11**, 270.
Pressman, B. C. & Lardy, H. A. (1952). *J. biol. Chem.* **197**, 547.
Ringer, S. (1883). *J. Physiol.* **4**, 29.
Sanadi, D. R. & Littlefield, J. W. (1951). *J. biol. Chem.* **193**, 683.
Schwarzenbach, G. & Ackermann, H. (1947). *Helv. chim. acta*, **30**, 1798.
Slater, E. C. (1950). *Nature, Lond.*, **166**, 982.
Slater, E. C. (1953*a*). *Biochem. J.* **53**, 521.
Slater, E. C. (1953*b*). *Annu. Rev. Biochem.* **22**, 17.
Slater, E. C. & Cleland, K. W. (1952). *Nature, Lond.*, **170**, 118.
Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **53**, 557.
Slater, E. C. & Holton, F. A. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 43.
Slater, E. C. & Holton, F. A. (1953*a*). *Biochem. J.* **55**, 530.
Slater, E. C. & Holton, F. A. (1953*b*). *Biochem. J.* (in the Press).
Steenbock, H., Kletzien, S. W. F. & Halpin, J. G. (1932). *J. biol. Chem.* **97**, 249.
Swingle, K. F., Axelrod, A. E. & Elvehjem, C. A. (1942). *J. biol. Chem.* **145**, 581.

The Estimation of Small Amounts of Formaldehyde Liberated during the Oxidation of Carbohydrates and other Substances with Periodate

By J. F. O'DEA and R. A. GIBBONS
Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1

(*Received* 7 *April* 1953)

In the course of investigations into the chemistry of the blood-group mucoids and closely related polysaccharides, it was necessary to measure the formaldehyde produced on oxidation of these materials with periodate. The gravimetric estimation of the aldehyde as its dimedon derivative was used by Aminoff & Morgan (1951) in their studies on blood-group A substance, but the method has two disadvantages. First, relatively large amounts (30–50 mg.) of the material to be oxidized are required in order to yield a suitable amount of the dimedon derivative, and secondly, other aldehydes such as acetaldehyde will also give a dimedon derivative and the quantitative separation of these non-specific materials, without loss of the formaldehyde derivative, may be difficult. Furthermore, the reagent blank is appreciable (Courtois, 1951).

A method for the determination of small amounts of formaldehyde was therefore sought which was specific for formaldehyde, and which was applicable to small amounts of sugars, polysaccharides, mucoids, or other materials yielding formaldehyde on oxidation with periodate. Eegriwe (1937) observed that formaldehyde reacted with chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid) on heating in strong sulphuric acid to yield a highly coloured compound, and that the reaction appeared to be specific for formaldehyde.