The Effect of Tonicity of the Medium on the Respiratory and Phosphorylative Activity of Heart-muscle Sarcosomes

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Since the work of Lehninger & Kennedy (1948) on the effect of tonicity of the medium on the activity of the fatty acid oxidase system of liver mitochondria, it has been repeatedly emphasized that approximately isotonic conditions are necessary for optimal oxidative phosphorylation activity of the mitochondria. The present study was undertaken as part of an investigation of the optimal conditions for oxidative phosphorylation in heart-muscle sarcosomes, the granules which correspond to the mitochondria in other tissues (Cleland & Slater, 1953*a*).

It has been known since the time of Kölliker (1888) that sarcosomes undergo a series of structural changes when placed in hypotonic media. These changes have been studied in detail by Cleland & Slater (1953*b*). In the present paper, we are not concerned with the most striking morphological changes, which occur below an osmolar concentration of 0·05. The only change which occurs between isotonic (osmolar concentration of 0·32) and 0·05-osmolar media is a considerable but largely reversible swelling of the sarcosome, and the present paper deals with the effect of this swelling on the respiratory and phosphorylative activity of the sarcosomes.

Some of this work was presented at the Second International Congress of Biochemistry in Paris, 21–27 July 1952 (Slater & Cleland, 1952*a*).

METHODS

Isolation of sarcosomes. The method described in the previous paper (Cleland & Slater, 1953*a*) was used. Different types of preparations are designated according to the composition of the isolation medium, as described in the previous paper.

Keilin & Hartree heart-muscle preparation. This was prepared according to the procedure of Keilin & Hartree (1947).

Activity of α-ketoglutaric oxidase system. The activity of this system (defined as the system of enzymes catalysing the aerobic oxidation of α-ketoglutarate to succinate) was measured by determining the rate of oxygen uptake in the presence of the components listed in Table 1. Two reaction mixtures were used, hypotonic (omitting fluoride) and isotonic. The activity was considerably higher in the former for two reasons: (i) fluoride inhibits the α-ketoglutaric oxidase system (Slater & Holton, 1952); and (ii) the system is more active under hypotonic conditions (see below). Hexokinase, 20–100 units (Berger, Slein, Colowick & Cori, 1946) per mg. protein of the sarcosomal preparation, and 0·6–1·5 mg. (protein) of the sarcosomal preparation were added in addition to the components listed in Table 1. In the case of the isotonic medium, the final concentration was made approx. isotonic with KCl or sucrose. The final volume was either 3 ml. in 15 ml. Warburg flasks or 1 ml. in 7 ml. Warburg flasks. Measurements were made over 30–45 min. at 25°. The enzyme activities are expressed as Q_{O_2} (μl. O_2/mg. protein/hr.).

Table 1. *Reaction mixtures for measuring activity of α-ketoglutaric oxidase system*

(All components were adjusted to pH 7·4 (glass electrode), with KOH or HCl.)

	Concentration (M)	
Component	Isotonic mixture	Hypotonic mixture
Phosphate (Sørensen)	0·03–0·05	0·015–0·03
Malonate, sodium or potassium	0·01	0·01
Fluoride, sodium or potassium	0·04	—
Glucose	0·01–0·03	0·02
Versene	0·001–0·002	0·001–0·002
Adenosinediphosphate	$4·4–6·7 \times 10^{-4}$	$6·7 \times 10^{-4}$
Adenosine-5-phosphate	$4·7–6·0 \times 10^{-4}$	$6·0 \times 10^{-4}$
$MgCl_2$	0·005	0·005
α-Ketoglutarate, potassium	0·003–0·01	0·003–0·01
Cytochrome *c*	$3·5–5·5 \times 10^{-5}$	$3·5–5·5 \times 10^{-5}$

The conditions described in Table 1 are optimal for oxidative phosphorylation (Slater & Holton, 1953).

Measurement of yield of oxidative phosphorylation. The method described by Slater (1953) was used. The yield (P:O) is the number of molecules of hexosemonophosphate (HMP) synthesized per atom of oxygen consumed. The HMP formed (ΔHMP) was corrected for myokinase activity, as described by Slater (1953).

Activity of succinic oxidase system. Unless phosphorylation accompanying the oxidation of succinate was studied, the activity of the succinic oxidase system was studied under the conditions optimal for the Keilin & Hartree heart-muscle preparation (Slater, 1949*a*), except that the temperature was 25° instead of 38°. When oxidative phosphorylation was studied, concentrations were as follows: phosphate, 0·035 M; adenosinediphosphate (ADP), $4·5–5·4 \times 10^{-4}$ M; adenosinemonophosphate (AMP), $4·7–5·2 \times 10^{-4}$ M; NaF,

0·034–0·04 M; glucose, 0·0084 M; $MgCl_2$, 0·005 M; succinate, 0·023 M; cytochrome *c*, $4{\cdot}8 \times 10^{-5}$ M; 0·6 mg. (protein) sarcosomal preparation/ml.; 60–250 units hexokinase/mg. protein of sarcosomal preparation. Sufficient KCl was added to make the medium approximately isotonic. Activities are expressed as Q_{O_2} at 25°. The activity with this reaction mixture is considerably below the optimum, owing to inhibition of succinic dehydrogenase by fluoride (Slater & Bonner, 1952).

Succinic dehydrogenase. This was measured as in the previous paper (Cleland & Slater, 1953*a*).

Cytochrome oxidase. The cytochrome oxidase activity of sarcosomes was measured under conditions similar to those for the succinic oxidase system, except that ascorbic acid (0·025 M) replaced succinate. The cytochrome oxidase activity of the Keilin & Hartree preparation was measured as previously described (Slater, 1949*b*).

Myokinase and ATP-ase. The activities of myokinase and ATP-ase were measured as described in Slater (1953).

Protein. Protein was measured as in the previous paper (Cleland & Slater, 1953*a*).

Osmolar concentration of reaction media. The osmolar concentrations given in this paper were calculated as the total concentration of all species of solute present in the solution, assuming complete ionization of salts and disregarding the fact that some species are not osmotically active. Isotonicity is defined as 0·33-osmolar.

RESULTS

Effect of pH

As a preliminary to the study of the effect of tonicity of the medium on the activity, the effect of small changes of pH around neutrality was examined.

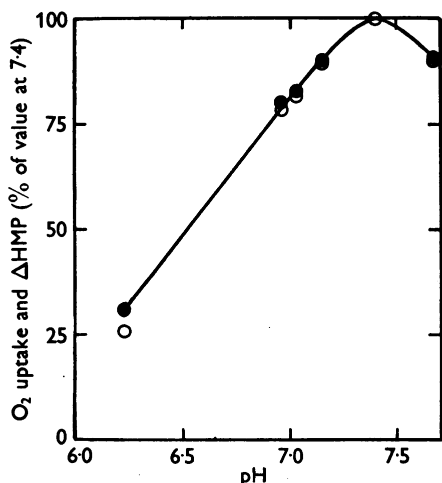


Fig. 1. Effect of pH on activity of α-ketoglutaric oxidase system and on accompanying phosphorylation. ●—●, O_2 uptake; ○—○, HMP synthesized.

Fig. 1 shows that the activity of the α-ketoglutaric oxidase system has quite a sharp optimum at pH 7·4. The accompanying phosphorylation follows practically the same curve except under definitely acid conditions. Thus the P:O ratio is independent of pH over a considerable range. The same activity was found in sodium phosphate buffer as in Sørensen buffer.

Effect of hypertonic sucrose on the activity of respiratory enzyme systems

In a preliminary experiment, summarized in Table 2, the effect of making the medium strongly hypertonic by the addition of 0·88 M-sucrose to the isotonic reaction mixture was studied. The results show that this concentration of sucrose inhibited all the oxidase systems studied. The α-ketoglutaric oxidase system was the most affected (94% inhibition) and cytochrome oxidase the least (59%). Oxidative phosphorylation, particularly that associated with the oxidation of succinate, was also greatly impaired. Myokinase was inhibited by 42% but the ATP-ase by only 14%.

Table 2. *Effect of hypertonic sucrose* (0·88 M) *on the activities of heart-muscle sarcosomes*

(Cat heart, phosphate-saline preparation. No Versene used, otherwise 'isotonic reaction mixture' (see Table 1).)

Activity	Without sucrose	With sucrose
Myokinase Q_P	3·6	2·1
ATP-ase Q_P	208	180
α-Ketoglutaric oxidase system		
Q_{O_2}	27·0	1·7
P:O	2·69	1·45
Succinic oxidase system		
Q_{O_2}	82	21
P:O	1·04	0·18
Cytochrome oxidase		
Q_{O_2}*	401	166

* Measured with suboptimal concentration of cytochrome *c* ($4{\cdot}8 \times 10^{-5}$ M).

The inhibitory effect of sucrose on the succinic oxidase system and cytochrome oxidase was further studied, using the Keilin & Hartree heart-muscle preparation as the source of these enzyme systems. The effect of different concentrations of sucrose on the activity of the succinic oxidase system, measured under different conditions, is shown in Table 3, from which the following conclusions are drawn: (1) The inhibition is less in 0·123 M-phosphate (optimum for the activity of the succinic oxidase system, Keilin & Hartree, 1949) than in 0·038 M, about the concentration used in the experiments on oxidative phosphorylation. (2) The inhibition is considerably greater in the absence of added cytochrome *c* than in its presence (cf. Schneider & Hogeboom, 1950), showing that sucrose affects the catalytic activity of the endogenous cytochrome *c* in the preparation. Other experiments showed that

Table 3. *Inhibition of succinic oxidase system of Keilin-Hartree heart-muscle preparation by sucrose*

[Phosphate] (M)	[KCl] (M)	[Cyt. c] (M × 10^5)	[Sucrose] (M)	Activity of succinic oxidase system (Q_{O_2})	Inhibition (%)
0·038	0·058	4·4	0	175	0
			0·05	153	13
			0·31	110	37
			0·78	39	78
0·123	0	4·4	0	368	0
			0·31	287	22
		0	0	292	0
			0·31	193	34

Table 4. *Inhibition of succinic dehydrogenase of Keilin-Hartree heart-muscle preparation by sucrose*

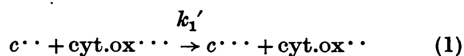
Enzyme system	H acceptor	[Succinate] (M)	[Sucrose] (M)	Q_{O_2}	Specific activity*	Inhibition (%)
Succinic oxidase	O_2	0·024	0	272	—	—
		0·024	0·52	147	—	46
Succinic dehydrogenase	Methylene blue	0·024	0	76	—	—
		0·024	0·52	49	—	36
Succinic dehydrogenase	Potassium ferricyanide	0·027	0	—	8·0	—
		0·027	0·59	—	6·8	15
		0·0005	0	—	3·6	—
		0·0005	0·59	—	3·1	15

* Measured by the spectrophotometric method.

the inhibition was completely reversed by dilution and was not affected by purifying the sucrose solution by treatment with the ion-exchange resin, Dowex 50.

Succinic dehydrogenase, when measured with methylene blue, was inhibited, although to a less degree than the succinic oxidase system, but when measured by the rate of reduction of potassium ferricyanide, there was only a slight inhibition (Table 4). Lowering the succinate concentration to a point which gave somewhat less than half the maximal activity did not affect the degree of inhibition; thus sucrose does not compete with succinate for succinic dehydrogenase.

The effect of 0·88 M-sucrose on the cytochrome oxidase activity measured with ascorbic acid and varying concentrations of cytochrome c is shown in Fig. 2. It can be seen that even at this high concentration, sucrose inhibits the cytochrome oxidase activity, measured at infinite cytochrome c concentration, by only 12 %, but has a marked effect on the K_m (cytochrome c), which is increased by 90 %. The reactions which are concerned in this measurement may be expressed in a simplified form:



$$c^{\cdot\cdot} + \text{cyt.ox}^{\cdot\cdot\cdot} \xrightarrow{k_1'} c^{\cdot\cdot\cdot} + \text{cyt.ox}^{\cdot\cdot} \quad (1)$$

$$\text{cyt.ox}^{\cdot\cdot} \xrightarrow{k_2'} \text{cyt.ox}^{\cdot\cdot\cdot}. \quad (2)$$

Overall reaction $c^{\cdot\cdot} \rightarrow c^{\cdot\cdot\cdot}$,

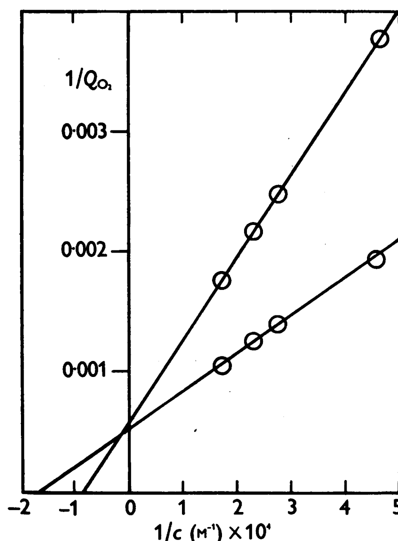


Fig. 2. Effect of sucrose (0·88 M) on cytochrome oxidase activity of Keilin & Hartree heart-muscle preparation. Abscissa is the inverse of the cytochrome c concentration and the ordinate is the inverse of the Q_{O_2}. Concentration of heart-muscle preparation, 0·27 mg. protein/ml. The inverse of the activity at infinite cytochrome c concentration is given by the point at which the straight lines cross the ordinate. The lines cross the abscissa at $1/c = -1/K_m$, where K_m is the Michaelis constant.

where $c^{\cdot\cdot}$ and $c^{\cdot\cdot\cdot}$ are reduced and oxidized cytochrome c respectively and cyt.ox$^{\cdot\cdot}$ and cyt.ox$^{\cdot\cdot\cdot}$ are reduced and oxidized cytochrome c oxidase respectively. k_2' is not a simple rate constant, but includes the concentration of O_2 which oxidizes reduced cytochrome c oxidase by an unknown mechanism. k_1' is also not a simple rate constant, since it will depend upon the accessibility of the cytochrome c to cytochrome c oxidase (see Slater, 1949*b*). It is assumed that sufficient ascorbic acid is present so that all the cytochrome c is kept in the reduced state. The rate of the reaction, v, is given by the expression

$$v = k_2' \,[\text{cyt.ox}]/(1 + K_m/[\text{cyt. } c]),$$

where [cyt.ox] is the total concentration of both reduced and oxidized cytochrome c oxidase, [cyt. c] the total concentration of reduced and oxidized cytochrome c and K_m, the Michaelis constant, equals k_2'/k_1'. At infinite cytochrome c concentration, obtained by the extrapolation shown in Fig. 2, the velocity equals k_2' [cyt.ox]. Since this is decreased by 12 % by 0·88 M-sucrose and K_m ($=k_2'/k_1'$) is increased by 90 %, it follows that k_1' has been decreased by 54 %. The effect of 0·88 M-sucrose is, therefore, to affect the accessibility of cytochrome c to its oxidase, without having an appreciable effect on cytochrome oxidase itself.

Thus the major effect of a high concentration of sucrose on the Keilin & Hartree heart-muscle preparation is to cause impaired accessibility both of endogenous cytochrome c to the other components of the succinic oxidase system and of added cytochrome c to cytochrome c oxidase. This is a characteristic of non-specific physical inhibitors, which inhibit the system not by combining with any one component, but by acting on the particle itself in such a way as to cause impaired mutual accessibility of the components of the system (Keilin & Hartree, 1949; Slater, 1949*c*). The lack of effect on succinic dehydrogenase, when this was measured with potassium ferricyanide compared with a distinct effect when methylene blue was the hydrogen acceptor, recalls the behaviour of narcotics, found by Stoppani (1949).

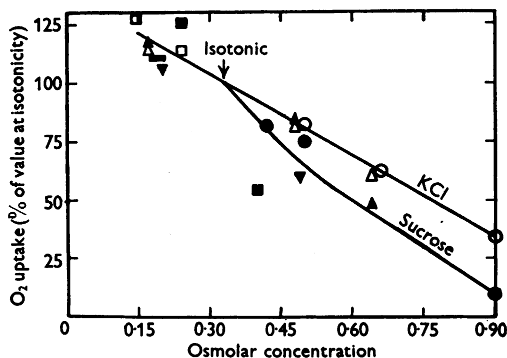


Fig. 3. Effect of tonicity of medium on activity of α-ketoglutaric oxidase system of rat-heart sarcosomes. The two curves have been drawn through points obtained in different experiments in which tonicity of the medium has been varied by the addition of KCl or sucrose. Activity in isotonic medium = 100. The same symbols have been used for the same preparation. Open symbols represent the effect of added KCl, blocked symbols the effect of added sucrose.

Effect of tonicity of medium on the α-ketoglutaric oxidase system and on P:O *ratio*

The effect of the tonicity of the reaction medium on the activity of the α-ketoglutaric oxidase system and on the accompanying phosphorylation in saline-Versene preparations is shown in Table 5.

Table 5. *Effect of tonicity on activity of α-ketoglutaric oxidase system, and on accompanying phosphorylation*

(Saline-Versene rat-heart preparations, hypotonic reaction mixture.)

Exp.	[KCl] (M)	[Sucrose] (M)	Osmolar concn.	O_2 uptake (μg.atoms)	ΔHMP (μmoles)	P:O
1	0·011	0	0·14	4·44	9·7	2·20
	0·059	0	0·24	3·95	7·6	1·93
	0·099	0	0·32	3·50*	6·5	1·86
	0·011	0·095	0·24	3·68	7·7	2·10
	0·011	0·175	0·32	2·93	5·4	1·83
	0·011	0·255	0·40	1·57	3·3	2·09
2	0·012	0	0·17	3·57	9·5	2·66
	0·089	0	0·32	3·16†	8·3	2·64
	0·169	0	0·48	2·59	6·4	2·46
	0·249	0	0·64	1·88	4·2	2·22
	0·012	0·154	0·32	3·04	8·2	2·69
	0·012	0·314	0·48	2·55	6·6	2·58
	0·012	0·474	0·64	1·46	3·4	2·30

* $Q_{O_2} = 63$. † $Q_{O_2} = 64$.

Table 6. *Comparison of effect of phosphate with that of* KCl *and sucrose on the activity of the α-ketoglutaric oxidase system, and on accompanying phosphorylation*

(Saline-Versene rat-heart preparations. Exp. 1, isotonic reaction mixture; Exp. 2, hypotonic reaction mixture.)

Exp.	Osmolar concn.	Tonicity increased by addition of	O_2 uptake (μg.atoms)	ΔHMP (μmoles)	P:O
1	0·33	—	3·20†	5·29	1·65
	0·50	Phosphate	2·87	3·10	1·08
	0·50	KCl	2·62	3·40	1·30
	0·50	Sucrose	2·38	4·15	1·74
2	0·15	—	4·44	9·7	2·20
	0·32	Phosphate*	4·46	6·7	1·50
	0·32	KCl	3·50‡	6·5	1·86
	0·32	Sucrose	2·93	5·4	1·83
	0·45	Phosphate* + sucrose	3·48	6·5	1·87

* The same concentration of phosphate in both cases.
† $Q_{O_2}=64$.
‡ $Q_{O_2}=63$.

In these experiments the hypotonic reaction mixture, containing a low concentration of phosphate and no fluoride, was used. Other experiments employing the isotonic reaction mixture, containing fluoride, gave essentially the same results for the effect of hypertonicity. Sucrose-Versene preparations behaved in the same manner. In Fig. 3, average curves drawn through points obtained in a number of experiments are shown.

Table 6 describes the effect of using Sørensen phosphate buffer, instead of KCl or sucrose, to increase the tonicity.

The following conclusions may be drawn from Tables 5 and 6 and Fig. 3.

(1) The greatest activity of the α-ketoglutaric oxidase system is obtained under hypotonic conditions. As the tonicity of the medium is increased by the addition of KCl or sucrose, the activity markedly decreases, sucrose being somewhat more inhibitory than KCl. Phosphate, on the other hand, has relatively little effect on the activity. It seems likely that these effects can be explained on the basis of tonicity *per se*, since it has been found that phosphate readily penetrates into the sarcosomes (Cleland, 1952), and so is not osmotically active. The slightly greater inhibition by sucrose compared with KCl can also be explained on this basis since KCl slowly penetrates into the sarcosomes, and sucrose penetrates much more slowly (Cleland, 1952; cf. Berthet, Berthet, Appelmans & Duve, 1951).

(2) Sucrose and KCl, except in relatively high concentration, have little effect on the P:O ratio, despite the marked effect on the rate of oxygen uptake. Thus, it seems that tonicity does not have any effect on the oxidative phosphorylation, until highly hypertonic conditions are reached. In other experiments, not shown in Table 5, 0·6 M-sucrose added to an isotonic reaction mixture decreased the P:O ratio by 58 %, while 0·32 M-KCl decreased it by 50 % (see also Table 2 for the effect of 0·88 M-sucrose). Phosphate, although it has relatively little effect on the oxygen uptake is markedly inhibitory of the oxidative phosphorylation activity (Table 6). This is further examined below. The addition of sucrose, together with phosphate (last line of Exp. 2, Table 6), decreased the effect of phosphate.

Because of the relatively large number of components in the reaction mixture, it was not possible to test the activity of the α-ketoglutaric oxidase system in a reaction mixture of less than 0·14-osmolar concentration, i.e. 0·42 × isotonic. In view of the irreversible structural changes which occur below 0·05-osmolar, it is possible that an optimal tonicity exists below 0·14-osmolar, but there is no indication of this in Fig. 3. It is frequently stated in the literature that optimal activity of liver mitochondria is obtained under isotonic conditions, but a calculation of the tonicities of the solutions which gave optimal activity suggest that these are, in fact, considerably hypotonic. For example, Lehninger & Kennedy (1948) studied the effect of added salts and sucrose on the fatty acid oxidase activity of liver mitochondria, using a reaction mixture of 0·045-osmolar, calculated according to the method of this paper. The activity without added salts or sucrose was very low. Optimal activity was obtained with between 0·05 and 0·12 M-KCl, i.e. at a total osmolar concentration of 0·145–0·285. With sucrose, a sharper optimum was obtained with 0·08 M-sucrose, i.e. at a total osmolar concentration of 0·125. With 0·20 M-sucrose, when the total osmolar concentration would be 0·245, i.e. slightly hypotonic, the activity was only about 45 % of the optimum. Similarly, oxidative phosphorylation associated with the oxidation of β-hydroxybutyrate was just as active at an osmolar concentration of 0·13 as

at 0·20 M (Lehninger, 1949), but much impaired at 0·05-osmolar. There is, therefore, not necessarily any discrepancy between the present study with heart-muscle sarcosomes and the earlier work on liver mitochondria.

Reversibility of effect on tonicity

The effect of tonicity of the medium is almost completely reversible over a wide range of tonicity. This was shown by incubating the preparation (saline) for 15 min. at 25° under hypotonic, isotonic and hypertonic conditions before adding to an isotonic reaction mixture. Versene (0·005 M) was added during the incubation, in order to prevent inactivation of the α-ketoglutaric oxidase system (Slater & Cleland, 1952*b*). The sample incubated under isotonic conditions gave slightly higher activity than the control which was added to the reaction mixture without previous incubation (Table 7). The sample incubated under hypotonic conditions gave the same activity as the control, while that incubated under highly hypertonic conditions was only slightly inactivated. As is shown below, sarcosomes isolated with strongly hypertonic (0·88 M) sucrose were very inactive.

Table 7. *Reversibility of effect of tonicity of medium on activity of the α-ketoglutaric oxidase system*

(Saline rat-heart preparation diluted fourfold in side bulb of Warburg flask, incubated 15 min. at 25° before tipping into main flask. Final concentrations after tipping were those of isotonic reaction mixture (Table 1).)

Concentrations during incubation				Activity of α-ketoglutaric oxidase system
[Phosphate] (M)	[KCl] (M)	[Versene] (M)	Osmolar concn.	(Q_{O_2})
0·005	0·040	0·005	0·11	63
0·005	0·146	0·005	0·32	69
0·005	0·326	0·005	0·64	59
Unincubated control				63

Treatment of sarcosomes under even more highly hypotonic conditions for a short period had only a minor effect on the respiratory activity. In the experiment, described in Table 8, sarcosomes isolated with isotonic saline or sucrose containing Versene were suspended in 15 vol. water for about 10 min. at 0°, collected by centrifugation, which required a further 15 min., and finally resuspended in isotonic medium. Although the tonicity was very low, microscopic examination showed that the irreversible changes of structure discussed by Cleland & Slater (1953*b*) did not occur to any appreciable extent, probably because the treatment was not sufficiently prolonged. While diluted in the water, the sarcosomes were 'transformed', with formation of vesicles (see Cleland & Slater, 1953*b*), but the vesicles largely disappeared when the sarcosomes were placed in the reaction mixture used for measurement of the activity. The activities of these water-treated preparations were measured in comparison with samples of the same sarcosomes, which had been treated simultaneously in an identical manner, except for the dilution with water. Hypotonic conditions were used for measuring the activity of the α-ketoglutaric oxidase system. The saline-Versene preparation suffered some loss of phosphorylative activity with a slight loss of α-ketoglutaric oxidase activity, while the sucrose-Versene preparation actually increased in oxidase activity, with no change in the P:O ratio.

Table 8. *Effect of pre-treatment of sarcosomes with water on activity of α-ketoglutaric oxidase system and on accompanying phosphorylation*

(Saline-Versene and isotonic sucrose-Versene preparations from two pooled rat hearts. Water-treated preparations: sample of washed sarcosomes of each preparation suspended in 15 vol. water and recentrifuged before suspending in appropriate medium. Untreated preparations: another sample suspended immediately in appropriate medium. Activities measured in hypotonic reaction mixture.)

	Saline-Versene	Sucrose-Versene
Untreated		
Q_{O_2}	56	55
P:O	2·56	2·96
Water-treated		
Q_{O_2}	48	76
P:O	2·08	2·94

Effect of phosphate concentration

The effect of phosphate concentration is further examined in Table 9. Over a range of concentrations examined in this experiment, the activity of the oxidase system slightly increased, either in the presence or the absence of fluoride. In other experiments, it was found that increasing the concentration beyond 0·06 M caused some decrease of

Table 9. *Effect of phosphate concentration on the activity of the α-ketoglutaric oxidase system and on accompanying phosphorylation*

(Saline-Versene rat-heart preparation. For other concentrations, see Table 1. Q_{O_2} with isotonic reaction mixture (Table 1) was 62.)

[NaF] (M)	[Phosphate] (M)	O_2 uptake (μg.atoms)	ΔHMP (μmoles)	P:O
0·04	0·01	3·33	7·42	2·23
0·04	0·02	3·50	9·87	2·82
0·04	0·03	3·59	10·73	3·00
0·04	0·04	3·73	11·07	2·97
0·04	0·06	4·05	11·47	2·83
0	0·01	3·40	8·26	2·44
0	0·02	6·15	15·56	2·53
0	0·04	6·29	16·46	2·62
0	0·06	—	15·46	—

activity, i.e. there is a flat optimum of phosphate concentration in the region of 0·06 M. The P:O ratio is greatest at about 0·03 M-phosphate, but the effect of a variation between 0·02 and 0·06 M is not very great. The depressing effect of lower phosphate concentrations was greater in the presence than in the absence of fluoride. In the case of 0·01 M-phosphate without fluoride, the oxygen uptake ceased suddenly and almost completely 15–20 min. before the end of the experiment (duration 45 min.), when the residual phosphate concentration was (according to HMP analysis) only 0·0017 M. This is in agreement with Ochoa's (1944) finding that inorganic phosphate is necessary for the oxidation of α-ketoglutarate (see also Lardy & Wellman, 1952).

Comparison of sucrose with saline preparations

Hypertonic sucrose preparations. For reasons which are considered in the Discussion, it seemed likely that the greatest yield of oxidative phosphorylation would be obtained if the sarcosome preparations resembled as closely as possible the form in the intact cell. Hogeboom, Schneider & Pallade (1948) found with liver mitochondria that 0·88 M-sucrose was the best medium for this purpose. Accordingly, the properties of a preparation made in such a way were compared with a preparation isolated with phosphate-saline, using isotonic reaction mixture (Table 10). Since the concentration of sucrose introduced with the preparation into the reaction medium is considerable, the activities of the hypertonic sucrose preparation in Table 10 have been corrected to correspond to an isotonic reaction mixture, using factors obtained from Table 5.

Table 10. *Comparison of phosphate-saline and hypertonic sucrose preparations*

Activity	Preparation: Phosphate-saline	Preparation: Hypertonic sucrose
α-Ketoglutaric oxidase system		
Q_{O_2}	27	4·4
P:O	2·69	2·58
Succinic oxidase system		
Q_{O_2}	82	65
P:O	1·04	0·16
Cytochrome oxidase		
Q_{O_2}*	401	550

* Measured with a suboptimal concentration of cytochrome *c* ($4·8 \times 10^{-5}$ M).

The following conclusions may be drawn: (1) The activities of the succinic oxidase system and particularly of the α-ketoglutaric oxidase system of the sucrose preparation are lower than those of the phosphate-saline preparation. (2) The phosphorylation associated with the oxidation of succinate was also very low in the sucrose preparation, but that associated with the oxidation of α-ketoglutarate was normal. (3) The cytochrome oxidase activity of the sucrose preparation was, in contrast, somewhat higher than the normal preparation.

It is clear from these experiments that the inhibitory effects of high concentrations of sucrose are largely irreversible and that hypertonic sucrose is unsuitable for the isolation of sarcosomes with high respiratory activity. This is in agreement with the morphological findings (Cleland & Slater, 1953*b*). Since, however, lower concentrations of sucrose were without effect on the P:O ratio of saline preparations (Table 5), the use of isotonic sucrose (containing 0·005 M-Versene) was studied.

Isotonic sucrose preparations. Table 11 summarizes the results of some preparations made with either saline-Versene or isotonic sucrose-Versene, examined with both a hypotonic and an isotonic reaction mixture. In the first three experiments, different portions of the same heart were used for the different preparations. If these three experiments are considered alone, it would appear that sucrose gives a preparation with somewhat higher P:O ratio than saline. However, in a large number of experiments with different rat hearts, the mean P:O ratio of isotonic saline-Versene preparations did not differ significantly from that of isotonic sucrose-Versene preparations.

Table 11. *Activities of α-ketoglutaric oxidase system and yield of oxidative phosphorylation with saline-Versene and isotonic-sucrose preparations of rat heart*

Exp.	Reaction mixture	Saline-Versene prepn.		Isotonic sucrose-Versene prepn.	
		Q_{O_2}	P:O	Q_{O_2}	P:O
1	Hypotonic	93	2·66	—	—
	Isotonic	64	2·44	69	2·99
2	Isotonic	67	2·62	51	2·73
3	Hypotonic	56	2·56	55	2·96
4	Hypotonic	115	2·57	—	—
	Isotonic	62	3·00	—	—
5	Hypotonic	—	—	55	2·52
	Isotonic	—	—	44	2·54

It has been shown above that the sucrose preparations are more stable to extensive dilution with water than the saline preparations. The preparations also differ in the effect of omission of cytochrome *c* from the reaction mixture.

Effect of cytochrome c. The omission of cytochrome *c* from the reaction mixture depressed the activity of the α-ketoglutaric oxidase systems of the saline-Versene and sucrose-Versene preparations by 78 and 14 % respectively (Table 12). The activity of saline-

Versene preparations in the absence of cytochrome *c*, however, differed widely from preparation to preparation. One preparation (from cat heart) contained enough cytochrome *c* for 72% of the full activity.

Table 12. *Effect of omission of cytochrome* c *from reaction mixture on activity of α-ketoglutaric oxidase system and accompanying phosphorylation*

(Preparations from the same rat heart; isotonic reaction mixture.)

Preparation	Without added cyt. *c*		With added cyt. *c*	
	Q_{O_2}	P:O	Q_{O_2}	P:O
Saline-Versene	15	2·62	67	2·62
Isotonic sucrose-Versene	44	2·59	51	2·79

The requirement for cytochrome *c* by saline preparations is probably simply a reflexion of the ease with which cytochrome *c* is extracted from heart muscle by salt (Tsou, 1952). Maximum activity, which was not further increased by the addition of cytochrome *c*, was obtained if the supernatant obtained in the first high-speed centrifugation of the preparation of the sarcosomes (see Cleland & Slater, 1953*a*) was added to the sarcosomes (Table 13).

Table 13. *Activation of the α-ketoglutaric oxidase system of sarcosomes isolated with phosphate-saline by the soluble fraction of the homogenate*

(Pig preparation. No Versene or malonate used, otherwise 'isotonic reaction mixture' (Table 1).)

Addition	O_2 uptake (μl./hr.)
None	32
Cytochrome *c*	90
Supernatant	94
Supernatant (dialysed)	82
Supernatant + cytochrome *c*	92

The low activity of the α-ketoglutaric oxidase system of the saline preparation in the absence of cytochrome *c* was not accompanied by any change in the P:O ratio. The slight fall of the P:O ratio of the sucrose preparation by the omission of cytochrome (Table 12) is probably within the experimental error and was not confirmed by other experiments. In a number of experiments with saline preparations, the addition of cytochrome *c* either had no effect on the P:O ratio or increased it (e.g. see the table of Slater, 1950). A very high concentration of some samples of cytochrome *c* ($1·5–2·5 \times 10^{-4}$ M) causes some inhibition of the phosphorylation without affecting the oxygen uptake (Slater, 1950). Cytochrome *c*, purified by the method of Margoliash (1952), caused no inhibition even in a concentration of 6×10^{-4} M.

Effect of histidine

Bonner (1951) has shown that the addition of histidine greatly activates the succinic oxidase system of the Keilin & Hartree heart-muscle preparation. In the present study, it was found that histidine (0·09 M) had no effect on the oxygen uptake or on the P:O ratio of a saline-Versene sarcosome preparation with α-ketoglutarate as substrate either in the presence or in the absence of added cytochrome *c*.

DISCUSSION

Oxidative phosphorylation and organization

In previous papers from this laboratory (Keilin & Hartree, 1940, 1949; Slater, 1949*c*) it has been emphasized that the activity of complex oxidase systems, which consist of a number of components firmly bound to insoluble particles, depends not only upon the activity of each individual component but also upon the physical state of the particle itself, which determines the spatial interrelationship and mutual accessibilities of the components of the system. Since an additional complexity enters with the introduction of phosphorylation, one might expect that oxidative phosphorylation would be even more susceptible to changes of the physical state of the particle. With this possibility in mind, we set out to prepare granules resembling as nearly as possible those in the intact cell.

Use of sucrose for the isolation of mitochondria and sarcosomes. Hogeboom *et al.* (1948) claim that strongly hypertonic (0·88 M) sucrose was required for the isolation of liver mitochondria with the morphological characteristics of mitochondria in the cell. In particular, this was the only medium which gave the rod-like form of mitochondria which they considered was characteristic of those in the intact cell. Accordingly, this hypertonic medium was used for isolating heart-muscle sarcosomes. Although such preparations contained a considerable proportion of rod-like forms (Cleland & Slater, 1953*b*), their catalytic activity was very low. Further microscopic studies led to the conclusion that undamaged sarcosomes should become spherical when in free suspension and that the isolation of a sarcosome as a rod indicated that it had been altered. 0·88 M-Sucrose added to the reaction medium was found to be strongly inhibitory of the respiratory enzymes, even in the less highly organized Keilin & Hartree heart-muscle preparation.

The suitability of 0·88 M-sucrose for the isolation of liver mitochondria led Hogeboom *et al.* (1948) to suggest that the osmotic pressure within the living hepatic cell at the mitochondrial membrane might be higher than the blood osmotic pressure. (This has again been suggested by Lehninger, 1951*a* and Harman & Feigelson, 1952*a*.) Later work from this

group has, however, indicated that mitochondria suspended in hypertonic sucrose possessed low catalytic activities, for example Schneider (1948) found that such preparations did not oxidize octanoic acid. In agreement with this, Harman (1950) found that liver mitochondria isolated with 0·50 M-sucrose were less active than those isolated with 0·25 M-sucrose or 0·12 M-KCl (see also Harman & Feigelson, 1952*b*). Kennedy & Lehninger (1949) were, however, able to obtain mitochondria which actively oxidized fatty acids by the use of 0·88 M-sucrose, but the final preparation was suspended in isotonic saline. The usual procedure now followed in the American laboratories is to isolate with isotonic sucrose but to suspend in isotonic saline (e.g. Potter, Lyle & Schneider, 1951; Green, 1951). Thus, the conception of the special advantage of hypertonic sucrose appears to have been dropped and our findings with heart-muscle sarcosomes are in agreement with this. Moreover, it is apparent that it is no longer considered that even isotonic sucrose has any special advantages compared with saline for preserving the integrity and activity of liver mitochondria. Lehninger (1951*b*) has explained that the purpose of using sucrose as the isolation medium is to prevent the agglutination of these granules which occurs in the presence of salts and makes difficult the separation of the mitochondria from nuclei and intact cells. In the case of heart-muscle sarcosomes, sucrose would not serve such a purpose, since there is no agglutination of intact sarcosomes even in the presence of salt. There is, in fact, no significant difference in respiratory activity or in phosphorylative ability between sarcosomes isolated with isotonic sucrose or saline. Those isolated with sucrose are considerably more active in the absence of cytochrome *c*, probably because salt extracts cytochrome *c* from intact sarcosomes as well as from water-washed heart-muscle mince (Tsou, 1952). Sarcosomes prepared with sucrose also withstand treatment with water better than saline preparations and are morphologically more stable (Knocke, 1909; Cleland & Slater, 1953*b*).

Effect of tonicity of reaction medium. An unexpected finding was that the α-ketoglutaric oxidase system was more active under strongly hypotonic conditions than under isotonic. Since phosphate, which largely penetrates the sarcosomes (Cleland, 1952), contributed about 30% of the osmolar concentration of the hypotonic reaction mixture, the mixture is in effect considerably more hypotonic than it appears. Our findings are in agreement with those of Harman & Feigelson (1952*b*), published since the completion of our experiments. It has been shown above that the published data of Lehninger and his co-workers indicate that liver mitochondria are also more active under hypotonic conditions. It has been suggested in the previous paper (Cleland & Slater, 1953*a*) that many of the enzymes concerned in hydrogen transport are situated in the membrane of the sarcosome. When the sarcosome swells in hypotonic solution, this membrane will be stretched and it is conceivable that this stretching might increase the mutual accessibility of the components of the hydrogen-transferring system. The fact that the P:O ratio is not affected suggests not only that the phosphorylating enzymes are in excess, but that their accessibility to the hydrogen-transferring system is not a limiting factor in the overall oxidative phosphorylation reaction.

This effect of tonicity on the activity of the sarcosomes was reversible to a considerable degree (see Table 7). Even treatment of a sucrose preparation with water for 25 min. at 0° caused no inactivation. Harman & Feigelson (1952*b*) have recently found that treatment of heart-muscle mitochondria with water caused inactivation, but their treatment was probably more prolonged than ours. It seems probable that the heart-muscle sarcosomes are more stable than liver mitochondria to this treatment, since Lehninger (1951*c*) has found that treatment of the latter preparations with water for only 5 min. at 0° cause impaired phosphorylation associated with the oxidation of β-hydroxybutyrate. It should be noted that all the present experiments were made after treatment with Versene which prevents the morphological alterations and enzymic inactivation which rapidly occurs under isotonic conditions at room temperature with preparations not containing Versene (Slater & Cleland, 1952*b*; Cleland, 1952), but does not prevent the swelling which occurs under hypotonic conditions.

Relative sensitivity of oxidase system and phosphorylation. Contrary to the expectations expressed in the first paragraph of this Discussion, it was found that the P:O ratio was relatively less susceptible to changes in physical state of the particles than was the oxidative capacity. For example, over a range of tonicities which caused a threefold variation in the activity of the α-ketoglutaric oxidase system (Exp. 1, Table 5), the P:O ratio was unchanged. Similarly, variation of pH markedly affected the oxidative activity but not the P:O ratio. Other examples of large effects on the oxidase activity, accompanied by little change of the P:O ratio, are found in the comparison of phosphate-saline with hypertonic sucrose as isolation medium (Table 10) and the effect of omission of cytochrome *c* from the reaction mixture (Table 12). Another paper (Slater & Cleland, 1953) shows that incubation of the sarcosomes in the absence of the reaction mixture or Versene causes large inactivation of the α-ketoglutaric oxidase system with only minor changes in the P:O ratio.

These examples indicate that the phosphorylative enzymes are normally in excess of purely

oxidative enzymes. This conclusion is of importance for the biochemical investigations of oxidative phosphorylation, since it suggests that measurements of the yield of oxidative phosphorylation on isolated tissue preparations will not give low values, due to a deficiency of phosphorylating enzymes, in comparison with the yields occurring within the intact cell. Although the population of sarcosomes in any one preparation is probably not uniform, the differences will probably lie in the activity of the α-ketoglutaric oxidase system rather than the P:O ratio. This makes α-ketoglutarate particularly suitable as substrate for measurements of the yield of oxidative phosphorylation. Succinate is less suitable for this purpose since treatments which cause loss of phosphorylative ability do not affect the relatively more stable succinic oxidase system (Cross, Taggart, Covo & Green, 1949; Slater & Cleland, 1953).

SUMMARY

1. The α-ketoglutaric oxidase system of heart-muscle sarcosomes has a pH optimum at 7·4. The yield of oxidative phosphorylation (P:O ratio) is unchanged between pH 6·2 and 7·7.

2. Hypertonic sucrose (0·88 M) is an inhibitor of the succinic oxidase system in the Keilin & Hartree preparation. Its major effect appears to be on the accessibility of both the endogenous and added cytochrome *c* to the other components of the system.

3. Maximum activity of the α-ketoglutaric oxidase system of heart-muscle sarcosomes is obtained under the most highly hypotonic conditions studied, equivalent to about one-third isotonic. Under these conditions, sarcosomes are swollen, but shrink again when placed in isotonic medium. The effect of tonicity on the activity of the α-ketoglutaric oxidase system is also reversible.

4. As the tonicity is increased by saline, sucrose or phosphate, the activity of the α-ketoglutaric oxidase system decreases.

5. The P:O ratio is not affected over a wide range of sucrose concentrations which have a marked effect on the activity of the α-ketoglutaric oxidase system. This and other examples where the oxidase system is more sensitive than the P:O ratio to variations of the conditions indicates that the phosphorylative enzymes are normally in excess of the purely oxidative enzymes and increases the likelihood that measurements of the yield of oxidative phosphorylation on isolated tissue preparations represent the state of affairs in the cell.

6. Phosphate, in high concentration, decreases the P:O ratio; the optimal concentration is 0·03 M.

7. Hypertonic sucrose is unsuitable for the isolation of sarcosomes. There is, however, no significant difference between sarcosomes isolated with isotonic sucrose and isotonic saline, except that the latter are deficient in cytochrome *c*.

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Chemical Action of X-Rays on Nucleic Acids and Related Substances in Aqueous Systems

1. DEGRADATION OF NUCLEIC ACIDS, PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES BY X-RAYS AND BY FREE RADICALS PRODUCED CHEMICALLY

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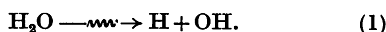
It is now generally considered that the chemical action of ionizing radiations on aqueous solutions is indirect and involves the primary formation of free radicals which are produced from the water according to the net process (Weiss, 1944, 1947*a*, *b*; Stein & Weiss, 1949):

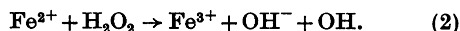


$$H_2O \rightsquigarrow H + OH. \quad (1)$$

There are also some definite indications that the formation of these radicals *in vivo* may be responsible for some of the biological effects of these radiations (cf. Barron, Dickman, Muntz & Singer, 1949; Barron & Dickman, 1949). As evidence has accumulated to emphasize the important role which the nucleic acids play in growth processes it appeared of interest to study the chemical action of X-radiation on these substances in aqueous systems.

Several investigators have dealt with the action of X-rays on solutions of deoxyribonucleic acids (DNA) and have observed physical effects such as a loss of viscosity and of streaming birefringence (Wegmüller, 1942; Sparrow & Rosenfeld, 1946; Taylor, Greenstein & Hollaender, 1948). The results obtained indicated that the effect of the radiation was to break the long asymmetric molecules into shorter fragments.

We have previously reported our preliminary observations on the chemical effects of X-rays on aqueous solutions of both DNA and RNA (ribonucleic acid) (Scholes, Stein & Weiss, 1949; Scholes & Weiss, 1950) and have interpreted the mechanism of this action as an indirect one. This we confirmed by showing that chemically produced OH radicals effected similar changes in the nucleate complex. The OH radicals were generated in solution by the hydrogen peroxide-ferrous salt (Fenton's) reagent, where they are formed according to the following reaction (Haber & Weiss, 1934):



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH. \quad (2)$$

During the preparation of this paper several publications bearing on the work have appeared. Butler (1949), by irradiation of DNA with X- and γ-rays and measurements of the viscosity changes in the presence of protective agents, has also concluded that the mechanism of the attack is of an indirect nature. Further evidence is the observed depolymerization of DNA with chemically produced free radicals (Butler & Smith, 1950; Smith & Butler, 1951; Limperos & Moscher, 1950), and the inability of X-rays to depolymerize the nucleate in the dry state, in ethylene glycol and in a frozen aqueous solution (Limperos & Moscher, 1950).

Some investigations have also been made on the influence of oxygen in the irradiation of DNA solutions (Butler & Conway, 1950) and on the molecular sizes, as determined by sedimentation and diffusion-constant techniques, of the degraded nucleate fragments (Conway, Gilbert & Butler, 1950). These studies have shown that after irradiation, the mean molecular weight of DNA is considerably lowered and that the material becomes much more inhomogeneous. Although molecular oxygen does not appear to have any influence on the immediate effects of the irradiation these authors have shown that in the presence of oxygen the DNA suffers a further progressive fall in anomalous