Production of Thiol Groups and Retention of Calcium Ions by Cardiac Mitochondria

Eric J. HARRIS* and Harold BAUM†

**Department of Biophysics, University College London, Gower Street, London WC1E 6BT, U.K., and*

†*Department of Biochemistry, Chelsea College, London SW3 6LX, U.K.*

(Received 26 July 1979)

Efflux of Ca^{2+} from previously Ca^{2+} -loaded heart mitochondria was measured after inhibiting respiratory activity. The efflux was increased by *p*-chloromercuribenzoate, methylmercuric chloride, Cu^{2+} , Fe^{2+} , 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (uncoupler), 1,1,1-trifluoro-3-(2-thienylacetone and indomethacin; after such increase it could be diminished by dithiothreitol. The induced loss of the Ca^{2+} was accompanied by a loss of endogenous adenine nucleotide. Methylmercuric chloride was particularly effective, since it was active at ratios of about 1 nmol/mg of mitochondrial protein. The non-respiring mitochondria were found to regenerate bound thiol groups after their original complement had reacted with thiol-blocking reagent. This regeneration was diminished by the Ca^{2+} -efflux-stimulating agents that were not themselves thiol-blocking reagents, such as thyroxine, uncoupler, trifluorothienylacetone and indomethacin. The external exposure of thiol groups was also diminished by thyroxine, uncoupler and trifluorothienylacetone. The results support the proposal made previously that the membrane is maintained in a state of low permeability by adenine nucleotide and Mg^{2+} being bound to thiol-dependent sites.

Recent work has shown that the leakage of preloaded Ca^{2+} from heart mitochondria can be modified by a number of agents: adenine nucleotides diminish efflux (Harris, 1979*a*), whereas mersalyl and other thiol-blocking reagents and thyroid hormones increase it (Harris *et al.*, 1979). The effects of the latter agents could be prevented or reversed either by adding adenine nucleotide (with Mg^{2+} ions if efflux was high) or by adding a source of thiol groups. The stimulated loss of Ca^{2+} was associated with a net loss of adenine nucleotide, and both losses were inhibited by bongkreikic acid (Harris *et al.*, 1979). This set of observations led to the proposal that thiol groups were involved in keeping ADP and Mg^{2+} ions bound to the membrane, in which condition permeability was low. The finding that low Ca^{2+} efflux is favoured by a reduced state of the nicotinamide nucleotides obtained alternatively by favourable substrate (Lehninger *et al.*, 1978) or by respiratory inhibition (Harris, 1979*b*; Harris *et al.*, 1979) suggested that there is a passage of reducing equivalents from NADH to generate NADPH and so feed substrate to glutathione reductase to provide a source of new thiol groups. Respiratory inhibition is effective in this regard only if there is endogenous

ATP to provide energy for the transhydrogenase reaction.

We have now investigated and describe below the action on mitochondrial Ca^{2+} retention of some further substances, and have also tested their effects on the generation or exposure of groups that react with thiol-blocking reagents. This process presumably depends on a continuous reduction of disulphide groups, but may also involve a conformational change, as occurs, for example, in phosphofructokinase (Holloway *et al.*, 1978). A potentially complicating feature is that induced increases of membrane permeability might permit access to thiol groups in the internal compartment by hitherto impermeable thiol-blocking reagents.

Methods

Rat heart mitochondria were prepared by a modification of the previous procedure (Harris & Zaba, 1977; Harris *et al.*, 1979), which was introduced after a suggestion made by Vercesi *et al.* (1978). The minced tissue obtained from the Climex mincer was digested with 1 mg each of trypsin and collagenase per g of tissue in 5 ml of medium at 0°C for 15 min.

This medium contained 75 mM-KCl, 150 mM-mannitol, 10 mM-Tris/HCl buffer, pH 7.2, and 1 mM-Tris/EGTA. The digest was filtered through a nylon gauze (0.2 mm) and the tissue was rinsed with 10 ml of medium. The tissue was then lightly homogenized in a medium containing 300 mM-mannitol, 10 mM-Tris/HCl buffer, pH 7.2, 5 mM-KCl, 1 mM-Tris/EGTA and 0.1% serum albumin. About 20 ml of medium was used per heart homogenized. The homogenate was put in tubes in the centrifuge rotor (radius 10 cm) and the machine was run up to 1000 *g* and then switched off and allowed to coast down. This treatment brought down the cellular debris; the supernatant was re-centrifuged at 9000 *g* for 4 min to sediment the mitochondria. The pellets were resuspended and combined in about 30 ml of the same medium, and again sedimented. For the next wash the resuspension was in a similar medium without EGTA. The pellet from this centrifugation was swirled lightly with 300 mM-mannitol in 10 mM-Tris/HCl buffer, pH 7.2, to remove fluffy material, which was discarded, and the residual heavy material was resuspended in a portion of similar medium at about 30 mg of protein/ml. The protein was measured by a biuret reaction (Layne, 1957) standardized with serum albumin.

The uptake of Ca^{2+} (to introduce the required Ca^{2+} loading) and the subsequent release of Ca^{2+} after the addition of Ruthenium Red (0.4 μM) to inhibit active uptake were carried out as described previously by using the metallochrome indicator Arsenazo III (Harris, 1977, 1979a). The changes of absorbance between 685 and 665 nm were recorded on an Aminco D.W.2 spectrophotometer. In most experiments the Ca^{2+} load was applied while the mitochondria were utilizing pyruvate + malate as substrates; a respiratory inhibitor was added along with the Ruthenium Red after the loading was complete. The reasons for inhibiting respiration were that the efflux is thereby diminished and subsequent effects of agents being tested cannot then be ascribed to respiratory effects.

A series of measurements of Ca^{2+} efflux were made with mitochondria that had been treated during preparation with ^{14}C -labelled ADP as described previously (Harris *et al.*, 1979). In these experiments the volume of suspension in the spectrophotometer cuvette was increased to 3.5 ml so that 3×0.5 ml samples could be withdrawn during the course of Ca^{2+} discharge. The samples were separated by centrifugation for 1 min in a Coleman Microfuge (Harris & van Dam, 1968) so that the relative amounts of ^{14}C -labelled nucleotide associated with the pelleted protein could be measured. The pellets were washed superficially with ice-cold mannitol solution and then dissolved in a solution of urea (30%, w/v) in dilute ammonia [aq. ammonia (sp.gr. 0.880)/water, 1:19, v/v]. The solubilized protein

was transferred to counting vials containing Unisolve 1 (Koch-Light Laboratories).

To investigate the effects of agents on the production of reactive thiol groups two methods were employed. Before application of either method the mitochondria were treated to remove the existing reactive thiol groups, as suggested by the work of Pedersen & Coty (1975). For each 20 mg of mitochondrial protein in suspension in about 10 ml of the EGTA wash medium, at the first washing stage of the preparation, additions of 1 μmol of mersalyl and then 2 μmol of *N*-ethylmaleimide were made (omission of the latter reagent left more free thiol groups exposed); after 5 min at 0°C the excess reagents were removed by centrifugal separation, and the pellet was resuspended in the EGTA-free wash medium (30 ml) and re-separated; the pellet was finally resuspended in the buffered 300 mM-mannitol at about 20 mg of protein/ml. The treated mitochondria would still take up a limited quantity of Ca^{2+} (approx. 40 nmol/mg of protein) when energized with succinate; the rate of uptake and the maximum quantity were increased on adding dithiothreitol and phosphate to provide an anion made capable of penetrating as a result of the thiol compound removing the bound mercurial. It is relevant to add that an addition of ATP (10 μM) to a suspension of the treated mitochondria in the presence of Ca^{2+} and succinate would increase the rate of Ca^{2+} uptake. This is probably attributable to formation of internal phosphate from the ATP as it enters, since oligomycin removed the ATP effect. Addition of ATP at this concentration to untreated mitochondria supplemented with phosphate and succinate had no perceptible effect on the rate of Ca^{2+} uptake.

The treated mitochondria were used in incubations (at about 1 mg of protein/ml) with either ^{14}C -labelled *N*-ethylmaleimide at 12 μM (8.4 $\mu\text{Ci}/\mu\text{mol}$) or with dithiobis-(4-nitrobenzoic acid) at 100 μM . From the incubations with the ^{14}C -labelled *N*-ethylmaleimide samples of protein were separated in the bench centrifuge at intervals so that the attached radioactivity could be assayed. The course of the reduction of dithiobis-(4-nitrobenzoic acid) to thiolnitrobenzoic acid was followed spectrophotometrically using the absorbance difference between 412 and 520 nm in the dual-wavelength spectrophotometer.

Experiments on Ca^{2+} efflux and thiol-group reaction were carried out at 20°C in a medium containing 120 mM-KCl, 20 mM-Tris/HCl buffer, pH 7.2, 3% poly(ethylene glycol) (mol. wt. 8000) and other additions specified in the text.

The KCl was recrystallized from a solution in 1 mM-EDTA. The Arsenazo III was purified as described previously (Harris, 1979a).

Results

The release of Ca^{2+} in response to mersalyl and

N-ethylmaleimide has been described already (Harris *et al.*, 1979; Lofrumento & Zanotti, 1978). Other metal or organometal ions that react with thiol groups have similar effects; the most striking were obtained with methylmercuric chloride and with Cu^{2+} . The Ca^{2+} efflux from non-respiring mitochondria was increased by $1\text{ }\mu\text{M}$ -methylmercuric chloride (2.5 nmol/mg) or by $0.5\text{ }\mu\text{M}$ - CuSO_4 (Fig. 1). Titration of the agents showed (1) that at these low doses the effects were transient, as if protective thiol groups were being titrated, and (2) that the effect of Cu^{2+} , unlike that of the mercurial, seemed to be limited, as if access to the important groups were restricted. The increased efflux in response to methylmercuric chloride could be diminished again by dithiothreitol. Phenylmercuric acetate behaved like methylmercuric chloride by giving transient responses to low quantities added, but was less effective (not illustrated). *p*-Chloromercuribenzoate also stimulated the efflux of Ca^{2+} , and the effect could be reversed by adding dithiothreitol in excess (Fig. 2). Since more of this agent is required in order to elicit the response, the response is not transient, and is presumably limited by slow access instead of by actual removal by chemical combination.

Addition of FeSO_4 (made up in deoxygenated water) had effects resembling those of CuSO_4 at low micromolar concentrations, and the efflux could be diminished by either dithiothreitol or by ascorbate, which presumably removes the Fe^{2+} by chelation (Fig. 3).

Another compound that was found to behave like those agents that remove thiol groups is 2,5-dibromothymoquinone; Fig. 4 (curve *B*) records its action in releasing Ca^{2+} . This substance has been used in photosynthetic studies to inhibit plastoquinone reduction (Sadewasser & Dilley, 1978) and is noted by Flohé *et al.* (1978) to inhibit mitochondrial electron flow at the level of the cytochrome *b*-ubiquinone pool. A reaction between thiol groups and quinones is general (Joceyln, 1972). As with the more potent agents already mentioned, if the dibromothymoquinone was added at low concentration its effect on Ca^{2+} efflux was transient, in agreement with our finding (described below) that it removes thiol groups. The action of a conventional uncoupler on Ca^{2+} retention is illustrated in Fig. 4 (curve *A*) for comparison.

The compound trifluorothienylacetone is known to be an inhibitor of succinate dehydrogenase

Fig. 1. Stimulation of Ca^{2+} efflux from Ca^{2+} -loaded mitochondria by Cu^{2+} and by methylmercuric chloride

The mitochondria, while respiring, were loaded with 60 nmol of Ca^{2+} /mg of protein. Respiration was then inhibited with rotenone (Rot) at $1\text{ }\mu\text{g/ml}$ and active Ca^{2+} uptake was inhibited with Ruthenium Red (RR) at $0.4\text{ }\mu\text{M}$. When the efflux had declined to a steady low value, the agents were added, Cu^{2+} as CuSO_4 at 0.5 , 1.0 , 1.5 , 2.5 and $4\text{ }\mu\text{M}$ and methylmercuric chloride at 1 , 2 and $3\text{ }\mu\text{M}$. The numbers on the respective curves are the Ca^{2+} efflux in nmol/min per mg of protein at the steepest part of each response. The transient nature of the responses and the limited effect of the Cu^{2+} are discussed in the text. The protein concentration was 0.4 mg/ml . Ca^{2+} movement was followed spectrophotometrically by recording the absorbance changes between 685 and 665 nm from a suspension containing Arsenazo III indicator (see the Methods section). The sensitivity to Ca^{2+} was calibrated by making an addition of CaCl_2 .

Fig. 2. Stimulation of Ca^{2+} efflux from Ca^{2+} -loaded mitochondria by *p*-chloromercuribenzoate

The mitochondria were first loaded with 40 nmol of Ca^{2+} /mg of protein, and rotenone (Rot) and Ruthenium Red (RR) were then added as in Fig. 1. To one experiment two successive additions of the mercurial (PCMB) were made to 10 and 20 μM , and finally the stimulated efflux was diminished by adding dithiothreitol (DTT) to 100 μM . The protein concentration was 0.48 mg/ml. The other details were as described for Fig. 1.

Fig. 3. Stimulation of Ca^{2+} efflux from Ca^{2+} -loaded mitochondria by Fe^{2+}

The mitochondria were first loaded with 40 nmol of Ca^{2+} /mg of protein, and then antimycin (Am) at 1 $\mu\text{g}/\text{ml}$ and Ruthenium Red (RR) were added to inhibit respiration and active Ca^{2+} uptake. In the two experiments additions of 10 μM - (curve A) or 4 μM - FeSO_4 (curve B) were made. The efflux of Ca^{2+} was inhibited either by dithiothreitol (DTT) at 100 μM or by ascorbate (Asc) at 300 μM . The protein concentration was 0.4 mg/ml. The other details were as described for Fig. 1.

(Tappel, 1960) and was tested in view of the sensitivity of the Ca^{2+} efflux to the redox condition of the respiratory chain. As shown by curve A in Fig. 5, the compound stimulates Ca^{2+} efflux and its effect is lessened by dithiothreitol. The possibility that this effect might implicate an iron-sulphur centre in the maintenance of membrane thiol groups has already been discussed (Baum & Harris, 1979).

As an example of the anti-inflammatory compounds found by Al-Shaikhaly & Baum (1979) to cause swelling of non-energized mitochondria in the presence of Ca^{2+} , the effect of indomethacin was tested, and this is illustrated (Fig. 5, curve B).

Loss of adenine nucleotide accompanying loss of Ca^{2+}

Experiments made on mitochondria that had first been exposed to [^{14}C]ADP to label their endogenous adenine nucleotides (see the Methods section, and also Harris *et al.*, 1979) provided information on the proportion of internal nucleotide that was lost with the stimulated efflux of Ca^{2+} . Table 1 shows that the three agents tested in this way all gave rise to loss of adenine nucleotide; this is analogous to the effects of mersalyl and thyroxine (Harris *et al.*, 1979).

Effects of the agents on thiol-group production or efflux

Fig. 6 illustrates some results obtained by the use of [^{14}C]labelled *N*-ethylmaleimide applied to mitochondria previously treated with mersalyl and unlabelled *N*-ethylmaleimide (with the mersalyl added

Fig. 4. Stimulation of Ca^{2+} efflux from Ca^{2+} -loaded (37 nmol/mg of protein) mitochondria by tetrachlorotrifluoromethylbenzimidazole (TTFB) at $1.2 \mu\text{M}$ and the diminution of the stimulated efflux in response to dithiothreitol (DTT) at $100 \mu\text{M}$ and Mg^{2+} ions (at $20 \mu\text{M}$) (curve A), and stimulation of Ca^{2+} efflux from Ca^{2+} -loaded (37 nmol/mg of protein) mitochondria by dibromothymoquinone (DBTQ) added successively at 10 and $20 \mu\text{M}$ (curve B)

For curve A, the respiration was inhibited after giving the Ca^{2+} load with rotenone (Rot) at $1 \mu\text{g/ml}$ and Ruthenium Red (RR) was added at $0.4 \mu\text{M}$. For curve B, after Ca^{2+} loading the respiration had been inhibited with antimycin (Am) at $1 \mu\text{g/ml}$ and Ruthenium Red (RR) was added at $0.4 \mu\text{M}$. For both experiments the protein was 0.47 mg/ml . The other details were as described for Fig. 1.

first). The pellets were washed superficially with ice-cold mannitol solution and solubilized in 30% urea for counting of radioactivity in Unisolve 1. It is only the attachment of the reagent to the protein that is measured, and production of soluble thiols to react with the reagent would not affect the result. In these and similar experiments the rates of incorporation of labelled *N*-ethylmaleimide into the protein fraction in the presence of certain agents were compared with the control rate to give the percentages collected in Table 2.

If the preparation of mitochondria was exposed only to unlabelled *N*-ethylmaleimide before the washing and addition of the labelled compound, the initial uptake and the subsequent rate of uptake of the ^{14}C -labelled compound were lessened. Con-

versely, if the preparation was treated only with mersalyl (at $1 \mu\text{mol}/20 \text{ mg}$ of protein) both the initial uptake and the rate of uptake of ^{14}C -labelled *N*-ethylmaleimide were increased. It was confirmed, in agreement with the results in Table 2 of Coty & Pedersen (1975), that exposure to dithiothreitol of the mersalyl- and *N*-ethylmaleimide-treated protein increased the groups available to bind labelled *N*-ethylmaleimide by 0.9–1 nmol/mg of protein.

The alternative method for following the generation of available thiol groups depended on spectroscopic monitoring of the thiolnitrobenzoic acid produced by exchange between externally exposed thiol groups and dithiobis-(4-nitrobenzoic acid). Here all available thiol groups emerging from the mitochondria, whether soluble or externally bound, will be

Fig. 5. Stimulation of Ca^{2+} efflux from Ca^{2+} -loaded (40 nmol/mg of protein) mitochondria by trifluorothienylacetone (TTA) added at 10 and $20 \mu\text{M}$ or by indomethacin (IM) added at 40 and $80 \mu\text{M}$

The respiration was inhibited after giving the Ca^{2+} load with rotenone (Rot) at $1 \mu\text{g/ml}$ and Ruthenium Red (RR) was added at $0.4 \mu\text{M}$. The efflux after stimulation by the test agents was subsequently diminished by adding dithiothreitol (DTT) at $100 \mu\text{M}$. The protein was 0.4 mg/ml and other details were as described for Fig. 1.

Table 1. Adenine nucleotide losses accompanying stimulated efflux of Ca^{2+} from previously Ca^{2+} -loaded mitochondria. The endogenous adenine nucleotide had been labelled by exposing the mitochondria to $[^{14}\text{C}]\text{ADP}$ during their preparation. They were loaded with 25 nmol of Ca^{2+}/mg of protein before inhibition of respiration with rotenone ($1 \mu\text{g/ml}$) and inhibition of active Ca^{2+} uptake with Ruthenium Red at $0.2 \mu\text{M}$.

Agent added to stimulate Ca^{2+} efflux	Rate of induced Ca^{2+} efflux (nmol/min per mg)	Approximate quantity of Ca^{2+} lost at time of sampling (nmol/mg)	% of internal nucleotide lost
<i>p</i> -Chloromercuribenzoate			
29 μM	9.8	6.9	69
40 μM	5.0*	6.7	68
Dibromothymoquinone			
16 μM	3.8	8.2	81
10 μM	2.0*	11.0	64
Methylmercuric chloride			
3 μM	3.5	6.1	54
3 μM	1.1*	7.6	56

* Experiments made with a different preparation from that used for the others.

taken into account. A test for thiol groups in the supernatant failed to show any time-dependent increment. As before, most of the available thiol groups initially present were removed by the mersalyl-plus-*N*-ethylmaleimide treatment before the experiment

was started. A difficulty is that the rate of exposure of thiol groups gradually diminished over the 10–15 min observation time, so that minor decelerating effects are difficult to distinguish (curve A in Fig. 7). The inhibitory effect (compared with the control)

Fig. 6. Time course of reaction of N -ethylmaleimide with the proteins of intact mitochondria

The mitochondria had first been treated with $1\mu\text{mol}$ of mersalyl and $2\mu\text{mol}$ of N -ethylmaleimide per 20mg of protein. After a washing and resuspension they were incubated with ^{14}C -labelled N -ethylmaleimide at $12\mu\text{M}$ at 20°C in the presence of $1\mu\text{g}$ of rotenone/ml. The attachment of labelled material to the protein was followed by separating samples by centrifugation for 1min in a Coleman Microfuge. The pellet was washed superficially with cold saline, and solubilized and assayed for ^{14}C radioactivity (see the Methods section). (a) Curve A is a control without further additions, curve B was obtained with $125\mu\text{M}$ -trifluorothienylacetone added after the first sample and curve C relates to an incubation with addition of uncoupler (tetrachlorotrifluoromethylbenzimidazole) at $5\mu\text{M}$ after the first sample. The protein concentration was 0.6mg/ml . (b) Curve D is the control without additions, curve E was obtained with thyroxine present throughout the experiment at $8\mu\text{M}$ and curve F with indomethacin present throughout at $67\mu\text{M}$. The protein concentration was 0.8mg/ml .

Fig. 7. Records of exposure of thiol groups as measured by reduction of dithiobis-(4-nitrobenzoic acid) at $100\mu\text{M}$ from mitochondria incubated with the reagent and with various additions

The left-hand portions of the records show the gradual reduction of the reagent before any further additions, and the corresponding rate of production of thiol groups in nmol/min per mg of protein is marked on the line. The additions made in curves B–F were: curve B, trifluorothienylacetone (TTA) at $100\mu\text{M}$; curve C, bongkreikic acid (BKA) at $80\mu\text{M}$; curve D, ADP at $40\mu\text{M}$ and then at $80\mu\text{M}$; curve E, thyroxine (T_4) at $50\mu\text{M}$; curve F, uncoupler (tetrachlorotrifluoromethylbenzimidazole; TTFB) at $5\mu\text{M}$. The protein concentration was 0.4mg/ml , and rotenone was present throughout at $1\mu\text{g/ml}$.

hydrogenase and remove the ATP needed for the transhydrogenase, so that they inhibit the production of NADPH, which is a substrate for glutathione reductase. It was not possible to obtain a record after dibromothymoquinone addition because this compound reacted directly with the thiol-nitrobenzoic acid. Indomethacin at $160\mu\text{M}$ had no perceptible effect on exposure of thiol groups. Although not illustrated, it is notable that dibucaine at $300\mu\text{M}$ doubled the rate of exposure of thiol groups, and butacaine at $800\mu\text{M}$ had a similar effect.

Discussion

We have proposed (Harris *et al.*, 1979) that the maintenance of the mitochondrial membrane in a condition of low permeability is associated with the binding of ADP and Mg^{2+} , and that this, in turn, requires the presence of thiol groups. It follows that any reagent that removes thiol groups, such as mercurials, metal ions such as Cu^{2+} and Fe^{2+} and, it now appears, dibromothymoquinone, will cause dissociation of nucleotide and give rise to unspecific permeability. The action of the mercurials and metal ions has previously been found to include an induc-

produced by trifluorothienylacetone is shown in curve B in Fig. 7. Compounds such as ADP and bongkreikic acid, which we have shown diminish the rate of Ca^{2+} leakage and which induce a contraction of the structure (Stoner & Sirak, 1973a,b), also lessen the rate of exposure of thiol groups (curves C and D in Fig. 7). In this condition presumably a higher internal thiol concentration will prevail and will favour preservation of membrane thiol groups and ADP binding. Both thyroxine and tetrachlorotrifluoromethylbenzimidazole (uncoupler) inhibit exposure of thiol groups (curves E and F in Fig. 7); these compounds respectively inhibit the trans-

Table 2. Rates of combination of ^{14}C -labelled *N*-ethylmaleimide (at $12\text{ }\mu\text{M}$) with the protein of mitochondria that had first been exposed to mersalyl and unlabelled *N*-ethylmaleimide (see the Methods section) compared with and without added compounds

The protein was used at about 1 mg/ml . The control rates varied between preparations from 0.07 to $0.10\text{ nmol of thiol/min per mg}$. All tests were made with rotenone present at $1\text{ }\mu\text{g/ml}$.

Agent added	Concn. (μM)	Rate of <i>N</i> -ethylmaleimide incorporation (% of control)
Trifluorothienylacetone	33	71
Trifluorothienylacetone	100	56
Tetrachlorotrifluoromethylbenzimidazole	5	42
Methylmercuric chloride	5	18
Dibromothymoquinone	8	32

tion of permeability towards K^+ (Brierley, 1976; Zaba & Harris, 1976); it includes stimulation of Ca^{2+} efflux and loss of nucleotides (Harris *et al.*, 1979; Table 2 of the present paper). The present measurements show that agents that interfere with the production of protein-bound thiol groups also stimulate Ca^{2+} and nucleotide loss. The exposure of thiol groups can, however, be lessened by agents that diminish permeability, such as ADP and bongkrekic acid, suggesting that the important thiol groups are essentially internal. Since some of the reactions in the chain leading to production of thiol groups are themselves sensitive to thiol-blocking reagents (for example the transhydrogenase), the control of the permeability will depend critically on removal and regeneration of thiol groups.

The observation that the local anaesthetics enhance the rate of liberation of thiol groups may be related to the inhibition of Ca^{2+} efflux by these compounds that has been observed by A. P. Dawson (unpublished work).

Thanks are due to the British Heart Foundation for a grant for expenses and to Dr. M. C. W. Evans of the Department of Botany, University College London, for the use of the D.W.2 spectrophotometer. The dibromothymoquinone was a gift from Dr. P. Heathcote (Department of Botany, University College London), and the tetrachlorotrifluoromethylbenzimidazole was a gift from Dr. R. B. Beechey (Shell Research, Sittingbourne, Kent, U.K.).

References

- Al-Shaikhaly, N. & Baum, H. (1979) *Biochem. Soc. Trans.* **7**, 215–216
- Baum, H. & Harris, E. J. (1979) *Proc. Int. Conf. Proteins of Iron Metabolism 4th*, Davos, p. 46
- Brierley, G. P. (1976) *Mol. Cell. Biochem.* **10**, 41–62
- Coty, W. A. & Pedersen, W. W. (1975) *J. Biol. Chem.* **250**, 3515–3521
- Flohé, L., Loschen, G., Azzi, A. & Richter, C. (1978) in *Superoxide and Superoxide Dismutase* (Michelson, A. M., ed.), pp. 324–334, Academic Press, London
- Harris, E. J. (1977) *Biochem. J.* **168**, 447–456
- Harris, E. J. (1979a) *Biochem. J.* **178**, 673–680
- Harris, E. J. (1979b) *Biochem. Soc. Trans.* **7**, 770–775
- Harris, E. J. & van Dam, K. (1968) *Biochem. J.* **106**, 759–766
- Harris, E. J. & Zaba, B. (1977) *FEBS Lett.* **79**, 284–290
- Harris, E. J., Al-Shaikhaly, M. & Baum, H. (1979) *Biochem. J.* **182**, 455–464
- Holloway, M. R., Rodriguez, H. B., Hood, K., Simpson, I. & Nunn, J. D. (1978) *Proc. FEBS Meet. 12th* **52**, 377–388
- Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, p. 69, Academic Press, London
- Layne, E. (1957) *Methods Enzymol.* **3**, 45
- Lehninger, A. L., Vercesi, A. & Bababunmi, E. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1690–1694
- Lofrumento, N. E. & Zanotti, F. (1978) *FEBS Lett.* **87**, 186–190
- Pedersen, W. A. & Coty, P. L. (1975) *J. Biol. Chem.* **250**, 3515–3521
- Sadewasser, D. A. & Dilley, R. A. (1978) *Biochim. Biophys. Acta* **501**, 208–216
- Stoner, C. D. & Sirak, H. D. (1973a) *J. Cell Biol.* **56**, 51–64
- Stoner, C. D. & Sirak, H. D. (1973b) *J. Cell Biol.* **56**, 65–73
- Tappel, A. L. (1960) *Biochem. Pharmacol.* **3**, 289–296
- Vercesi, A., Reynafarje, B. & Lehninger, A. L. (1978) *J. Biol. Chem.* **253**, 6379–6385
- Zaba, B. N. & Harris, E. J. (1976) *Biochem. J.* **160**, 707–714