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^{31}P-NMR SPECTROSCOPIC INVESTIGATIONS AND MITOCHONDRIAL STUDIES ON THE CARDIOPROTECTIVE EFFICIENCY OF 2-MERCAPTOPROPIONYLGLYCINE

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Abstract—Contents of high energy phosphates in the isolated perfused rat heart were followed during ischemia and reperfusion using ^{31}P NMR spectroscopy. Application of 2-mercaptopropionylglycine resulted in significantly higher content of ATP in the reperfusion phase whereas during ischemia no differences between control and therapy hearts were found. Analysis of postischemic mitochondrial function reveals that improved ATP level is paralleled by an increased respiratory control index and a reduced ATPase activity.

It is suggested that 2-mercaptopropionylglycine may cause increase of high energy phosphates during reperfusion by improving mitochondrial oxidative phosphorylation.

Cardioprotective action of various drugs or buffer compositions has been demonstrated with ^{31}P NMR spectroscopy in the ischemic myocardium [1, 2].

During recent years several investigations on the action of 2-mercaptopropionylglycine (MPG) have shown that the drug may improve oxidative phosphorylation in damaged mitochondria [3–5]. Moreover, it was revealed that ATPase activity was reduced, oligomycin sensitivity was increased and ATP–P_i exchange was stimulated in oligomycin-sensitive (OS-) ATPase preparations [6, 7].

In particular, MPG was found to protect the ischemic working rat heart preparation [5, 8], and cardioplegic dog hearts [9]. Protection of the ischemic canine myocardium by MPG was also reported very recently [10].

In the present paper ^{31}P NMR spectroscopy was used as a non-destructive method to evaluate further action of MPG on the isolated perfused rat heart, and to compare post-ischemic high energy phosphates with mitochondrial function data.

MATERIALS AND METHODS

Animals. Female Wistar rats (200–250 g) (*Mus rattus* GmbH, Brunnthal, F.R.G.) fed *ad libitum* with Hope Standard diet (Hope B.V., Woerden, The Netherlands) were used.

The rats were anesthetized with diethylether and 500 IU heparin (Liquemine®) were injected into the v. iugularis interna before thoracotomy. Control and therapy groups were randomized, Student's *t*-test and Holm's procedure [11] were applied for statistical evaluation.

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Perfusion conditions. Isolated, spontaneously beating rat hearts were perfused in the retrograde way via the aorta according to the Langendorff method [12] with a modified Krebs–Henseleit buffer [13] containing 124 mM NaCl, 19.5 mM $NaHCO_3$, 4.7 mM KCl, 1.16 mM KH_2PO_4, 1.17 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 0.25 mM Ca–Na_2EDTA.

Due to the flow rate of 40 l. carbogen/hr the pH of the buffer was 7.40. The buffer (300 ml) was recirculated at a constant perfusion pressure of 100 cm H_2O, the aortic pressure was monitored using a Gould P 230 b Statham transducer and a Siemens oscilloscope. The rat hearts were placed into a 20 mm NMR class tube 1 cm above ground and coronary flow was maintained at the level of the aortic cannula. Throughout the experiments buffer vessels and NMR glass tube were held at a constant temperature of 37°.

Five to ten minutes after the start of the retrograde perfusion the experiments were initiated. During phases I–III ^{31}P NMR spectra were continously recorded.

Phase I: Normoxic conditions, buffer gassed with 40 l./hr of carbogen (O_2/CO_2 95/5%) (Messer Griesheim, Frankfurt) perfusion pressure 100 cm H_2O. Duration of phase I 30 min, 4 spectra were taken, coronary flow: 8–10 ml/min.

Phase II: Ischemic conditions, the retrograde flow was completely stopped. Duration of phase II 15 min, 2 spectra were obtained. Aortic pressure amplitude decreased within 5–10 min to zero.

Phase III: Reperfusion phase, see conditions in phase I. Duration of phase III 30 min, 4 spectra were taken.

In the therapy experiments, neutralized MPG (1 mM) was added initially from the beginning of the experiment onward into the perfusion buffer.

We did not observe any alterations of the spectra due to addition of MPG in normoxic hearts.

Spectrum I_{30}	Phase $I_{23-30\,min}$*
Spectrum II_7	Phase $II_{0-7\,min}$
Spectrum II_{15}	Phase $II_{7-15\,min}$
Spectrum III_7	Phase $III_{0-7\,min}$
Spectrum III_{30}	Phase $III_{23-30\,min}$

* There were no significant changes of P_i pattern prior to phase I_{23-30}.

^{31}P *NMR spectroscopy*. ^{31}P NMR spectra were taken at 121.46 MHz on a Bruker CXP 300 NMR spectrometer. Four hundred and eight scans of 45 degree flip angle (20 μsec) were accumulated in 1-sec intervals. FIDs (free induction decay) were treated by a 20 Hz line broadening before Fourier transformation. Peak intensities were normalized to the first accumulated FID. Chemical shifts are referenced to creatine phosphate.

Isolation of mitochondria. An identical experimental setup as described under perfusion conditions was used for investigations on mitochondrial function. The hearts were used immediately after termination of the experiments. Hearts were homogenized for 2 sec by means of an ultraturrax (Janke and Kunkel, F.R.G.), the homogenates were cryoprotected as described by Fleischer [14]. Thereafter, mitochondria were isolated according to Mela and Seitz [15], omitting the nagarse treatment. The mitochondrial pellets were studied for integrity of the membrane by electron microscopy and by biochemical techniques.

Determination of mitochondrial parameters. Mitochondrial respiration was measured oxypolarographically as described by Estabrook [16] using an YSI oxygen monitor (Yellow Springs Instruments, U.S.A.) and ATPase activity was determined enzymatically according to Pullman *et al.* [17]. Protein concentrations were measured using the Folin reagent [18].

ST_4 = oxygen uptake of mitochondria in the presence of substrate (n atoms oxygen uptake/mg mitochondrial protein/min); ST_3 = oxygen uptake of mitochondria in the presence of substrate and ADP

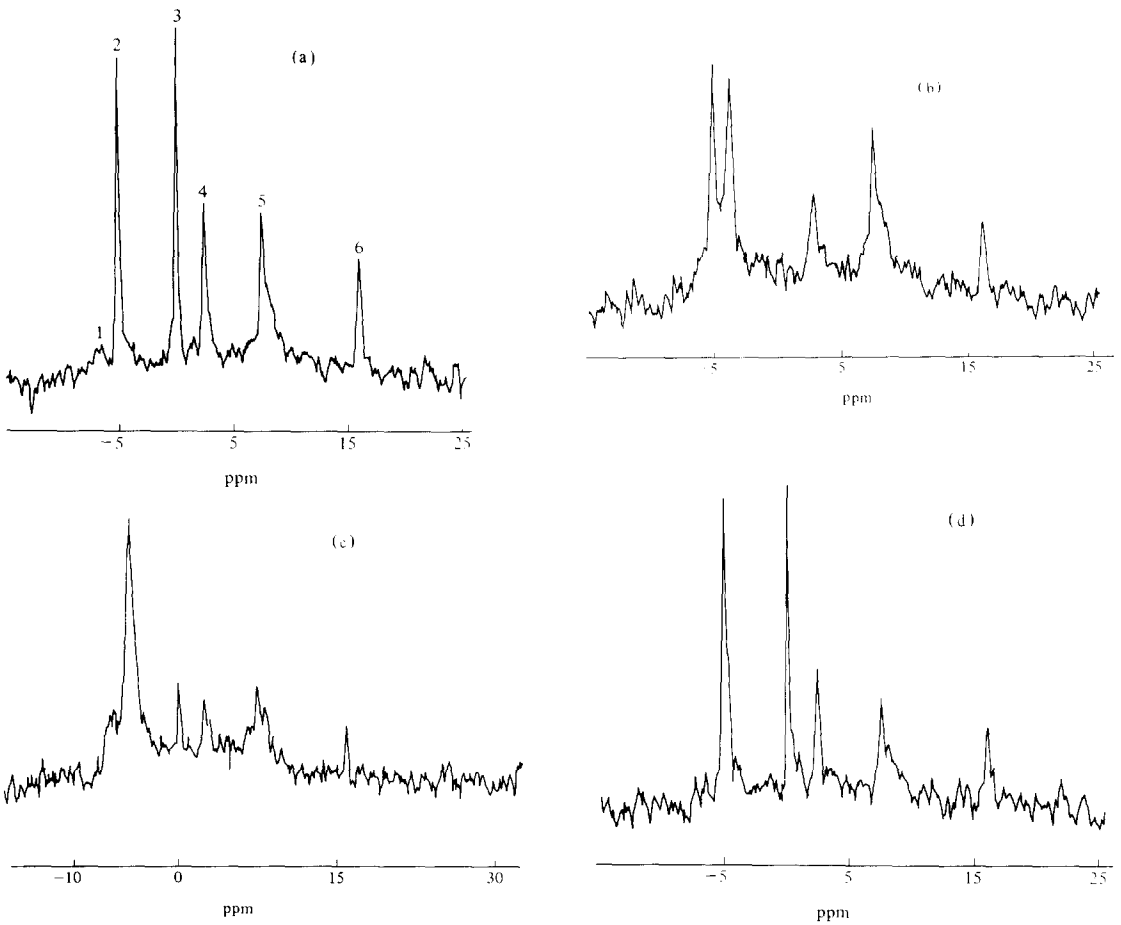

Fig. 1. ^{31}P NMR spectroscopy of isolated, perfused rat hearts. ^{31}P-NMR spectra (408 scans, 20 μsec pulse duration, 1 sec repetition time) of the isolated perfused rat heart in normoxia (phase I_{30}) (a), ischemia (phase II_{15}) (b), and reperfusion (phase III_{30}) of a control (c) and a therapy (d) group. Representative spectra are shown. In the control group (N = 9) 6 hearts, in the therapy group (N = 6) 5 hearts exhibited spectra similar to the ones presented; concentration of MPG: 1 mM, added into the perfusion buffer from the beginning of the experiment onward; numeration of peaks: 1 sugar phosphates; 2 inorganic phosphate; intensity of this peak is mainly due to buffer phosphate (1 mM); 3 phosphocreatine; 4 alpha ATP; 5 gamma ATP; 6 β ATP.

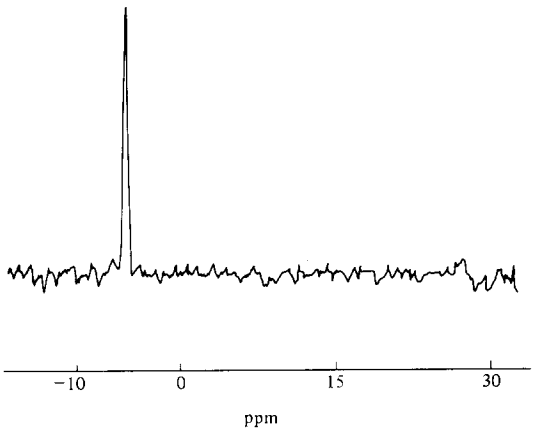

Fig. 2. ^{31}P NMR control spectrum of Krebs–Henseleit buffer. For experimental conditions see Fig. 1 legend. The NMR glass tube contained Krebs–Henseleit buffer gassed with carbogen, pH 7.40, 37°.

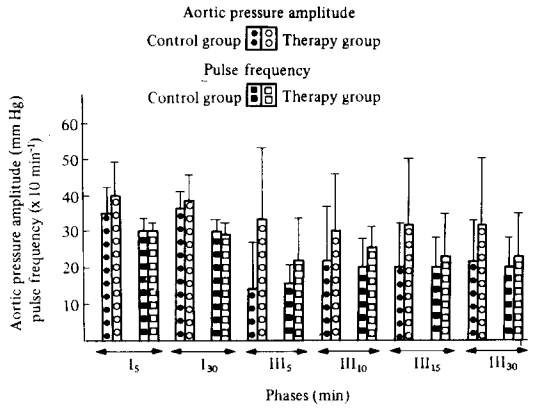

Fig. 3. Hemodynamic parameters (aortic pressure amplitude, pulse frequency) recorded during normoxia and reperfusion. Identical experimental set up as described in Figs. 1 and 2 legend. Numbers of hearts investigated: N = 9 control group, N = 6 therapy group. Mean values ± SD are shown during phases I and III. Differences in aortic pressure amplitude and pulse frequency during phase III are not significant.

(n atoms oxygen uptake/mg mitochondrial protein/min); RCR = respiratory control rate (ratio of oxygen consumed in the presence of ADP in relation to oxygen consumed in the absence of ADP); ADP/O = ratio nmol ADP consumed to n atom oxygen used. In the case that all ADP is used for ATP production ADP/O ratio is equivalent to: nmol ATP formed /n atom oxygen consumed; OPR = oxidative phosphorylation rate: (nmol ATP produced during ST_3/mg mitochondrial protein/min).

Reagents. 2-Mercaptopropionylglycine was a gift from Fresenius, Bad Homburg, F.R.G. Other reagents and buffer substances were either analytical grade or the finest quality available.

RESULTS

Representative spectra of control and therapy groups during normoxia ischemia and reperfusion phase are shown in Fig. 1. Characteristic patterns of phosphocreatine (CP) and adenosine triphosphate are found in normoxic hearts (Fig. 1a). High intensity of the P_i peak is due to extracellular (buffer) phosphate; see control spectrum (Fig. 2).

Compared to normoxia, in the ischemic phase II the ATP content is decreased, CP is no longer detectable (Fig. 1b). Concomitantly, we observe a splitting and shift of the P_i peak, which is due to intracellular phosphate. Since intracellular pH decreased under the ischemic conditions, we arrive at higher amounts of $H_2PO_4^-$, thus obtaining a signal with intra- and extracellular components.

During reperfusion, the above described changes during ischemia appear more pronounced in spectra obtained from control hearts (Fig. 1c). By contrast, pharmacologically protected hearts (Fig. 1d) reveal spectra similar to that shown in Fig. 1a. It should be noted that the shift and splitting of the P_i peak is completely reversed under the conditions of therapy. Vitality of the isolated perfused rat heart preparation under the experimental conditions was controlled

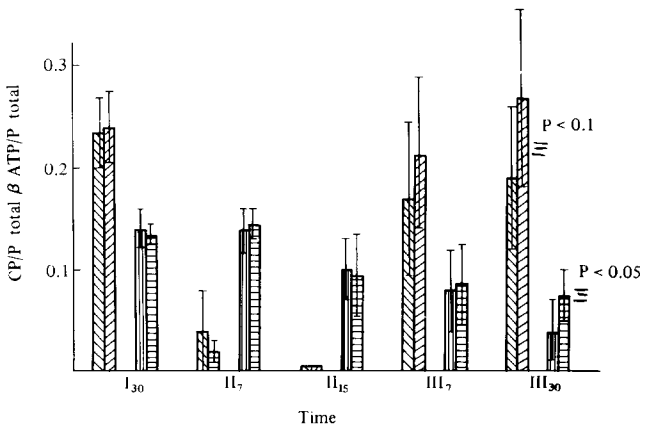

Fig. 4. Quantitative evaluation of all spectra. Peak areas of all control and MPG spectra are quantitatively evaluated in Fig. 2. The peak areas of β ATP and CP were calculated and CP/phosphate and β ATP/phosphate ratios were determined. The error bars denote ± SD: ▨ control, phosphocreatine; ▨ therapy, phosphocreatine; ▥ control, ATP; ▤ therapy, ATP; controls N = 9 therapy N = 6.

Table 1. Mitochondrial oxidative phosphorylation during reperfusion (phase III_{30}) of the ischemic, isolated perfused rat heart

ST_4	ST_3	ADP/O	OPR	RCR*	
49.9	315.0	3.0	970.6	6.7	Control
±14.0	±68.0	±0.2	±242.8	±1.7	NAD coupling
41.4	380.5	3.1	1205.1	9.9	Therapy, 1 mM MPG
±12.8	±90.9	±0.2	±287.1	±3.7	NAD coupling
133.0	251.8	1.2	297.1	1.9	Control
±29.0	±46.7	±0.3	±59.6	±0.3	FAD coupling
124.0	311.4	1.4	449.4	2.6	Therapy, 1 mM MPG
±50.0	±109.0	±0.3	±150.4	±0.5	FAD coupling

Oxygen consumption was measured in mitochondria, isolated from hearts in phase $III_{30\,min}$. For control and therapy groups, respectively, 8 hearts were investigated. Means were calculated from duplicate measurements in each heart.

Sequence of additions: (1) 0.48–1.03 mg of mitochondria, suspended in 1 ml medium (5); (2) 10 mM NAD-dependent substrate or 3.3 mM FAD dependent substrate + 1 μg rotenone; (3) 300 μM ADP. The temperature was 37°.

RCR values (therapy compared with control) differ significantly with $P < 0.05$ for NAD- and $P < 0.01$ for FAD coupling site; means ± SD are shown.

by measuring intraaortic pressure amplitude (130 mm Hg systolic and 80 mm Hg diastolic) and pulse frequency (Fig. 3).

A quantitative evaluation of all control and therapy data is shown in Fig. 4. Generally, towards the end of phase II_{15}, there are no differences between controls and MPG treated hearts. In the early reperfusion phase (III_7) phosphocreatine rapidly increases. By contrast ATP contents further decrease. This tendency is enhanced in phase III_{30}, representing late reperfusion. At this stage, however, control and therapy groups differ significantly (for ATP $P < 0.05$, compare also Figs. 1c and 1d). Moreover, mitochondrial function parameters determined in phase III_{30} show significant differences. In the MPG-treated group the coupling degree RCR is significantly higher at the NAD ($P < 0.05$) and FAD ($P < 0.01$) coupling sites (Table 1).

Mitochondrial ATPase activities of the control group increase during reperfusion (phase III_{30}) when compared to normoxic values (Table 2). In the therapy group activation of the ATPase–ATP-synthase system into the ATP-splitting direction is significantly ($P < 0.05$) lower and sensitivity toward oligomycin is maintained.

DISCUSSION

A sequence similar to the one observed previously for the decrease of high energy phosphates during ischemia [19, 20] is shown in Figs. 1 and 4. Significant decrease of ATP contents only becomes apparent at very low phospocreatine levels.

Rapid increase of phosphocreatine in the early reperfusion phase as shown in Fig. 4 is considered to be due to reoccurring ATP-synthesis and via mitochondrial creatine kinase [23]. ATP synthesis and production of phosphocreatine is possibly linked by compartmentation and/or by some sort of coupling of mitochondrial ATP-synthesis to creatine kinase [21, 22, 24, 25].

During ischemia and reperfusion intensity of the ^{31}P signal decreases. This may be due to either compartmentation of ^{31}P components or binding to membranes or, as suggested by [26] to a loss through leaky cellular membranes [27]. To account for this loss in signal intensity, CP/P total and β-ATP/P total ratios were calculated.

The slight overshoot in CP production in the late reperfusion phase was also noted by other authors [23, 28]. As found by Bailey *et al.* [23] this observation is characteristic for hearts regaining mechanical function after ischemia. In the presence of 1 mM MPG (the dose–response relationship of MPG had revealed 1 mM as the most effective concentration, Evers and Zimmer, in preparation) significant improvement of myocardial ATP levels is found in the reperfusion phase. Hemodynamic parameters, however, do not significantly differ between both groups (Fig. 3). This discrepancy is explained by a

Table 2. Mitochondrial ATPase activities during normoxia (phase I_{30}) and reperfusion (phase III_{30}) after ischemia in isolated, perfused rat hearts

	−Oligomycin* (μmol ADP/mg/min)	+Oligomycin* (μmol ADP/mg/min)
Phase I_{30} control	1.2 ± 0.1	0.4 ± 0.1
Phase III_{30} control	2.2 ± 0.4	0.6 ± 0.1
Phase III_{30} therapy (1 mM MPG)	1.4 ± 0.1	0.4 ± 0.1

For each determination 30 μg of mitochondrial protein was used. For control and therapy groups respectively, four hearts were investigated. The assays were done in triplicate for ATPase activities and oligomycin sensitivities; means ± SD are shown.

* Values of controls (phase III_{30}) and therapy (phase III_{30}) differ significantly $P < 0.05$.

lack of sensitivity of the perfused Langendorff (not working) heart to myocardial dysfunction after short ischemic periods compared to the isolated working rat heart (own observations). The cardiac function parameter aortic flow (obtained from the isolated working rat heart) is a very sensitive indicator of post-ischemic metabolic perturbation [5, 8], whereas aortic pressure amplitude (isolated perfused, not working rat heart) just may serve to indicate gross viability of the preparation.

It is suggested that protection of the high energy phosphate content in reperfused rat hearts is caused by an improvement of mitochondrial oxidative phosphorylation. It was emphasized [29] that the ability of the ischemic heart to recover is closely linked to mitochondrial capacity for sufficient ATP production. A close correlation between mitochondrial dysfunction and disturbed tissue performance in ischemia or after an ischemic stress was observed and may be causally related, but this point was discussed controversially by different groups [29–35].

In the therapy group (phase III) the mitochondrial function data reveal an improved coupling of oxidative phosphorylation and a reduced ATPase activity as well as satisfactory sensitivity toward oligomycin. These biochemical data indicate ameliorated mitochondrial function during late reperfusion by MPG and correlate well with increased content of ATP in phase III_{30}.

Mechanism of action of the drug should comprise (a) restoration of ischemically decreased myocardial SH/SS ratio, and (b) free radical scavenging [9, 36, 37]. The importance of free myocardial SH-groups for subcellular structure and function has been stressed a long time ago by Mendez [38]. Guarnieri *et al.* [36] found disturbances in cellular SH/SS redox equilibrium and diminution of membrane bound SH-groups during myocardial ischemia. Meerson *et al.* [39] on the other hand consider free radicals to constitute one main cause for ischemic damage. The latter view is shared by other investigators [40]. MPG which is a very reactive substance in SH–SS-interchange reactions [7–9] and an effective free radical scavenger [9, 37] may act in both ways and thus is considered to be a potentially useful drug for a cardioprotective therapy.

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REFERENCES

1. R. L. Nunnally and P. A. Bottomley, *Science* **211**, 177 (1981).
2. P. B. Garlick, G. K. Radda and P. J. Seeley, *Biochem. J.* **184**, 547 (1979).
3. G. Zimmer, G. Ohlenschläger and I. Berger, *Arzneimittel-Forsch./Drug Res.* **28**, 961 (1978).
4. G. Zimmer, M. Schneider and H. Hoffmann, *Arzneimittel-Forsch./Drug Res.* **28**, 811 (1978).
5. J. Fuchs, L. Mainka and G. Zimmer, *Arzneimittel-Forsch./Drug Res.* **35**, 1394 (1985).
6. G. Zimmer, L. Mainka and B. M. Heil, *FEBS Lett.* **150**, 207 (1982).
7. G. Zimmer, L. Mainka and G. Ohlenschläger, *FEBS Lett.* **94**, 223 (1978).
8. J. Fuchs, P. Veit and G. Zimmer, *Basic Res. Cardiol.* **80**, 231 (1985).
9. G. Zimmer, F. Beyersdorf and J. Fuchs, *Molec. Physiol.* **8**, 495 (1985).
10. S. E. Mitsos, K. M. Walden, G. D. Abrams, M. A. Schork and B. R. Lucchesi, *Circulation* **70**, II–259 (1984).
11. S. Holm, *Scand. Statist.* **6**, 65 (1979).
12. J. R. Neely and M. J. Rovetto, *Meth. Enzymol.* **39**, 43 (1975).
13. H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.* **210**, 33 (1932).
14. S. Fleischer, *Meth. Enzymol* **55**, 28 (1979).
15. L. Mela and S. Seitz, *Meth. Enzymol.* **55**, 39 (1979).
16. E. W. Estabrook, *Meth. Enzymol.* **10**, 41 (1967).
17. M. E. Pullman, H. S. Penefsky, A. Datta and E. Racker, *J. biol. Chem.* **235**, 3322 (1960).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. N. S. Dhalla, J. C. Yates, D. A. Walz, V. A. McDonald and R. E. Olson, *Can. J. Physiol. Pharmac.* **50**, 333 (1972).
20. P. M. Matthews, G. K. Radda and D. J. Taylor, *Biochem. Soc. Trans.* **9**, 236 (1981).
21. R. L. Nunnally and D. P. Hollis, *Biochemistry* **18**, 3642 (1979).
22. V. A. Saks, V. V. Kupriyanov, G. V. Elizarova and W. E. Jacobus, *J. biol. Chem.* **255**, 755 (1980).
23. I. A. Bailey, A. M. L. Seymour and G. K. Radda, *Biochim. biophys. Acta* **637**, 1 (1981).
24. S. Erickson-Viitanen, P. Viitanen, P. J. Geiger, W. C. T. Yang and S. P. Bessman, *J. biol. Chem.* **257**, 14395 (1982).
25. S. Erickson-Viitanen, P. J. Geiger, P. Viitanen and S. P. Bessman, *J. biol. Chem.* **257**, 14405 (1982).
26. W. E. Jacobus, G. J. Taylor IV, D. P. Hollis and R. L. Nunnally, *Nature, Lond.* **265**, 756 (1977).
27. C. E. Ganote, J. Worstell and J. P. Kaltenbach, *Am. J. Pathol.* **84**, 327 (1976).
28. I. A. Bailey and A. M. Seymour, *Biochem. Soc. Trans.* **9**, 234 (1981).
29. W. G. Nayler, E. Fassold and C. Yepez, *Cardiovasc. Res.* **12**, 152 (1978).
30. T. Nagao, M. A. Matlib, D. Franklin, R. W. Millard and A. Schwartz, *J. Molec. Cell. Cardiol.* **12**, 29 (1980).
31. L. R. Bush, M. Shlafer, D. W. Haack, and B. R. Lucchesi, *Bas. Res. Cardiol.* **75**, 555 (1980).
32. M. Shlafer, M. Kish, B. R. Lucchesi, A. D. Slater and S. Warren, *Bas. Res. Cardiol.* **76**, 250 (1981).
33. S. Mittnacht, S. C. Sherman and J. L. Farber, *J. biol. Chem.* **254**, 9871 (1979).
34. S. Mittnacht and J. L. Farber *J. biol. Chem.* **256**, 3199 (1981).
35. D. J. Hearse, M. S. Humphrey and G. R. Bullock, *J. Molec. Cell. Cardiol.* **10**, 641 (1978).
36. C. Guarnieri, F. Flamigni and C. Rossoni-Caldarera, *Biochem. Biophys. Res. Commun.* **89**, 678 (1979).
37. T. F. Slater, in *Recent Advances in 2-MPG Treatment of Liver Diseases* (Eds. R. Williams, G. Gasbarrini and M. Davis) pp. 83–88. Editrice Compositori, Bologna (1981).
38. R. Mendez, *J. Pharmac. Exp. Ther.* **81**, 151 (1944).
39. F. Z. Meerson, V. E. Kagan, Y. P. Kozlov, L. M. Belina and Yu. V. Arkhipenko, *Basic Res. Cardiol.* **77**, 465 (1982).
40. M. L. Hess and N. H. Manson, *J. Molec. Cell. Cardiol.* **16**, 969 (1984).