Biochemical Pharmacology, Vol. 26, pp. 1021–1026. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF P13, A POLYFLAVANE COMPOUND, ON PIG MYOCARDIUM ATP-SYNTHESIS, ATPases AND CREATINE KINASE

ATTILIO DI PIETRO, CATHERINE GODINOT and DANIÈLE C. GAUTHERON

Laboratoire de Biochimie Dynamique et Laboratoire de Biologie et Technologie des membranes, du CNRS, Université Claude Bernard de Lyon I, 43, Bd du 11 Novembre 1918, 69621 Villeurbanne, France

and

PIERRE FOULHOUX

Centre de recherches Pierre Fabre, 17, avenue Jean Moulin, 81106 Castres, France

(*Received* 9 *August* 1976; *accepted* 16 *November* 1976)

Abstract—The effects of P13, were examined on energy stores of the pig myocardial cell. The synthesis of ATP by oxidative phosphorylation was not modified in intact mitochondria. ATP hydrolysis by the various cell ATPases was inhibited in varying degrees: sarcolemma > sarcoplasmic reticulum > mitochondria > myosin. The inhibition depended on the accessibility of P13 to the enzyme since P13 strongly inhibited submitochondrial particle ATPase activity without modifying the activity in intact mitochondria. P13 acted competitively with respect to ATP, but a mixed type inhibition was observed when the enzyme was bound to a membrane. Myosin ATPase activity was almost unaffected by P13. Creatine phosphate utilization by creatine kinase was strongly inhibited by P13. Therefore, P13 helps to maintain high levels of energy stores: ATP and creatine phosphate.

For a long time *Poterium spinosum L.* (Rosaceae) has been known for its hypoglycaemic effect ever since diabetic Bedouin used decoctions prepared from the roots of this plant and were thus cured of their illness without any secondary effect [1]. The experiments conducted in several laboratories showed that the active principle was located in the main root bark [2]; it only acted on hyperglycaemic patients without affecting normal people [3–6]; it was dialysable, soluble in *n*-butanol and not very soluble in acetone [6]. Carraz *et al.* thought that the hypoglycaemic effect might be assigned to pentacyclic triterpenes [7]. An extensive study of the bark root constituents of *Poterium Spinosum L.* has revealed the presence of tannins [8, 9], flavonoids, triterpenes [10] and tormentoside [11].

The Centre de Recherches Pierre Fabre has isolated an active principle belonging to the tannins class, named P13. Besides its hypoglycaemic effect on hyperglycaemic dogs, the product has also shown a variety of effects on the myocardial cell: coronarodilatation, protection against anoxia [12], membrane stabilization, effect on cation transmembraneous exchanges during action potential of myocardial cells* [13], simultaneous antiarythmic [14] and cardiotonic effects* which constitute a new therapeutic class. These effects lead one to think that P13 might act on the energy stores of the cardiac cell and their utilization.

The present paper describes the effects of P13 first on ATPsynthesis by oxidative phosphorylation and creatine phosphate synthesis by creatine kinase, two main sources of myocardium energy, and secondly, on their utilization by the various ATPases of the cell or by creatine kinase.

MATERIAL AND METHODS

P13 was prepared by the Centre de Recherche Pierre Fabre according to the Brevet d'Invention (patent) No. 70 27189 (23 July 1970): crushed roots of *Poterium spinosum* were extracted with methanol. Extracted tannins were complexed with dimethyl aminoethanol and repeatedly precipitated by ethyl acetate or chloroform, filtrated and dried under vacuum. Gel permeation chromatography demonstrated the absence of terpenes, flavonoids and low mol. wt tannins. Absence of tannins was confirmed by thin layer chromatography; P13 is a polymer of 3,3′,4,4′,5,7-heptahydroxyflavane with a mol. wt of 2000–3000, as described by Bäisset *et al.* [14]. The same compound can be extracted from other plants: *Tieghemella, Entandrophragma, Schinopsis lorentzii, Acacia mollissima, Uncaria gambir, Rhus coriara*, but it is purer and more active when prepared from *Poterium spinosum*.

The purity of the ATP used in kinetic studies was checked by high pressure liquid chromatography using 'Permaphase' ABX resin (DuPont): it was shown that ATP contained no more than 2% ADP. The concentration of ATP was also verified by its absorption at 259 *nm*.

Preparation of the various cell ATPase fractions. Known procedures were applied to pig heart.

ETP (electron transport particles or submitochondrial particles) and soluble ATPase F_1 were prepared from mitochondria [15] as previously described [16].

* Y. M. Gargouïl, personal communication.

Myosin was obtained by the method of Perry [17], improved by ammonium sulfate fractionation according to the Kielley and Bradley method [18]. Pure myosin was stored at −20° in 50% glycerol −0.3 M KCl [19].

Sarcoplasmic reticulum was prepared either by the method of Mead *et al.* [20] in 0.1 M KCl, 5 mM histidine-HCl, pH 7.2 buffer or by the method of Smoly *et al.* [21] in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 buffer. Enzyme preparation was frozen in liquid nitrogen. Their purity was checked by measuring marker enzyme activities: NADPH-cytochrome c reductase (EC 1.6.2.4) and rotenone-insensitive NADH-cytochrome c reductase [22] (sarcoplasmic reticulum), and cytochrome c oxidase (EC 1.9.3.1) [23] (mitochondria).

Sarcolemma were obtained either by the method of Matsui and Schwartz [24] in 1 mM EDTA, pH 7.5 or by the Pitts and Schwartz procedure [25], omitting the last step, in 25 mM imidazole, 1 mM EDTA, pH 7.0 buffer. Enzyme preparation was kept frozen in liquid nitrogen. Marker enzyme tested was 5'-nucleotidase (EC 3.1.3.5) [26].

Marker enzymes tested had the following activities: cytochrome oxidase: 3.3 μatom O/min/mg protein in purified mitochondria; NADPH-cytochrome c reductase: 250 nmoles NADPH oxidized/min/mg protein in sarcoplasmic reticulum while the corresponding activity was 41 in mitochondria; rotenone-insensitive NADH-cytochrome c reductase: 96 nmoles/min/mg protein in sarcoplasmic reticulum and 48 in mitochondria; 5'-nucleotidase: 1,4 μmole Pi released/hr/mg protein in sarcolemma instead of 0.35 in the starting homogenate.

Assay of ATPase activity. ATPase activity was determined either by a spectrophotometric method, using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes and measuring the rate of NADH disappearance as previously described [16], or by a colorimetric method. In the latter case, the reaction time was first of all 1 min at 30° for mitochondria, ETP and F_1, secondly 5 min at 25° for myosin and at 37° for sarcoplasmic reticulum or sarcolemma. The incubation medium (0.8 ml) contained ATP-Mg at variable concentrations and 0.25 M sucrose, 31 mM Tris–HCl, pH 7.4 buffer for mitochondria and ETP, 31 mM Tris–HCl, pH 7.4 buffer for F_1 and sarcolemma, 20 mM His, 0.1 M KCl, pH 7.2 buffer for sarcoplasmic reticulum and 30 mM ammonium acetate, 50 mM Tris–HCl, pH 7.5 plus 5 mM calcium acetate [19] to measure Ca^{2+}-dependent activity or 0.6 M ammonium acetate, 2 mM EDTA, pH 7.5 [18] to measure NH_4^+-dependent activity of myosin.

All ATPase fractions showed a basal Mg^{2+}-dependent activity except myosin, where Mg^{2+} was replaced by Ca^{2+} or by NH_4^+. In all cases the ATP/cation ratio was kept constant to 1 (in initial rate determination).

Sarcolemmal ATPase activity was considerably enhanced by 120 mM Na^+ + 24 mM K^+; 0.1 mM ouabain specifically inhibited this $(Na^+ + K^+)$-dependent activity without affecting the Mg^{2+}-dependent activity. Therefore the $(Na^+ + K^+)$-dependent activity was calculated by substracting the values obtained in the presence of $(Na^+ + K^+)$ plus ouabain from the values in the absence of ouabain. The assays were initiated by addition of the enzyme. At the indicated times, the reaction was stopped by adding 75 μl ice-cold 50% trichloracetic acid; the inorganic phosphate released was estimated by the method of Sumner [27]. One unit corresponds to one μmole ATP hydrolysed/min/mg proteins.

Estimation of ATPsynthesis by oxidative phosphorylation. Mitochondrial respiration and the coupled ATPsynthesis were measured by oxypolarography [28] incubating 2.4 mg intact mitochondria in 2 ml 16 mM Tris HCl, 5 mM potassium phosphate, 112 mM KCl, 1 mM EDTA, pH 7.4 medium [29] at 28°. The respiratory substrate was either 10 mM glutamate or 10 mM succinate or 10 mM α-ketoglutarate of 15 mM β-hydroxybutyrate or 10 mM pyruvate plus 1 mM malate. ADP (0.2 mM) was added to initiate state 3 as defined by Chance and Williams [28].

Creatine kinase activity. The effects of P13 were measured either on purified cytoplasmic creatine kinase (EC 2.7.3.2), purchased from Sigma (Type I), or on the enzyme associated with the mitochondria using intact freshly prepared mitochondria.

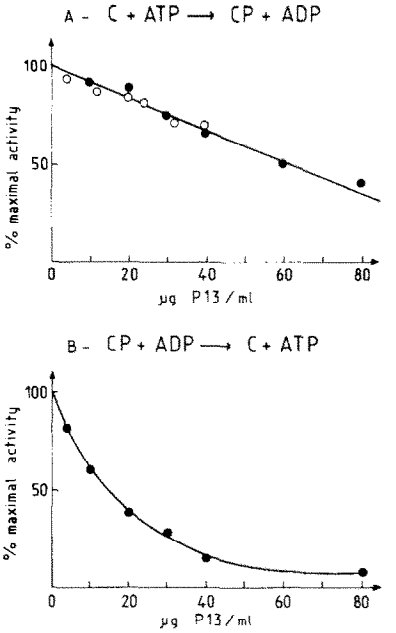

Fig. 1. P13 inhibition of creatine kinase activities.
A Creatine phosphate synthesis: 0.52 μg cytoplasmic pure enzyme (●——●) was incubated in 0.5 ml final vol of 0.44 M glycine, 4 mM ATP, 4 mM $MgCl_2$, 33 mM creatine, 10 mM reduced glutathione, 0.4 mM phosphoenol pyruvate, 0.25 mM NADH, 20 μg pyruvate kinase, 20 μg lactate dehydrogenase, pH 7.6; NADH disappearance rate was measured at 340 nm. The mitochondrial enzyme (○—— ○) activity was determined as described in Methods. B—ATPsynthesis: 0.52 μg cytoplasmic enzyme was added to 0.5 ml 0.1 M imidazole-acetate, 1 mM ADP, 10 mM Mg-acetate, 35 mM creatine phosphate, 1 mM reduced glutathione, pH 7.0. The rate of ATP formation was measured by the luciferin-luciferase system in arsenate buffer [34]. Reaction temperature was 30°. Optimal activity (100 per cent) was 54 μmoles creatine phosphate synthetized/min/mg protein or 200 μmoles ATP synthetized/min/mg for the cytoplasmic enzyme and 6.4 μatomes O consumed/hr/mg protein for the mitochondrial enzyme. C = creatine; CP = creatine phosphate.

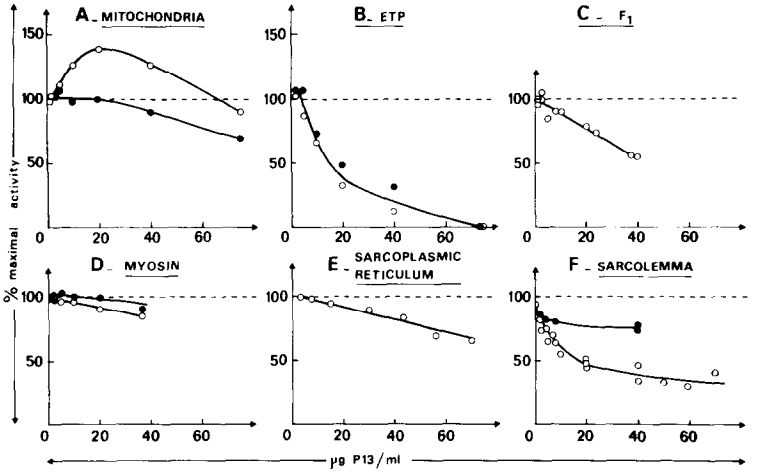

Fig. 2. P13 effects on ATPase activities. Each ATPase fraction was incubated with various P13 concentrations at a fixed saturating ATP concentration. ATPase activity was measured either by the colorimetric method or by the enzymatic method; the same results were obtained in both cases.

A—mitochondria (600 µg): 3 mM ATP-Mg; no addition (○—○) plus 1.25 µM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (●—●). B—ETP (100 µg): 3 mM ATP-Mg; no addition (○—○), plus 1.25 µM CCCP (●—●). C—F_1 (3 µg): 3 mM ATP-Mg. D—myosin (55 µg): 5 mM ATP; NH_4^+-dependent activity (○- - -○), Ca^{2+}-dependent activity (●- - -●). E—Sarcoplasmic reticulum (20 µg): 4.8 mM ATP. F—Sarcolemma (100 µg): 3.2 mM ATP-Mg; Mg^{2+} basal activity (●—●), ($Na^+ + K^+$)-dependent activity (○- - -○). 100 per cent maximal activity was respectively equal to 0.09 µmoles/mn/mg for mitochondria and 0.23 in the presence of CCCP, 1.5–1.7 for ETP, 56 for F_1, 0.80 (○- - -○) and 0.085 (●—●) for myosin, 0.69 for sarcoplasmic reticulum, 0.043 (●- - -●) and 0.20 (○- - -○) for sarcolemma.

Creatine phosphate synthesis by cytoplasmic creatine kinase was measured in the standard medium of Forster *et al.* [30] using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes and measuring the rate of NADH disappearance.

Creatine phosphate utilization by the cytoplasmic enzyme was determined by incubation in the medium described by Forster *et al.* [30] and measuring the amount of ATP formed by the luciferin-luciferase system [31]. Creatine phosphate synthesis by the mitochondrial enzyme was studied by adding 20 mM creatine and 6 mM $MgCl_2$ to mitochondria in 15 mM glutamate (state 4 respiration) as described by Vial *et al.* [32] and calculating the increase of the final state 4.

Protein estimation. Protein was estimated by a biuret method [33] or by the Lowry *et al.* procedure [34].

Calculations. Kinetic constants were calculated in individual experiments using the weighted least square method of Wilkinson [35] with an Olivetti Programma 101 desk top computer. When more than two experiments were performed, an average value was calculated and expressed together with the standard error of the mean.

RESULTS

Effects of P13 on ATPsynthesis by oxidative phosphorylation. All experiments with freshly prepared intact mitochondria showed no effect of P13 from 0.5 to 80 µg/ml on RCR, whatever the substrate was. A weak inhibition (7–20 per cent) of state 4 respiration was observed with all substrates except glutamate but an equal inhibition of state 3 was obtained; therefore, the RCR value (respiratory control ratio [27]) was not affected by any of the tested P13 concentrations. The ADP/O ratio was also unaffected by P13 for every substrate studied. It is worthwhile to note that cytochrome oxidase was not significantly affected by P13 either when measured with intact mitochondria or with ETP.

P13 inhibition of creatine kinase activities. Figure 1 shows that P13 inhibited both creatine phosphate and ATP synthesis by creatine kinase. Figure 1A demonstrates that P13 inhibited creatine phosphate synthesis by the purified enzyme with an I_{50} value of 60 µg P13/ml, when studied by the spectrophotometric technique. It is interesting to note also that the same inhibition pattern and I_{50} were obtained with the mitochondrial bound enzyme, when studied by the oxypolarographic technique.

ATPsynthesis was even more strongly inhibited by P13 than creatine phosphate synthesis with an I_{50} value of 15 µg/ml; 95 per cent inhibition was observed at 80 µg P13/ml (Fig. 1B). P13 inhibition did not depend on the P13/protein ratio but only on the P13 concentration in the assay (protein concentration ranging from 0.26 to 1.3 µg/assay).

P13 inhibition of ATPase activities. P13 inhibited all the ATPase activities of the different fractions tested in varying degrees (Fig. 2). The ($Na^+ + K^+$)-dependent activity of sarcolemma was the most affected; it was inhibited by low P13 concentrations with an approximate I_{50} value of 15 µg P13/ml. Low P13 concentration (5 µg/ml) inhibited by 20 per cent the Mg^{2+} basal ATPase activity but increasing the P13 concentration did not further modify this residual activity (Fig. 2F).

ATPase activity of sarcoplasmic reticulum was not very sensitive to P13 action: 5 per cent inhibition for 15 µg/ml and 30 per cent for 70 µg/ml (Fig. 2E).

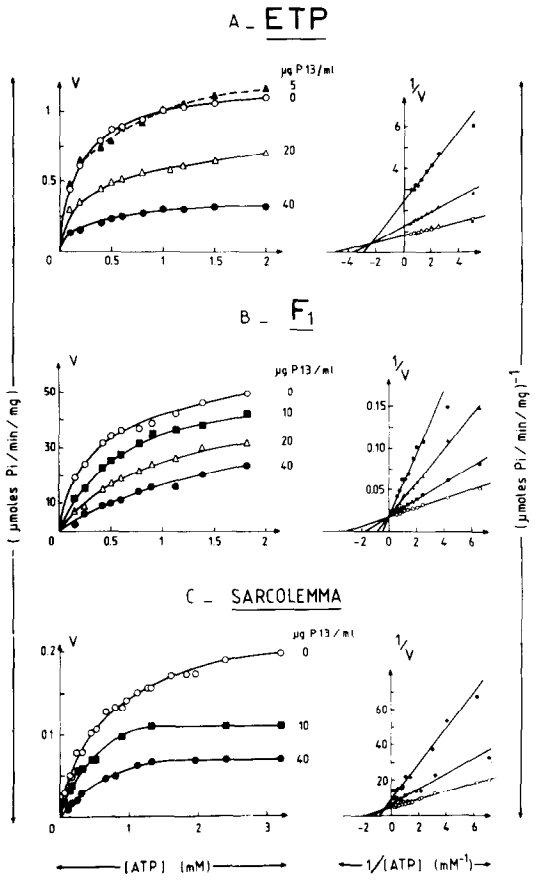

Fig. 3. Kinetics of P13 ATPase inhibition. On the left: initial rate of ATPase activities as a function of ATP concentration in the presence of various concentrations of P13: ○—no P13; ▲—5 μg/ml; ■—10 μg/ml; △—20 μg/ml; ●—40 μg/ml. On the right, reciprocal plots of the same data. A—ETP = 100 μg; B—F_1 = 3 μg; C—Sarcolemma = 100 μg. Experimental conditions are described in Methods.

P13 effects on mitochondrial ATPase varied with the activity and the accessibility of the enzyme. ATPase activity of intact, well coupled mitochondria was enhanced by 40 per cent with 20 μg P13/ml; in contrast with uncoupled mitochondria, which showed a high ATPase activity, P13 showed no effect up to 20 μg/ml and a weak inhibition at higher concentration: about 30 per cent for 75 μg/ml (Fig. 2A). When mitochondria were disrupted by sonication to form essentially inside-out [36] particles named ETP, the mitochondrial ATPase activity became strongly inhibited by P13 with I_{50} value of about 15 μg/ml and 100 per cent inhibition for 75 μg/ml (Fig. 2B). When the same enzyme was solubilized and purified, an intermediate inhibition was obtained, increasing linearly with respect to P13 concentration, with an approximate I_{50} value of 40 μg/ml (Fig. 2C). Myosin, which has an Mg^{2+}-independent ATPase activity was almost unaffected by P13: 5–10 per cent inhibition of Ca^{2+}-dependent activity and 15 per cent inhibition of NH_4^+-indepent activity at 38 μg/ml (Fig. 2D).

Kinetics of P13 inhibition. Data given in Fig. 2 were obtained at a fixed saturating ATP concentration. In contrast Fig. 3, shows P13 inhibition at various ATP concentrations obtained with ETP, F_1 or sarcolemma.

Calculated kinetic constants for ETP were: V_m = 1.21 ± 0.01 μmoles/min/mg protein and $K_{m(ATP)}$ = 0.20 ± 0.01 mM. P13 inhibition appeared to be of the mixed type with an apparent *Ki* value of 55 ± 5 μg/ml (Fig. 3A), as calculated from the graphs (average of 3 experiments).

The same parameters determined for F_1 were: V_m = 56.3 ± 0.2 μmoles/min/mg protein and $K_{m(ATP)}$ = 0.32 ± 0.03 mM. P13 inhibition was competitive with respect to ATP, and gave a graphically calculated *Ki* value of 10 ± 2 μg/ml (Fig. 3B).

The study carried out with sarcolemma gave: V_m = 0.225 ± 0.005 μmoles/min/mg protein and $K_{m(ATP)}$ = 0.53 ± 0.04 mM. P13 inhibition was of the mixed type with an apparent *Ki* value of 41 ± 4 μg/ml as calculated from the graphs.

Reversal of inhibition. Sarcolemmal Mg^{2+}-dependent and ($Na^+ + K^+$)-dependent activities were inhibited by 50 μg P13/ml. Table 1 shows that a simple dilution abolished the inhibition since the same levels of optimal activity as those obtained by 1 μg P13/ml in the incubation medium without preincubation were obtained (see Fig. 2F). Therefore it can be concluded that P13 inhibition of ATPase activity was reversible.

Table 1. Reversal of P13 inhibition of sarcolemma ATPase activity

	Mg^{2+}-dependent activity		($Na^+ + K^+$)-independent activity	
	μmoles/min/mg prot. × 10^3	% maximal activity	μmoles/min/mg prot. × 10^3	% maximal activity
No. P13	43.3 (7)	100	200 (7)	100
50 μg P13/ml in incubation medium	29.0 ± 2.2 (4)	67	66.7 ± 3.3 (4)	33
50 μg P13/ml during preincubation and final dilution to 1 μg/ml	36.0 ± 1.7 (7)	83	167 ± 8.3 (7)	83

Sarcolemma were preincubated with 50 μg P13/ml (inhibiting concentration) at 0 4° during 1 min to several hr. The ATPase reaction was initiated by adding appropriate aliquots of enzyme–P13 solution which brought P13 concentration to 1 μg/ml (not inhibitory range). Controls were made without P13 and by adding 50 μg P13/ml in the reaction medium. ATPase activity was measured by the enzymatic method. The number of experiments are indicated in parentheses.

DISCUSSION

The present study shows that P13 effects on the energetic metabolism are in good agreement with the macroscopic results observed in therapeutics.

P13 tends to lower the rate of oxidation of substrates by mitochondria without affecting coupling between oxidation and phosphorylation; therefore the yield in synthetized ATP is not modified. Since according to Hearse *et al.* [37] "with the anoxic isolated perfused rat heart, reoxygenation after the onset of major enzyme release may greatly exacerbate the release and extend ultrastructural damage", P13 seems to act by preventing excessive reoxygenation.

P13 inhibits all measured ATPase activities but the myosin one and inhibits creating kinase activity; therefore, it does not seem to be very specific towards enzymes whose substrate in ATP. However, P13 does not affect enzymes involved in the respiratory chain of mitochondria such as cytochrome oxidase and has a barely significant effect on the rate of respiration of various substrates: glutamate, succinate, α-ketoglutarate, β-hydroxybutyrate or pyruvate + malate. P13 seems then to interfere mainly with enzymes concerned with ATP utilization.

As mentioned previously, P13 inhibits all measured ATPase activities but the myosin one as tested with isolated myosin. Thus it seems to save ATP for contraction. This effect combines with the unaffected ATPsynthesis to increase the ATP level in the cell. P13 also diminishes creatine kinase activity. At saturating substrate concentrations, ATPsynthesis from creatine phosphate and ADP is more drastically decreased than creatine phosphate synthesis from creatine and ATP. The differential effect of P13 on these two creatine kinase activities must result in an increased level of creatine phosphate. As a decrease in creatine phosphate immediately follows the onset of infarct myocardium [38], it is not excluded that the protective effect of P13 against anoxia [12] could be related to this effect of P13 on creatine kinase activity. Through ATP and creatine phosphate, P13 saves the myocardial cell energy stores. If myosin ATPase is not more inhibited *in situ* than it is when purified, (15 per cent maximum) P13 would preserve a higher level of ATP for contraction. However, the effects of P13 should be checked on actin–myosin interactions and on the whole contractile system. The slight decrease in respiratory activity and the saving of energy stores in myocardial cell are in agreement with the results of Auclair *et al.* [12] showing that P13 prolonged the life of animals submitted to hypoxic conditions.

The inhibition of F_1-ATPase by P13 was of the competitive type indicating an effect only on the affinity for ATP. In contrast the inhibition observed when F_1 was bound to the mitochondrial membranes (ETP) was of the mixed type; therefore P13 not only affected the binding of ATP to the enzyme, but due to the membrane interaction, it interfered with the catalytic properties. It cannot be excluded that the P13 used in these experiments was not homogeneous in size. It is a polymer of an undefined length and light subfractions may exist in solution. The two inhibitory effects could then be attributed to different sizes of polymer affecting two different sites. However one should keep in mind that oligomycin and uncouplers affect F_1 catalytic properties only by indirect actions at the level of membrane subunits. The binding of P13 on the membrane did not modify unspecifically the membrane-bound enzymes since cytochrome oxidase activity was not affected by P13 whatever the membrane orientation was; moreover P13 does not seem to exert its respiratory effects at the level of this enzyme. The inhibition of the sarcolemmal ATPase activity was also of the mixed type, affecting V_m and K_m; this may come from a dual effect of P13 binding to the enzyme and to the membrane. It must be emphasized that the sarcolemmal ATPase activity is the one most affected by P13. As the role of this enzyme is to maintain the ion gradient in the cell, P13 must have an effect on the permeation of ions through the membranes. This has been observed by measuring the electrical activity of a myocardial fiber: P13 diminished the K^+ permeability of this fiber.* Other effects of P13 on ion permeability of membranes are under investigation.

Acknowledgements– Thanks are due to the French CNRS for financial help.

* Y. M. Gargouïl, personal communication.

REFERENCES

1. E. F. Steinmetz, *Q. J. Crude Drug Res.* **4**, 582 (1964).
2. E. F. Steinmetz, *Acta Phytoterapeutica* **12**, 41 (1965).
3. G. O. Schluetz and J. Venulet, *Biochem. Pharmac.* **12**, 274 (1963).
4. G. O. Schluetz, *Experientia* **20**, 78 (1964).
5. J. Mishkinski, E. Menszel and F. G. Sulman, *Archs. int. Pharmacodyn. Thér* **161**, 306 (1966).
6. T. M. Quisenberry and G. Gjerstad, *Q. J. Crude Drug Res.* 7, 957 (1967).
7. G. Carraz, A. Boucherle and A. Dardas, *C.r. Acad. Sci. Paris*, **266**, 293 (1968).
8. Z. Blazek, *Dt. Apotheker. ztg* **108**, 1434 (1968).
9. I. Fouraste, G. Moulis and E. Stanislas, *Plantes médicinales et phytothérapie* **VI**, 259 (1972).
10. D. Z. Shukjourov and A. A. Nasoudari, *Akad. Nauk azerojdzch. S.S.R. dokl.* **26**, 84 (1970).
11. A. Pourrat, H. Pourrat and P. Potier, *Anns Pharmaceutiques françaises* **31**, 321 (1973).
12. M. C. Auclair, S. Dechezlepretre, H. Lauressergues, M. Lemeignan and P. Lechat, *Thérapie* **31**, 647 (1976).
13. M. C. Auclair, M. Lemeignan and A. Rodallec, *C.R. Acad. Sci., serie D* **283**, 695 (1976).
14. A. Baïsset, P. Foulhoux and P. Montastruc, *Thérapie*, in press (1976).
15. D. C. Gautheron, R. Durand, N. Pialoux and Y. Gaudemer, *Bull. Soc. Chim. biol.* **46**, 645 (1964).
16. A. Di Pietro, C. Godinot, M. L. Bouillant and D. C. Gautheron *Biochimie* **57**, 959 (1975).
17. S. V. Perry, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. II, p. 582. Academic Press, New York (1955).
18. W. W. Kielley and L. B. Bradley, *J. biol. Chem.* **218**, 653 (1956).
19. H. R. Trayer and I. P. Trayer, *FEBS Lett.* **54**, 291 (1975).
20. R. J. Mead, M. B. Peterson and J. D. Welty, *Circulation Res.* **29**, 14 (1971).
21. J. M. Smoly, T. Wakabayashi, A. D. F. Addink and D. E. Green, *Archs. Biochem. Biophys.* **143**, 6 (1971).

22. G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand. *J. cell Biol.* **32**, 415 (1967).
23. B. Maïsterrena, J. Comte and D. C. Gautheron. *Biochim. biophys. Acta* **367**, 115 (1974).
24. H. Matsui and A. Schwartz, *Biochim. biophys. Acta* **128**, 380 (1966).
25. B. J. Pitts and A. Schwartz, *Biochim. biophys. Acta* **401**, 184 (1975).
26. C. S. Song and O. Bodansky, *J. biol. Chem.* **242**, 694 (1967).
27. J. B. Sumner, *Science* (*Washington*) **100**, 413 (1944).
28. B. Chance and G. R. Williams, *J. biol. Chem.* **217**, 383 (1955).
29. C. Godinot, C. Vial, B. Font and D. C. Gautheron, *Eur. J. Biochem.* **8**, 385 (1969).
30. G. Forster, E. Bernt and H. U. Bergmeyer, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer). Vol. II, p. 784. Academic Press, New York (1974).
31. B. L. Strehler and J. R. Totter, *Archs. Biochem. Biophys.* **40**, 28 (1952).
32. C. Vial, C. Godinot and D. C. Gautheron, *Biochimie* **54**, 843 (1972).
33. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
34. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
35. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
36. E. Racker, in *Essays in Biochemistry* (Ed. P. N. Campbell and F. Dickens). Vol. VI, p. 1. Academic Press, New York (1970).
37. D. J. Hearse, S. M. Humphrey, W. G. Nayler, A. Slade and D. Border. *J. mol. cell. Cardiol.* **7**, 315 (1975).
38. S. Gudbjarnason, *Cardiology* **56**, 232 (1971).