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Some Effects of Fructose-1,6-Diphosphate on Rat Myocardial Tissue Related to a Membrane-Stabilizing Action

L. GALZIGNA, V. RIZZOLI, M. BIANCHI, M. P. RIGOBELLO AND R. SCURI

Institute of Biological Chemistry, University of Padova, 35131 Padova and Centro Ricerche Consorzio Biomedica Foscama IRFI, Ferentino, Italy

This study aims at elucidating the mechanism of action of extracellular fructose-1,6-diphosphate (FDP). FDP is able to inhibit Ca^{++} entry into the myocardial tissue with an IC_{50} value of 11·5 mM and in addition, it is bound by rat heart slices, the binding being activated by Zn and conditions of chemical hypoxia induced by KCN and iodoacetate. The overall effect of extracellular FDP includes an increase of frequency and amplitude of contraction of perfused heart at concentration below 1 mM, and, in general, a stimulation of the oxygen consumption of the tissue. The antihaemolytic effect of FDP suggests its action as a membrane stabilizer. The effects of extracellular FDP on the myocardial cell can be interpreted both on the basis of a limited permeability of the cell membrane to it and as a purely extracellular effect transduced through the cell membrane with a final response consisting of an increase in the intracellular FDP.

KEY WORDS—Fructose-1,6-diphosphate; myocardium; tissue slices; Ca entry; membrane stabilization.

INTRODUCTION

The use of fructose-1,6-diphosphate (FDP) as an extracellular drug has been advocated after clinical studies showing a number of effects following its parenteral administration.[1,2] The effect of FDP, specific for it and not shared by other phosphorylated sugars or by fructose plus phosphate, was also studied *in vitro* on different types of cells, i.e. red blood cells[3,4] spermatozoa.[5] All results pointed to an interaction of FDP with the cell membrane inducing a perturbation of the ion transport. FDP also maintains better haemodynamics during hypoxemia[6] but the problem of the mechanism of action of this phosphorylated sugar is still open.

The intracellular fate of FDP is quite well known and its degradation to triose phosphates or to a monophosphate derivative is textbook knowledge. On the contrary, what can happen when FDP impinges on the cell membrane from outside is not so well understood.

Addressee for correspondence: Lauro Galzigna, Professor of Biochemistry, University of Padova, Via F. Marzolo 3, 35131 Padova, Italy.

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So far we have been unable to prove that FDP can penetrate through normal cell membrances[7] although other authors[8] suggest that it does. What we have observed on the effect of FDP is substantially the following: (1) FDP favours the cellular uptake of K^+[9] and the release of H^+.[4] The latter event induces an activation of the intracellular phosphofructokinase and hence an increase of the intracellular FDP,[3] ATP and 2,3-diphospoglycerate;[10] (2) FDP disappears from the circulating blood of rats after parenteral administration and the amount disposed of by the serum phosphatases is only a fraction of the amount disappearing.[11] FDP is hydrolysed to some extent by the cell membrane phosphatases[7] and the released phosphate is taken up by the cells; (3) The effect of FDP on ion translocation is not restricted to K^+ and H^+ since an inhibition of Ca^{++} uptake was also observed,[5] as demonstrated by using different types of cells.

In this paper we describe some effects of FDP on perfused rat heart and rat myocardial tissue slices with particular reference to the inhibition of Ca^{++} uptake considered as a generalized symptom of cellular dysfunction.[12] Our purpose is to

understand the mechanism of action of the extracellular FDP and find a rationale for its *in vitro* effects.

MATERIALS AND METHODS

Chemicals

^{45}Ca, [^{14}C](U)-FDP and [^{14}C](U)-inulin were from the Radiochemical Centre (Amersham, U.K.), and chlorpromazine from Specia (Paris, France). All other reagents were from Sigma (St. Louis, U.S.A.). FDP (Esafosfina) was obtained from Biomedica Foscama (Roma, Italy) and used without further purification.

Instruments

Beckman Liquid Scintillation Spectrometer, Perkin-Elmer Lambda Spectrophotomer, Beckman Expandomatic pH-meter with a Ca-specific electrode, Clark platinum electrode assembled with an oxygraph.

Perfused Rat Heart

Rat hearts were perfused at 37° C by the non-recirculating Langendorff procedure[13] with a medium of 0·115 M NaCl, 0·025 M $NaHCO_3$, 4 mM KCl, 0·9 mM KH_2PO_4, 1 mM $MgCl_2$, 1·5 mM $CaCl_2$ and 11 mM glucose at pH 7·4; the solution was saturated with 95 per cent 0_2 and 5 per cent CO_2 at 37° C. Before experiments the hearts were equilibrated for at least 20 min to allow haemodynamic and metabolic stabilization.

Tissue Slices

Hearts were excised from Wistar male rats (200 g) immediately after decapitation and 0·5 mm slices were obtained from the ventricles by a hand operated four blade tool. The incubation medium was a Krebs–Ringer composed of 145 mM NaCl, 1·5 mM KH_2PO_4, 3 mM $NaHCO_3$, 6 mM KCl, 10 mM Hepes and 2 mM glucose at pH 7·4 (final).

$^{45}Ca^{++}$ Uptake

Slices (100–150 mg wet weight) were incubated in vials with 1 ml of Krebs–Ringer medium at 30° C, under constant shaking with 0·06 to 0·05 mM $^{45}Ca^{++}$ (specific activity 1033 to 698 dpm nion^{-1}). The incubation was interrupted by filtration through Whatman GF/B filters under vacuum, the slices were washed three times with saline containing 0·1 mM EDTA and then homogenized for 3 min with a glass pestle in 1 ml of bidistilled water and 200 µl of 50 per cent (w/v) trichloroacetic acid. After centrifugation at 2000 *g* for 10 min the supernatant was transferred into 10 ml of Instagel (Packard) and the radioactivity measured by scintillation.

[^{14}C]-FDP Binding

The binding of [^{14}C]-FDP was measured by incubating 100 to 150 mg of slices in 1 ml of Krebs–Ringer medium at 30° C under shaking. The slices were washed three times with the Krebs–Ringer, ruptured and deproteinized as above, and the residual radioactivity counted in Instagel.

The measurement of the extracellular space was carried out with the non-permeant molecule [^{14}C]-inulin, as described by Sartorelli *et al.*[14] to assess whether FDP is taken up by the tissue by simple diffusion into the extracellular space. Additional experiments included the comparative titration of Ca^{++} with EGTA and FDP to check the extent of Ca^{++} chelation by FDP. The measurement of the O_2 consumption of the slices by an oxygraph with a Clark's electrode assembly in the presence of FDP and the checking of the effects of different compounds on the [^{14}C]-FDP binding were done to investigate the possible correlation between binding of FDP and stimulation of 0_2 consumption. We also tested the antihaemolytic effect of FDP on rat red blood cells according to Seeman and Roth[15] to investigate the possible behaviour of FDP as a membrane stabilizer.

RESULTS

The measurement of the consumption of the oxygen dissolved in the medium by the slices incubated in 2 ml of Krebs–Ringer with glucose showed that they consumed, in the same period of 10 min, 95 per cent of the total oxygen in the vessel in the presence of 10 mM FDP and only 60 per cent in its absence (Table 1).

Figure 1 shows the titration of 1 mM Ca by EGTA and FDP in 1 ml of Krebs–Ringer at 20° C. The chelating power of FDP is quite low and this allows us to attribute only a small part of the

Table 1. Effect of 10 mM FDP or equimolecular amounts of other compounds on oxygen consumption of 60 mg of rat heart slices suspended in Krebs–Ringer medium. Mean ± S.D. of eight experiments.

Addition	Oxygen consumption (nat min^{-1})
None	2·72 ± 0·31
FDP	3·22 ± 0·32*
Fructose + phosphate	1·80 ± 0·32
Fructose-6-phosphate	2·39 ± 0·28

*Increase significant by Student's *t*-test ($p < 0{\cdot}01$).

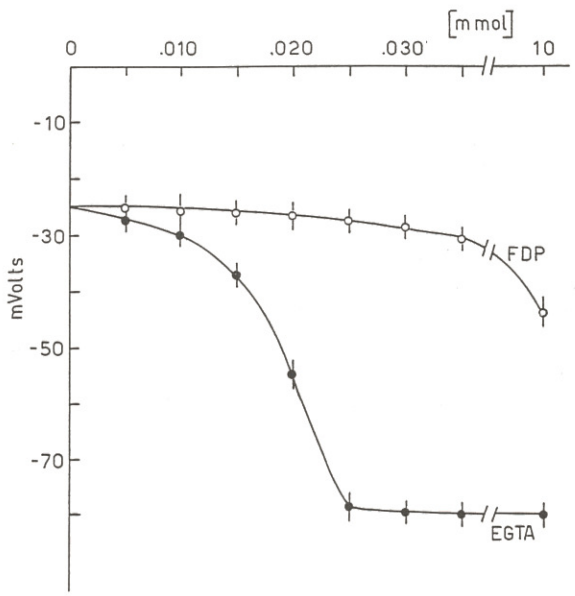

Figure 1. Titration curve of Ca^{++} with increasing concentrations of EGTA or FDP. 1 mM Ca^{++} was in 2 ml of Krebs–Ringer at 20° C and its signal was recorded by a pH-meter to a Ca-specific electrode at increasing concentrations of the chelating agents. Mean ± S.E. of three experiments.

following observed effect of FDP to Ca^{++} chelation.

Figure 2 shows the effect of 10 mM FDP on the uptake of $^{45}Ca^{++}$ as a function of time. The effect is independent of Ca^{++} concentration and the presence of FDP eliminates the fall in tissue counts of the $^{45}Ca^{++}$ with time. An IC_{50} of 11·5 mM can be calculated for the inhibitory effect of different concentrations of FDP on Ca^{++} uptake and such an effect was much lower with equimolecular fructose + phosphate (Table 2). Under the same conditions a typical Ca-antagonist such as diltiazem showed an IC_{50} value of 0·75 mM.

Figure 3 shows the binding of [^{14}C-]FDP as a function of the FDP concentration which is linear up to a concentration of 30 mM. The apparent lack of saturation may be due to the large extracellular space of the slices (see Figure 4).

The binding of [^{14}C]-FDP was also studied in the presence and absence of oxygen (with N_2 passed into the medium). The absence of oxygen as well as a low temperature (0° C) slightly (*ca.* 6·5 per cent) stimulated the binding (not shown).

The binding of [^{14}C]-FDP at 15 min is significantly ($P < 0{\cdot}01$) higher than that due to the amount which can diffuse into the extracellular space of the slices, as is shown by Figure 4, from which it appears that 85 per cent of the [^{14}C]-FDP bound can be accounted for by diffusion into the extracellular space. On the other hand the negligible amount of radioactivity (12 per cent higher than the background) found in third washing of the slices before determination of [^{14}C]-FDP indicates that either its binding to the

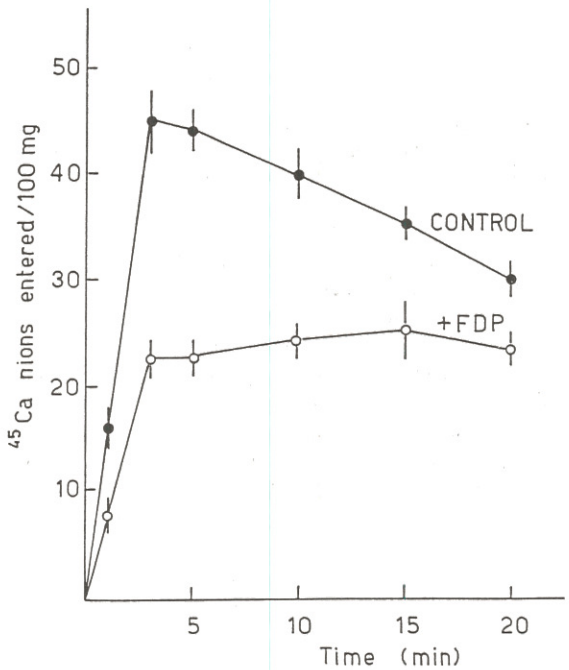

Figure 2. Efffect of 10 mM FDP on the entry of $^{45}Ca^{++}$ as a function of the incubation time (see Material and Methods; 100 mg refers to wet weight). Mean ± S.E. of four experiments.

Table 2. Effect of 10 mM FDP or equimolecular fructose + phosphate on the uptake of $^{45}Ca^{++}$ by slices incubated with either 0·06 or 0·5 mM $^{45}Ca^{++}$. Mean ± S.D. of six experiments.

Addition	$^{45}Ca^{++}$ entry(nions min^{-1} $(100\,mg)^{-1}$) 0·5 mM Ca^{++}	0·06 mM Ca^{++}
None	12·8 ± 2·2	7·37 ± 1·8
FDP	5·7 ± 2·5*	4·06 ± 2·0†
Fructose + phosphate	9·2 ± 3·1	5·45 ± 1·9

*Significant by Student's *t*-test ($p < 0·01$).
†Significant by Student's *t*-test ($p < 0·05$).

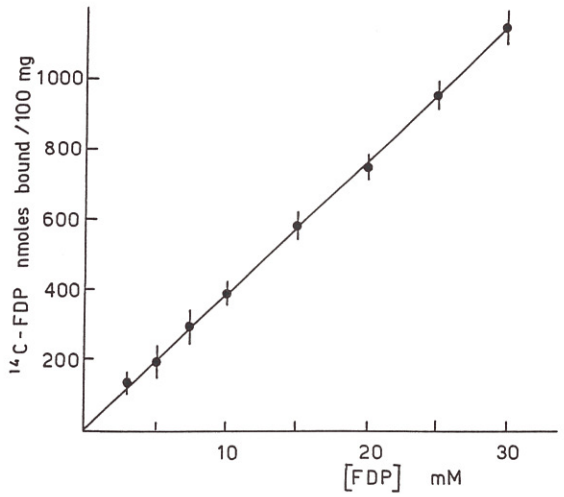

Figure 3. Binding of [^{14}C]-FDP as a function of the concentration. Incubation time was 60 min (see Materials and Methods). Mean ± S.E. of four experiments.

membrane is very stable or that [^{14}C]-FDP penetrates into the cells.

We have tested the effect of phosphatase inhibitors (heparin, fluride), phosphatase substrates (phenylphosphate), phosphate transport inhibitors (NEM); inhibitors of fructose-bisphosphatase (Zn)[16] and inducers of the so-called 'chemical hypoxia' (KCN and iodoacetate)[17] on the binding of [^{14}C]-FDP. Table 3 summarizes the results and the two significant changes observed, i.e. with Zn and KCN-iodoacetate, are concentration-dependent. Figure 5 illustrates the biphasic affect of FDP on the perfused heart which increased both amplitude and frequency up to 0·2 mM and decreases them thereafter.

The effect of FDP as a membrane stabilizer is shown in Table 4, which indicates that FDP, similarly to chlorpromiazine, acts as an inhibitor of hypotonic haemolysis. This non-myocardial effect of FDP is important to provide a rationale for its action at the membrane level.

DISCUSSION

The results of this study show that FDP, at concentrations close to those obtained after parenteral administration,[1] inhibits the entry of Ca^{++} into the myocardial tissue which results from its progressive deterioration. The effect of FDP parallels its ability to bind to myocardial slices, while the stimulating effect of hypoxia and that of Zn (Table 3) together with the linear behaviour shown in Figure 2 seem to indicate its ability to penetrate to some extent into cells.[18]

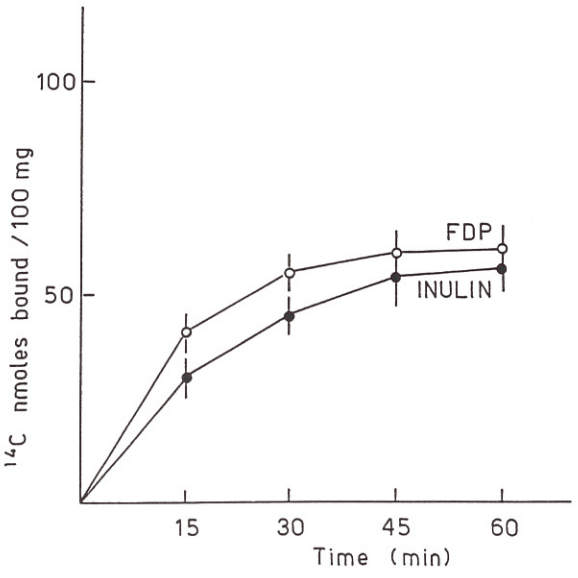

Figure 4. Comparison between the amount of [^{14}C]-FDP and [^{14}C]-inulin bound as a function of the time. FDP and inulin were 1 mM (131 dpm nmole $^{-1}$ specific activity). Mean ± S.E. of four experiments

Table 3. Effect of different compounds on the binding of [^{14}C]-FDP. A final concentration of 10 mM FDP (specific activity 6 dpm n mole^{-1}). Slices (100 mg) and an incubation time of 30 min were used. Mean ± s.d. of six experiments.

Addition	n moles of [^{14}C]-FDP bound
None	520 ± 20
Heparin (500 IU)	539 ± 15
NaF (50 mM)	540 ± 18
Phenylphosphate (50 mM)	503 ± 24
NEM (10 mM)	510 ± 11
$ZnCl_2$ (10 mM)	728 ± 36*
$ZnCl_2$ (20 mM)	929 ± 40*
KCN (5 mM) Iodoacetate (10 mM)	575 ± 25*
KCN (20 mM) Iodoacetate (40 mM)	686 ± 32*

*Significant by Student's t-test ($p < 0{\cdot}01$).

Table 4. Effect of chlorpromazine and FDP on hypotonic haemolysis as an index of membrane stabilization. Mean ± S.D. of five experiments.

Addition	Antihaemolytic effect (IC_{50})
FDP	$2(\pm 0{\cdot}6) \times 10^{-5}$M
Chlorpromazine	$1(\pm 0{\cdot}2) \times 10^{-5}$M

The hypoxic condition entails in any case a loss of the barrier effect of the membrane, while Zn, an inhibitor of the intracellular enzyme utilizing FDP as a substrate, increases the amount of FDP available for the phosphofructokinase activation.

The antihaemolytic effect of FDP, which is comparable to that of chlorpromazine, is compatible with the observed synergism between some effects of FDP and chlorpromazine.[19]

The fact that FDP seems to influence a number of different ionic channels in the cell membrane has suggested to us the possibility that it could be included in the class of compounds known as 'membrane stabilizers'.[20] FDP in fact influences the entry of Ca^{++} and possibly its recycling, together with the permeation of other cations such as K^+ and H^+ or anions such as PO_4^{--}, which may be an indiction of a general effect of membrane stabilization (expansion).

Membrane stabilization (expansion) is a phenomenon caused by various compounds, e.g. anaesthetics, tranquilizers, antihistaminics etc., resulting in the lipid portion of the membranes becoming more fluid. The phenomenon depends on the binding of the compounds to the membranes followed by a displacement of the Ca^{++} ions associated with the membrane proteins and a non-specific modification of different ion transport channels.[21]

The structure of FDP, on the other hand, could

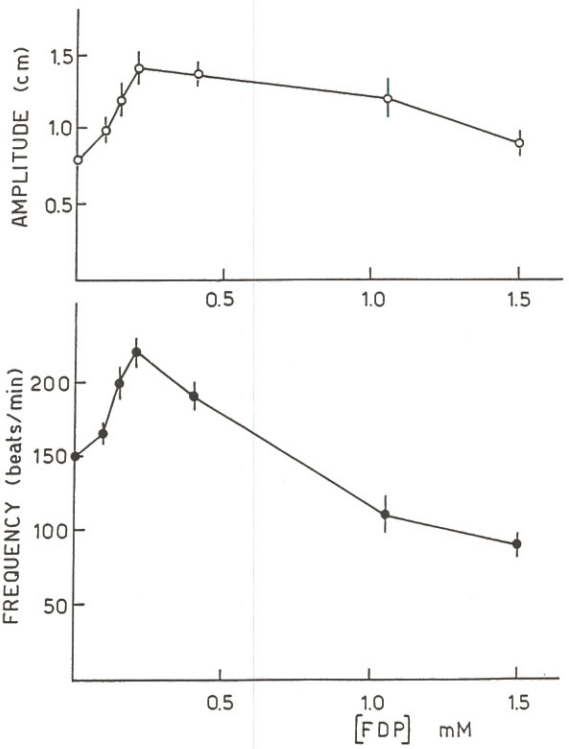

Figure 5. Effect of increasing concentration of FDP on the perfused heart. Mean ± S.E. of four experiments. A Hewlett-Packard model 775 4B polygraph was used with experimental conditions similar to those described by Pfaffman *et al.*[24] (see Materials and Methods). 1 cm = 12 mm Hg.

suggest its competition with the membrane phospholipids for the sites of their interaction with the proteins. A final result of this may be the creation of 'polar discontinuities'[22] of the membrane which might be associated with its partial permeability to the molecule, particularly enhanced when the membrane is damaged.

In terms of the so-called stimulus-response coupling[23] FDP, as an extracellular drug, after its interaction with the membrane could switch on a series of events which involve ionic fluxes as transducers, phosphofructokinase and FDP-bisphosphatase as effectors and intracellular FDP as a second messenger, in a cascade of effects having as a final target the aerobic glycolysis of the cell. The action of FDP can thus be explained both on the basis of a relative permeability of the damaged cell membrane to it, or as an action of the extracellular FDP at the membrane level which is transduced at the intracellular level. The biphasic effect of FDP on the perfused rat heart is consistent with what has been found by using rabbit heart[24] and the present results substantiate the claim that extracellular FDP, a membrane stabilizer, affects the ionic fluxes which induce the specific cellular responses observed.

REFERENCES

1. Manani, G., Galzigna, L., Costa, G., Tambuscio, B., Alati, G. L., Suma, V., Giovannoni, G., Volpe, A. and Giron. G. P. (1977). Clinical use of fructose-1,6-diphosphate. *Agressologie,* **18**, 207–212.
2. Markov, A. K., Neeley, W. A., Didlake, R. D., Bell, W. A. and Hellems, H. L. (1983). Biological and metabolic effects of fructose-1,6-diphosphate in man. *Clin. Res.,* **31**, 503A.
3. Lazzarino, G., Cattani, L., Costrini, R., Mulieri, L., Candiani, A. and Galzigna, L., (1984). Increase of intraerythrocytic fructose-1,6-diphosphate after incubation in whole human blood with fructose-1,6-diphosphate. *Clin. Biochem.,* **17**, 42–45.
4. Galzigna, L. and Rigobello, M. P. (1986). Proton and potassium fluxes in rat red blood cells incubated with sugar phosphates. *Experientia,* **42**, 138–139.
5. Fasolato, C., Deana, R. Rigoni, F. and Galzigna, L. (1987). Fructose-1,6-diphosphate improves the motility and other functions of bovine spermatozoa. *Med. Sci. Res. (Biochem.),* **15**, 203–204.
6. Markov, A. K., Oglethorpe, N., Blake, T. M., Lehan, P. H. and Hellems, H. K. (1980). Hemodynamic, electrocardiographic and metabolic effects of fructose-1,6-diphosphate on acute myocardial ischemia. *Am. Heart J.* **100**, 639–646.
7. Rigobello, M. P., Bianchi, M., Deana, R. and Galzigna, L. (1982). Interaction of fructose-1,6-diphosphate with some cell membranes. *Agressologie,* **23**, 63–66.
8. Chlouverakis, C. (1968). The lipolytic action of fructose-1,6-diphosphate. *Metabolism,* **17**, 708–716.
9. Cattani, L., Costrini, R., Lazzarino, G., Diana, M. and Galzigna, L. (1984). Fructose-1,6-diphosphate counteracts potassium effects on cardiac muscle. *Pharm. Res. Comm.* **16**, 785–793.
10. Magalini, S. I., Bondoli, A. and Scrascia, E. (1977) The action of phosphocreatine and fructose-1,6-diphosphate on blood *in vitro*. *Resuscitation,* **5**, 103–110.
11. Galzigna, L., Manani, G., Giron, G. P. and Burlina, A. (1977). Enzymatic assay of fructose-1,6-diphosphate for the measurement of its utilization by tissues. *Int. J. Vit. Nutr. Res.,* **47**, 88–91.
12. Fleckenstein, A. (1981). Pharmacology and electrophysiology of calcium antagonists. In: *Calcium Antagonism in Cardiovascular Therapy* (Zanchetti, A. and Krikler, D. M. eds) Amsterdam: Excerpta Medica, pp. 10–29.
13. Langendorff, D. (1985). Untersuchungen am uber lebenden Saugerthier herzen. *Pfluegers Arch.* **61**, 291–332.
14. Sartorelli, L., Ciman, M., Rizzoli, V. and Siliprandi, N. (1982). On the transport mechanisms of carnitine and its derivative in rat heart slices. *Ital. J. Biochem.* **31**, 261–268.
15. Seeman, P. and Roth, S. (1972). General anesthetics expand cell membrances at surgical concentrations. *Biochim. Biophys. Acta.,* **255**, 171–177.
16. Van Tol, A., Black, W. J. and Horecker, B. L. (1972). Activation of rabbit muscle fructose diphosphatase by EDTA and the effect of divalent cations. *Arch. Biochem. Biophys.* **151**, 591–596.
17. Lemaster, J. J., Di Guiseppi, J., Nieminen, A. L. and Herman, B. (1987). Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepaztocytes. *Nature,* **325**, 78–81.
18. Fisher, T. N. and Ginsberg, H. S. (1956). The reaction of influenza viruses with guinea pig polymorphonuclear leucocytes II. The reduction of white blood cell glycolysis by influenza viruses and receptor-destroying enzyme (RDE). *Virology,* **2**, 637–655.
19. Manani, G., Bossi, M., Tamuscio, B., and Galzigna, L. (1975). Analysis and interpretation of some metabolic effects of fructose-1,6-diphosphate (in Italian). *Acta Anaesth. Ital.,* **26**, 715–736.
20. Shanes, A. M. (1958). Electrochemical aspects of physiological and pharmacological action in excitable cells. Part I. The resting cell and its alteration by extrinsic factors. *Pharm. Rev.* **10**, 58–164.
21. Seeman, P., Staiman, A., Lee, T. and Chan-Wong, M. (1974). The membrane action of tranquilizers in relation to neuroleptic-induced parkinsonism and tardive dyskinesia. In: *The Phenothiazines and Related Drugs* (Forrest, T.S., Carr, C. J. and Usdin, E., eds) New York: Raven Press, pp. 137–148.
22. Watkins, J. C. Pharmacological receptors and general permeability phenomena of cell membranes. *J. Theoret. Biol.,* **9**, 37–50.
23. Roth, Z., Chayen, N. and Dikstein, S. (1983). The involvement of the intracellular redox state and pH in the metabolic control of stimulus-response coupling. *Int. Rev. Cytol.,* **85**, 39–61.
24. Pfaffman, M. A., Terry, J., Grillis, M. and Markov, A. (1983). The effects of fructose-1,6-diphosphate on the isolated rabbit heart. *Res. Comm. Chem. Pathol. Pharmacol.,* **41**, 211–225.

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