Evidence for Extracellular Enzymic Activity of the Isolated Perfused Rat Heart

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1. The dissimilation of a number of externally added hexose phosphates and 5'-nucleotides by the perfused rat heart is described, and non-specific esterase and 5'-nucleotidase activity associated with the superficial cell membrane or vascular system has been demonstrated. 2. The rate of production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glucose 6-phosphate suggests that oxidation occurred after hydrolysis to glucose. The incorporation of isotope from $[\text{U-}^{14}\text{C}]$ glucose 6-phosphate into glycogen was small, and similar to that obtained with $[\text{U-}^{14}\text{C}]$ glucose as substrate. 3. Glucose 6-phosphate was also partially isomerized to fructose 6-phosphate. Similarly, fructose 6-phosphate was converted mainly into glucose 6-phosphate, but also into glucose and inorganic phosphate. When fructose 1,6-diphosphate was added to the perfusate, a mixture of glucose 6-phosphate, fructose 6-phosphate and triose phosphates accumulated in the medium approximately in the equilibrium proportions of the phosphohexose-isomerase and triose phosphate-isomerase reactions, together with inorganic phosphate and some glucose. Glucose 1-phosphate was hydrolysed to glucose, but was not converted into glucose 6-phosphate. Leakage of enzymes out into the perfusion fluid did not occur. 4. This demonstration that phosphohexose isomerase, triose phosphate isomerase and aldolase may react with extracellular substrates at an appreciable rate suggests that these enzymes are attached to the cell membrane.

Various enzyme activities such as phosphatases, polysaccharide-splitting enzymes, dehydrogenases and oxidases have been identified on the cell surface of erythrocytes, yeast, bacteria and fungi (Rothstein, 1954). In particular, adenosine-triphosphatase activity has been shown to be associated with the membrane fraction of erythrocytes (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Whittam, 1962), brain tissue and nerve (Skou, 1962; Aldridge, 1962; Schwartz, Bachelard & McIlwain, 1962), liver (Emmelot & Bos, 1962), kidney cortex (Wheeler & Whittam, 1962) and cardiac muscle (Schwartz, 1962; Audittore, 1962), and is thought to be involved in the active transport of ions across the cell membrane. Relatively little work has been done on intact whole organ preparations, although Shaw & Stadie (1957, 1959) have presented evidence which was interpreted in favour of localization of the entire Embden-Meyerhof pathway on the surface of rat diaphragm muscle. Likewise, Beloff-Chain *et al.*

(1964) have reported that externally added glucose 6-phosphate and glucose 1-phosphate affected glycogen synthesis in the isolated rat diaphragm.

During the course of preliminary experiments on the effect of externally added hexose phosphates on the metabolism of glucose in the perfused rat heart, we observed powerful non-specific phosphatase and esterase activity, and certain other glycolytic enzymic activities which seemed to be associated with the external cell membrane or vascular cell surface. This work is the subject of the present report.

MATERIALS AND METHODS

Perfusion technique. The perfusion apparatus was based on the design of Bleehe & Fisher (1954) with the exception that a water-jacketed coarse 20 mm. fritted-glass filter (Fisher Scientific Co.) was used as a combined filter and upper reservoir. With this type of filtration, flow rates of 5–8 ml./min. were maintained for the duration of the perfusion. Male rats of the Sprague-Dawley strain, weighing 220–280 g., were used. The animals had unrestrained access to food and water before they were killed, and were anaesthetized with $\text{O}_2 + \text{CO}_2$ (1:1, v/v). The perfusion technique was similar to that described by Williamson & Krebs

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(1961). The basic perfusion fluid was Krebs bicarbonate medium (Krebs & Henseleit, 1932) containing half of the recommended calcium, and the perfusion temperature was 37°.

Reagents. Glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt) and glucose 1-phosphate (potassium salt) were obtained from Sigma Chemical Co., and had specified purity of 98–99%. Fructose 6-phosphate (sodium salt) was also obtained from Sigma Chemical Co., and contained 11% of glucose 6-phosphate and 1% of fructose 1,6-diphosphate. Glucose 6-phosphate and fructose 1,6-diphosphate were added to the basic perfusion fluid with no correction made for the increase of sodium. Glucose 1-phosphate was added to a modified Krebs bicarbonate medium containing one equivalent less of potassium as potassium chloride for each equivalent of potassium added with the glucose 1-phosphate.

[U-¹⁴C]Glucose 6-phosphate (U, uniformly labelled) was prepared from [U-¹⁴C]glucose as described by DiPietro & Weinhouse (1962), and was converted into the sodium salt before use. The specific activity of the [U-¹⁴C]glucose 6-phosphate in the perfusate was approx. 130 000 counts/min./μmole. All enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H. through the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Analytical methods. Glucose and glycogen were measured by the methods used by Williamson & Krebs (1961). Lactate was determined according to Horn & Bruns (1956), phosphate according to Lowry & Lopez (1946), and fructose by the method used for inulin by Fisher & Lindsay (1956).

Glucose 6-phosphate was determined by measuring the change in extinction at 340 mμ in a Beckman DU spectrometer after addition of glucose 6-phosphate dehydrogenase to cuvettes containing 0.5 ml. of diluted perfusate and 2.0 ml. of reaction mixture. The reaction mixture was freshly prepared and contained 0.04 M-glycylglycine, pH 7.5, 0.12 M-MgCl₂ and 0.6 mg. of NADP/ml. The glucose 6-phosphate dehydrogenase was free of hexokinase, phosphohexose-isomerase and phosphoglucumutase activity. Fructose 6-phosphate was assayed in the same reaction medium as glucose 6-phosphate by the addition of phosphohexose isomerase after the first reaction had reached completion, and glucose 1-phosphate was assayed in a similar fashion with the aid of phosphoglucumutase.

Dihydroxyacetone phosphate was assayed by measuring the change in extinction at 340 mμ upon the addition of α-glycerophosphate dehydrogenase to a reaction mixture of 1.0 ml. of diluted perfusate (1:5) and 1.5 ml. of assay buffer. The assay buffer contained 0.05 M-triethanolamine, pH 7.5, 5 mM-EDTA and 0.1 mg. of NADH/ml. Glyceraldehyde 3-phosphate was assayed in the same reaction media as above after the reaction had reached completion by measuring the additional decrease in extinction upon addition of triose phosphate isomerase. Fructose 1,6-diphosphate was assayed in a similar reaction medium to the above by using 0.2 ml. of diluted perfusate and 2.3 ml. of assay buffer. Triose phosphate isomerase and α-glycerophosphate isomerase were added first and the reaction was allowed to go to completion before the addition of aldolase. Calculations were based on a molar extinction 6.22×10^3 for NADH (NADPH) at 340 mμ for 1 cm. light-path.

Radioactive techniques. Radioactive solutions were counted at about 5° (40°F) in a Nuclear-Chicago liquid-scintillation spectrometer with 15 ml. of a dioxan-naphtha-

lane solvent per vial. The solvent was prepared by dissolving 100 g. of redistilled naphthalene and 40 g. of Cab-O-Sil (purchased from Raw Materials Co., 140 Federal Street, Boston 10, Mass., U.S.A.) in 1 l. of dioxan, followed by the addition of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene. The efficiency of counting was 57–60%. ¹⁴CO₂ was collected over 30 min. time-intervals by passing the effluent gas mixture from the heart-perfusion apparatus through 2 N-NaOH, and 0.5 ml. of this solution was used for counting. Total counts in the medium, and counts present in [¹⁴C]bicarbonate at different times during perfusion, were obtained from 0.5 ml. samples of perfusate diluted with 1 ml. of 0.1 N-NaOH. A portion (0.4 ml.) of this solution was used to obtain the total counts in the perfusate. Bicarbonate counts were obtained by volatilizing with acid the ¹⁴CO₂ from 1 ml. of the above solution into 0.2 ml. of 5 N-NaOH. The NaOH was diluted by the addition of 0.5 ml. of water and a 0.5 ml. portion was used for counting. At the end of the perfusion, the heart was flushed with 5 ml. of cold non-radioactive medium to remove radioactive perfusate from the extracellular space and capillary bed, and then treated in the normal manner for the determination of glycogen and dry weight (Williamson & Krebs, 1961). A portion of the neutralized glycogen hydrolysate was taken to obtain the incorporation of ¹⁴C into polysaccharide. An estimate of the total counts present in other fractions of the heart was obtained by counting a diluted portion of the ethanolic-KOH supernatant after glycogen precipitation.

RESULTS

Glucose 6-phosphate metabolism. Glucose 6-phosphate (5 mM) disappeared from the perfusion medium at a rapid rate over the first 15 min., but the rate of disappearance declined gradually during the succeeding 75 min. Removal of glucose 6-phosphate was associated with the production of fructose 6-phosphate and glucose, and a delayed but gradual increase of inorganic phosphate (Fig. 1). This pattern of glucose 6-phosphate metabolism was consistently seen with different hearts, although the magnitude of the changes varied somewhat from heart to heart. The hearts continued to beat well for the 90 min. perfusion period, and the visible performances of the hearts was in no way different from that of hearts perfused in the presence of glucose. During the first 60 min. of perfusion, the molar ratio fructose 6-phosphate:glucose 6-phosphate gradually increased to a constant value of 0.22. After the first 30 min. of perfusion, the disappearance of glucose 6-phosphate when corrected for the formation of fructose 6-phosphate closely paralleled the appearance of inorganic phosphate. There was no appearance of glucose 1-phosphate, fructose 1,6-diphosphate or triose phosphate in the perfusate, but some free fructose was detected during the first 30 min. of perfusion.

Other experiments showed that the rate of glucose 6-phosphate disappearance was independent of the presence of glucose, which was added to the perfusate over the concentration range 0.5–10 mM.

Likewise the disappearance of glucose 6-phosphate was not affected by insulin (2×10^{-3} units/ml.), although the accumulation of glucose in the medium under these circumstances was decreased by over 80%.

The metabolism of glucose 6-phosphate was also investigated with [U- 14 C]glucose 6-phosphate, as shown in Table 1. In this experiment, with an initial concentration of 3mm-glucose 6-phosphate, net formation of fructose 6-phosphate occurred only during the first 30min. of perfusion and fructose 6-phosphate disappeared at a slow rate along with glucose 6-phosphate over the succeeding 30 min. time-intervals. The concentration of glucose 6-phosphate at the end of perfusion was 1.5–2.0mm. The ratio of the concentration of fructose 6-phosphate to that of glucose 6-phosphate

increased from 6 to 22% during the first 30min. of perfusion, and thereafter remained constant. Glucose was formed throughout the perfusion, but the rate of glucose appearance declined towards the end of the experiment. Net lactate formation occurred only during the first 30min., and lactate was subsequently utilized. The counts disappearing from the perfusate after correction for [14 C]bicarbonate are expressed as hexose equivalents in the last column of Table 1. These remained constant at 11–12 μ moles/g. dry wt. over the three successive 30 min. intervals. From Table 1 it is apparent that there was a marked lag in the appearance of counts in the respiratory carbon dioxide, suggesting that [U- 14 C]glucose 6-phosphate was metabolized oxidatively by prior hydrolysis to [U- 14 C]glucose. Thus appearance of $^{14}\text{CO}_2$ over the first, second and third half-hour intervals accounted for 6, 43 and 70% respectively of the medium count decrease over these time-intervals. Of the total medium count decrease over the 90min. of the experiment (equivalent to a disappearance of 34.5 μ moles of [U- 14 C]glucose 6-phosphate/g. dry wt.), 39% appeared as $^{14}\text{CO}_2$, 9% in glycogen and 37% in the ethanolic-potassium hydroxide supernatant of the heart digest after polysaccharide precipitation.

In a similar experiment in which four hearts were perfused initially with 5mm-[U- 14 C]glucose, there was also a lag in the appearance of $^{14}\text{CO}_2$, though less pronounced than with [U- 14 C]glucose 6-phosphate. The production of $^{14}\text{CO}_2$ over the first, second and third half-hour intervals accounted on average for 19, 88 and 94% of the medium count decrease, which in turn represented 75–86% of the glucose uptake (45–55 μ moles/g. dry wt./30min.) over the three successive half-hour intervals. Of the total medium count decrease over the 90min. of the experiment (121 ± 6 μ moles/g. dry wt.), 53% appeared as $^{14}\text{CO}_2$, 8% in glycogen and 28% in the ethanolic-potassium hydroxide supernatant of the heart digest. Glucose 6-phosphate was not detected in the perfusate in these experiments.

Fructose 6-phosphate metabolism. When fructose 6-phosphate (3.3mm) was added to the perfusate, 183 μ moles of fructose 6-phosphate/g. dry wt.

Fig. 1. Metabolism of glucose 6-phosphate by the perfused rat heart. The heart was perfused with 20ml. of fluid containing 5mm-glucose 6-phosphate and 1ml. samples were taken for analysis at the times stated. O, Disappearance of glucose 6-phosphate; ●, appearance of fructose 6-phosphate; Δ, appearance of inorganic phosphate; ▲, appearance of glucose.

Table 1. Metabolism of [U- 14 C]glucose 6-phosphate by the perfused rat heart

Rat hearts were perfused with medium containing 3mm-[U- 14 C]glucose 6-phosphate. The initial perfusate volume was 15ml. and two 0.5ml. samples were removed for analysis and counting after 0, 30, 60 and 90 min. of perfusion. Values shown are means $\pm$ s.e.m. of four hearts, and are expressed in μ moles/g. dry wt.

Time-interval (min.)	Glucose 6-phosphate disappearance	Fructose 6-phosphate appearance	Glucose appearance	Lactate change	$^{14}\text{CO}_2$ appearance	Inorganic phosphate appearance	Medium count decrease (hexose equiv.)
0–30	69.4 ± 3.9	43.1 ± 1.8	8.8 ± 2.3	24.2 ± 3.3	0.7 ± 0.1	8 ± 5.1	11.7 ± 1.3
30–60	18.4 ± 1.6	-5.2 ± 1.2	7.3 ± 1.8	-16.3 ± 1.2	4.6 ± 0.6	45 ± 1.6	11.0 ± 1.4
60–90	17.1 ± 3.0	-5.7 ± 0.5	4.6 ± 2.1	-3.2 ± 1.7	8.3 ± 1.4	31 ± 2.6	11.8 ± 1.5

Fig. 2. Metabolism of fructose 6-phosphate by the perfused rat heart. The heart was perfused with 18 ml. of fluid containing 3.3 mM-fructose 6-phosphate and 1 ml. samples were taken for analysis at the times stated. O, Disappearance of fructose 6-phosphate; Δ , appearance of glucose 6-phosphate; $\bullet$, appearance of inorganic phosphate; $\blacktriangle$, appearance of glucose.

disappeared from the medium over the first 60 min. of perfusion and 143 μ moles of glucose 6-phosphate/g. dry wt. appeared (Fig. 2). The appearance of inorganic phosphate in the medium was delayed, as with hearts perfused initially with glucose 6-phosphate, but the total phosphate formation (46 μ moles/g. dry wt.) was approximately equal to the net hexose phosphate disappearance (40 μ moles/g. dry wt.). A small amount of glucose appeared in the medium. Fructose 1,6-diphosphate was not detected. This pattern of fructose 6-phosphate metabolism was confirmed in further experiments in which four hearts were perfused with medium containing 2.6 mM-fructose 6-phosphate for a total time of 90 min.

Glucose 1-phosphate metabolism. When glucose 1-phosphate (2.5 mM) was added to the perfusate it disappeared at a linear rate of 50 μ moles/g. dry wt./hr. after the first 15 min. of perfusion. Glucose accumulated in the medium and accounted for 44% of the glucose 1-phosphate disappearance (Table 2). There was a lag in the appearance of inorganic phosphate over the first 15 min. of perfusion, but the total phosphate formation was approximately equal to the glucose 1-phosphate disappearance over the 60 min. of perfusion. No significant conversion of glucose 1-phosphate into glucose 6-phosphate could be demonstrated.

Fructose 1,6-diphosphate metabolism. Fructose 1,6-diphosphate (2.5 mM) disappeared from the medium at an approximately constant rate of

Table 2. Metabolism of glucose 1-phosphate by the perfused rat heart

Rat hearts were perfused with medium containing 2.5 mM-glucose 1-phosphate. The initial perfusate volume was 15 ml. and 1 ml. samples were removed for analysis. Values shown are means $\pm$ S.E.M. of four hearts and are expressed in μ moles/g. dry wt.

Time of perfusion (min.)	Glucose 1-phosphate disappearance	Glucose appearance	Inorganic phosphate appearance
15	19.5 $\pm$ 1.8	7.3 $\pm$ 0.7	0.6 $\pm$ 5.7
30	31.2 $\pm$ 1.6	14.3 $\pm$ 1.2	17.0 $\pm$ 6.7
45	44.5 $\pm$ 2.6	20.5 $\pm$ 1.8	37.0 $\pm$ 8.4
60	57.2 $\pm$ 3.5	25.3 $\pm$ 2.2	51.6 $\pm$ 9.8

Table 3. Metabolism of fructose 1,6-diphosphate by the perfused rat heart

Rat hearts were perfused with medium containing 2.5 mM-fructose 1,6-diphosphate. The initial perfusate volume was 15 ml. and 1 ml. samples were removed for analysis after 0, 15, 30, 60 and 90 min. of perfusion. Values shown are means $\pm$ S.E.M. and are expressed in μ moles/g. dry wt.

Time of perfusion (min.)	No. of hearts	Fructose 1,6-diphosphate disappearance	Glucose 6-phosphate appearance	Fructose 6-phosphate appearance	Triose phosphate appearance	Inorganic phosphate appearance
15	4	27.9 $\pm$ 1.6	4.9 $\pm$ 0.3	1.3 $\pm$ 0.06	9.3 $\pm$ 1.6	1.9 $\pm$ 1.6
30	6	46.3 $\pm$ 3.3	8.2 $\pm$ 0.7	1.7 $\pm$ 0.2	14.3 $\pm$ 1.6	19.2 $\pm$ 4.5
60	6	75.7 $\pm$ 3.8	15.3 $\pm$ 1.0	2.8 $\pm$ 0.2	20.5 $\pm$ 2.2	59.1 $\pm$ 4.7
90	2	107.4	20.0	4.9	27.0	95.7

64 μ moles/g. dry wt./hr. after the first 15 min. of perfusion (Table 3). During the first 15 min., the rate was approximately twice as great as in succeeding 15 min. time-intervals. Removal of fructose 1,6-diphosphate from the medium was associated with the appearance of glucose 6-phosphate, fructose 6-phosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and inorganic phosphate. Glucose appearance was less than 1 μ mole/g. dry wt./hr. The mean concentrations of glucose 6-phosphate and fructose 6-phosphate in the medium after 60 min. of perfusion were 0.151 mM and 0.030 mM respectively and an approximate ratio of 80% of glucose 6-phosphate to 20% of fructose 6-phosphate was observed after 15 min. of perfusion. Dihydroxyacetone phosphate accounted for 83–95% of the triose phosphates during the course of the experiments. Formation of glucose 6-phosphate, fructose 6-phosphate and triose phosphates accounted for 50% of the fructose 1,6-phosphate removed from the perfusate.

Specificity of phosphatase activity. The previous experiments have demonstrated that the perfused rat heart will hydrolyse glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate and fructose 1,6-phosphate, when these compounds are added to the perfusate, with the production of 40–60 μ moles of inorganic phosphate/g. dry wt./hr. Table 4 shows that galactose 1-phosphate, phenyl phosphate and mannose 6-phosphate are similarly

hydrolysed. With all these compounds there was a lag in the production of inorganic phosphate over the first 15 min. of perfusion. Control experiments in which hearts were perfused with substrate-free medium show that 6 μ moles of phosphate/g. dry wt. were removed from the perfusate over the first 15 min., and that a similar amount of phosphate was gradually returned over the ensuing 45 min. Hence the lag in the appearance of inorganic phosphate in the medium with hearts perfused with phosphorylated compounds may be accounted for by an initial increase in the phosphate content of the heart.

Table 5 shows that a number of 5'-nucleotides, when added to the perfusate at an initial concentration of 1 mM, were rapidly hydrolysed with the production of 220–260 μ moles of inorganic phosphate/g. dry wt. within the first 10 min. of perfusion. The phosphate concentration of the medium increased by more than 2 mM during this time, indicating hydrolysis to monophosphorylated derivatives and nucleosides. AMP (1 mM) was 50% hydrolysed after 5 min. and 80% hydrolysed in 10 min.

Tests for stability of substrates and/or possible enzyme leakage into perfusate. Stability of the hexose phosphates under the experimental perfusion conditions was tested by allowing 15 ml. portions of perfusion medium containing each of the hexose phosphates used in this investigation to circulate

Table 4. *Phosphatase activity of perfused rat heart*

Hearts were perfused with 18 ml. of medium and 1 ml. samples were taken for phosphate analysis. Phosphate changes are expressed in μ moles/g. dry wt. Uptake is represented by a negative sign.

Substrate added to perfusate	Perfusion time (min.) No. of hearts	Formation of inorganic phosphate					
		... 5	10	15	30	45	60
None	2	0.6	–3.9	–6.0	–0.9	3.9	6.6
1 mM-Galactose 1-phosphate	3	2.1	1.3	2.9	15.3	32.2	37.0
2 mM-Phenyl phosphate	3	3.9	5.8	5.1	23.3	30.0	40.6
5 mM-Mannose 6-phosphate	2	–1.7	–1.2	2.4	7.8	19.8	32.5

Table 5. *Hydrolysis of nucleotides by the perfused rat heart*

Rat hearts were perfused with 15 ml. of medium containing the added nucleotide at a concentration of 1 mM.

Time of perfusion (min.) Additions to perfusate	Concn. of inorganic phosphate (mM)				Formation of inorganic phosphate (μ moles/g. dry wt.)		
	... 0	5	10	15	5	10	15
ATP	1.33	3.43	3.65	3.66	241	265	266
ITP	1.39	3.04	3.63	3.73	179	239	248
GTP	1.33	2.63	3.42	3.64	147	229	254
UTP	1.32	2.64	3.48	3.71	137	217	238
CTP	1.27	2.47	3.41	3.42	136	236	237
AMP	1.19	1.75	1.95	2.02	58	81	84

in the perfusion apparatus with gas equilibration but in the absence of a heart. Samples of fluid were removed for analysis after 30, 60 and 90 min. Changes in concentration of the hexose phosphates and inorganic phosphate in the medium were found to be very small (2% or less).

To assess the extent of enzyme leakage into the perfusate, hearts were removed from the circulation after 60 min. of perfusion, and the perfusate was allowed to recirculate for a further 30 min. Changes in concentration of hexose phosphates, triose phosphates and inorganic phosphate in the perfusate without the heart were compared to changes over the same time-interval (60–90 min.) in the presence of the heart. In all cases removal of the heart from the circulation virtually stopped all concentration changes, indicating that enzyme leakage from the heart into the medium during the perfusion could not account for the observed glycolytic activities. This demonstration of the integrity of the cell membrane of the rat heart during perfusion contrasts with the behaviour of the rat diaphragm and skeletal muscle, which release aldolase into the incubation medium (Zierler, 1957).

DISCUSSION

The present study has established that 5'-nucleotides and sugar phosphates are hydrolysed when added to the perfusion medium of the rat heart. In addition, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate in the perfusate undergo certain metabolic transformations similar to those of the Embden–Meyerhof pathway. Enzymes found to be accessible to their respective substrates in the extracellular compartment were phosphohexose isomerase, aldolase and triose phosphate isomerase. No phosphoglucotransferase was detected in this manner.

Three possibilities arise in the interpretation of these results: (1) certain enzymes of the normal glycolytic pathway are located on superficial cell membranes in such a manner that their active sites are accessible to substrate on both the intra- and extra-cellular sides of the membrane; (2) the phosphorylated hexoses and trioses can enter heart cells; (3) the observed metabolic transformations are caused by deterioration of the cell-membrane structure with consequent loss of intracellular enzymes into the perfusate. This last possibility may be discounted since control experiments showed that no further concentration changes of hexose phosphates or inorganic phosphate occurred in the perfusate once the heart had been removed from the perfusion apparatus. Although the experiments do not directly disprove penetration of glucose 6-phosphate into the cell, isomerization of fructose 6-phosphate in the intracellular com-

partment and diffusion of fructose 6-phosphate back into the perfusate, this possibility is highly unlikely for a number of reasons. In the first place the charge on the hexose phosphate makes free permeability of these compounds improbable. Secondly, if glucose 6-phosphate penetrated the cell, it would be expected that there would be a considerable incorporation of counts from [^{14}C]glucose 6-phosphate into glycogen, since glucose 6-phosphate is an activator of glycogen synthetase (Leloir, Olavarria, Goldemberg & Carminatti, 1959). In fact, however, glycogen synthesis from [^{14}C]glucose 6-phosphate was negligible. Possibly the most important argument against permeability of sugar phosphates is that if the fructose 6-phosphate which accumulated in the medium after the addition of glucose 6-phosphate was derived from intracellular fructose 6-phosphate, this would entail movement against a concentration gradient. In view of these considerations the most probable explanation for the experimental findings is that the enzymes in question are bound to the heart-cell membrane or perhaps to the walls of the vascular system. Localization of the so-called 'soluble' glycolytic enzymes has not been adequately investigated, but it is possible that all intracellular enzymes are bound to membranes, whether to the mitochondrial membrane, the membrane of the endoplasmic reticulum or to the cell membrane (Siekevitz, 1962).

The phosphatase activity demonstrated in the present investigation showed widely different activities towards 5'-nucleotide phosphates on the one hand and hexose phosphates on the other. Thus the nucleotides were hydrolysed with the production of 1600–2700 μmoles of inorganic phosphate/g. dry wt./hr., whereas glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate, galactose 1-phosphate, mannose 6-phosphate and phenyl phosphate yielded only 30–50 μmoles of inorganic phosphate/g. dry wt./hr. Whether these different rates represent different specificities of the same enzyme or separate enzymic sites is not at present clear.

Cytochemical and electron-microscope studies have revealed adenosine-triphosphatase and 5'-nucleotidase activities in the plasma membrane of liver (Essner, Novikoff & Masek, 1958), kidney (Spater, Novikoff & Masek, 1958) and probably diaphragm muscle (Padykula & Gauthier, 1963). Molbert, Duspiva & Deimling (1960) showed that the phosphatase activity of the plasma membrane of kidney tubules was non-specific, since activity was also demonstrated with phenyl phosphate. Muscatello, Andersson-Cedergren, Azzone & Decken (1961) describe three types of muscle adenosine triphosphatase: (1) a myofibrillar Mg^{2+} - Ca^{2+} -stimulated enzyme; (2) a mitochondrial Mg^{2+} -

stimulated enzyme; (3) a sarcotubular Mg^{2+} -stimulated and Ca^{2+} -inhibited enzyme, which is thought to be involved in the relaxation of muscle fibres. The phosphatase observed in the present experiments may be similar to the sarcotubular adenosine triphosphatase described by Padykula & Gauthier (1963) and Muscatello *et al.* (1961), since the cell membrane is known to be deeply invaginated (Siekevitz, 1959).

It is considered, however, that the biochemical significance of membrane-phosphatase activities of the type observed in the present experiments is obscure, and may not be related to known physiological events. The very high and non-specific nature of the activity towards 5'-nucleotides argues against it being involved in the active transport of cations.

If the present demonstrations of phosphatase activities on the cell surface of the rat heart apply to different muscle tissues, then the experiments of the other investigators who added hexose phosphates or adenine nucleotides to the external medium may need re-evaluation. The improved contractility of the rat diaphragm (Ellis, 1955), frog heart (Freedman, 1930; Lidner & Rigler, 1931) and rabbit heart (Gialdroni-Grassi, 1957), after addition of glucose 6-phosphate or glucose 1-phosphate, may for instance be caused by the increased inorganic phosphate concentration as a result of hydrolysis of the phosphate ester. Similarly, the stimulation of xylose transport in rat diaphragm muscle on addition of certain hexose phosphates and ATP observed by Newsholme & Randle (1961) was probably due to inorganic phosphate, which also stimulates xylose transport (Randle & Smith, 1958). The hydrolysis of ATP by rat diaphragm preparation has, in fact, been demonstrated by Marsh & Haugaard (1957). Shaw & Stadie (1957, 1959) observed that the specific activity of glucose and lactate was decreased after addition of hexose phosphates to rat diaphragm preparations incubated with [^{14}C]glucose. This demonstrates that some of the sugar phosphate was converted into glucose, a fact the authors neglected to consider carefully in their interpretation of their results.

In conclusion, it is thought that, although the surface enzymic activities described in the present work may be of little significance in the metabolism of the rat heart *in vivo*, the demonstration of the existence of these activities is of interest and may be of value in the interpretation of the results of others who are using this or similar muscle preparations *in vitro*.

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REFERENCES

- Aldridge, W. N. (1962). *Biochem. J.* **83**, 527.
 Audittore, J. V. (1962). *Proc. Soc. exp. Biol., N.Y.*, **110**, 595.
 Beloff-Chain, A., Betto, P., Catanzaro, R., Chain, E. B., Longinotti, L., Masi, I. & Pocchiari, F. (1964). *Biochem. J.* **91**, 620.
 Bleehen, N. M. & Fisher, R. B. (1954). *J. Physiol.* **123**, 260.
 DiPietro, D. L. & Weinhouse, S. (1962). *Biochemistry*, **1**, 455.
 Dunham, E. T. & Glynn, J. M. (1961). *J. Physiol.* **156**, 274.
 Ellis, S. (1955). *Amer. J. med. Sci.* **229**, 218.
 Emmelot, P. & Bos, C. L. (1962). *Biochim. biophys. Acta*, **58**, 375.
 Essner, E., Novikoff, A. B. & Masek, B. (1958). *J. biophys. biochem. Cytol.* **4**, 711.
 Fisher, R. B. & Lindsay, D. B. (1956). *J. Physiol.* **131**, 526.
 Freedman, N. E. (1930). *Amer. J. Physiol.* **92**, 107.
 Gialdroni-Grassi, G. (1957). *Arch. int. Pharmacodyn.* **121**, 401.
 Horn, H. D. & Bruns, F. H. (1956). *Biochim. biophys. Acta*, **21**, 378.
 Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
 Leloir, L. F., Olavarria, J. M., Goldemberg, S. H. & Carminatti, H. (1959). *Arch. Biochem. Biophys.* **81**, 508.
 Lidner, F. & Rigler, R. (1931). *Pflüg. Arch. ges. Physiol.* **226**, 697.
 Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
 Marsh, J. B. & Haugaard, N. (1957). *Biochim. biophys. Acta*, **23**, 204.
 Molbert, E. R. G., Duspiva, F. & Deimling, O. H. von (1960). *J. biophys. biochem. Cytol.* **7**, 387.
 Muscatello, U., Andersson-Cedergren, E., Azzone, G. F. & Decken, A. von der (1961). *J. biophys. biochem. Cytol.* **10** (suppl.), 201.
 Newsholme, E. A. & Randle, P. J. (1961). *Biochem. J.* **80**, 655.
 Padykula, H. A. & Gauthier, G. F. (1963). *J. Cell Biol.* **18**, 87.
 Post, R. L., Merritt, C. R., Kinsolving, C. R. & Albright, C. D. (1960). *J. biol. Chem.* **235**, 1796.
 Randle, P. J. & Smith, G. H. (1958). *Biochem. J.* **70**, 490.
 Rothstein, A. (1954). *Protoplasmatologia*, **2**, E-4, 1.
 Schwartz, A. (1962). *Biochem. biophys. Res. Commun.* **9**, 301.
 Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
 Shaw, W. N. & Stadie, W. C. (1957). *J. biol. Chem.* **227**, 115.
 Shaw, W. N. & Stadie, W. C. (1959). *J. biol. Chem.* **234**, 2491.
 Siekevitz, P. (1959). In *Regulation of Cell Metabolism*, p. 17. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
 Siekevitz, P. (1962). In *The Molecular Control of Cellular Activity*, p. 143. Ed. by Allen, J. M. New York, Toronto and London: McGraw-Hill Book Co.
 Skou, J. C. (1962). *Biochim. biophys. Acta*, **58**, 314.
 Spater, H., Novikoff, A. B. & Masek, B. (1958). *J. biophys. biochem. Cytol.* **4**, 765.
 Wheeler, K. P. & Whittam, R. (1962). *Biochem. J.* **85**, 495.
 Whittam, R. (1962). *Biochem. J.* **84**, 110.
 Williamson, J. R. & Krebs, H. A. (1961). *Biochem. J.* **80**, 540.
 Zierler, K. L. (1957). *Amer. J. Physiol.* **190**, 201.