anesthetized beagle and mongrel dogs in our studies was higher than reported in the literature(10). This discrepancy might be due to our use of an extraction ratio (percentage of dye removed by liver per unit of time determined by difference between arterial and hepatic venous dye concentration over an approximate 15-second time period) of 14% as reported by Ketterer *et al*(2) in anesthetized dogs. The same investigators have reported that normal, unanesthetized human subjects exhibit an average extraction of ICG of 74%(11). If the extraction ratio in the unanesthetized dog is significantly greater than the 14% used in our calculations, the estimated hepatic blood flow in the present series of experiments may be lower than we have observed.

Summary. The clearance rate of indocyanine green was determined in 24 beagle dogs and 12 mongrel dogs. The results indicate that in the unanesthetized state, the purebred animals exhibited an increased rate of clearance of the dye and decreased T-½ when compared with the mongrel dogs. Several possibilities were discussed to account for the differences noted.

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Penetration of ATP into the Myocardium.* (30242)

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It is generally assumed that nucleoside triphosphates do not enter intact into cells from the extracellular space. This has been shown to be the case for liver(1) and ascites tumor cells(2). During the course of studies on the effect of cardiac glycosides on myocardial nucleotide metabolism, it was of interest to determine whether ATP could penetrate into myocardial cells without chemical change.

The problem was studied by determination of the ratio of the C^{14}- to P^{32}-ATP in the myocardium after perfusion of the isolated guinea pig heart with a mixture of (8-C^{14})ATP and (β, γ, P^{32})ATP of known isotope ratio. Any change in this ratio in the isolated myocardial ATP would indicate that chemical change had taken place during incorporation of the molecule into the cell.

Materials and methods. (8-C^{14})ATP, disodium salt, with a specific activity of 0.75 mC/mM was purchased commercially. (β, γ, P^{32})ATP with an initial specific activity of 7 mC/mM was prepared by the procedure of Pressman(3). Male guinea pigs weighing 200-400 g were maintained on a diet of Purina pellets supplemented with lettuce and carrots. The isolated guinea pig heart was prepared and perfused in the apparatus de-

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scribed by Morgan *et al*(4) which provides for both recirculation and washout of the perfusion fluid. The perfusion fluid was Krebs-Henseleit bicarbonate buffer, pH 7.4 (5) containing glucose, 5.5 mM, and equilibrated with 95% O_2-5% CO_2.

The heart was first perfused with buffer for 5 minutes, following which the isotope-containing buffer was recirculated through it for the appropriate time interval. Samples of the perfusate leaving the coronary circulation were removed from time to time for analysis. At the end of the perfusion the heart was washed out with isotope-free buffer to remove isotopic material from the vasculature. It was then dropped into liquid nitrogen and the ATP content and specific activity determined.

The frozen tissue was transferred to a cold room at —10°C and pulverized under liquid nitrogen. The frozen powder was brought quickly into 3 ml of 0.3 N perchloric acid at 0°C and rapidly homogenized without allowing the powder to thaw before coming into contact with the acid. After centrifugation of the homogenate at 5000 × *g* for 5 minutes and washing of the precipitate, the combined supernatants were brought to pH 6.8-7.0 with KOH. The $KClO_4$ was removed by centrifugation after standing for 30 minutes at 0°C. Aliquots of the extract were used for paper chromatographic separation of the nucleotides.

Concentration of nucleotides and nucleosides in the acidified perfusate samples was accomplished by absorption on acid-washed Norite A followed by elution with 10% pyridine in water(6). Aliquots were then chromatographed on paper with carrier compounds.

The labeled compounds were separated on Whatman 1 and 3 MM papers using the solvent systems: methanol : isopropanol : 25% NH_4OH:H_2O (45:30:15:10)(7) and isobutyric acid: 1.0 M NH_4OH:0.1 M Na_2 EDTA (100:60:1.6)(8). In those experiments in which it was desired to separate inorganic phosphate which travels with ATP in the acidic solvent and with AMP in the basic solvent from other compounds, the dried chromatogram was run ascendingly in the second dimension in isopropyl ether : n-butanol : 98% formic acid (40:30:20)(7). The spots were localized on the paper by exposing the chromatograms to UV light (254 mμ). The UV-absorbing spots along with blank paper of the same size were eluted with 0.1 N HCl and absorption at 290, 280, 260, and 250 mμ was measured. The concentration was calculated using the molar absorbency indices of Bock *et al*(9). Phosphate was determined by the method of Dryer *et al*(10).

Radioactivity of the eluted spots was assayed in a Tri-Carb liquid scintillation counter. Solvents used were those of Okita *et al*(11) and Bruno and Christian(12). Quenching was determined by use of an internal standard. Simultaneous assay of P^{32} and C^{14} was performed by the screening technique of Okita *et al*(13). The accuracy of the determination of the two isotopes was within 5% when the isotope ratio C^{14}/P^{32} was kept greater than 0.67.

Recovery experiments showed that hydrolysis of the labeled ATP to ADP and AMP was less than 5% during the extraction and chromatographic procedures. Recoveries of amounts of ATP added to the extract ranged from 90-103%. Radiochemical purity was established with the two solvent systems, and the ultraviolet absorption ratios of the isolated compounds agreed with those of Bock *et al*(9). The molar ratio of adenine : ribose : phosphate was also established to be within experimental error.

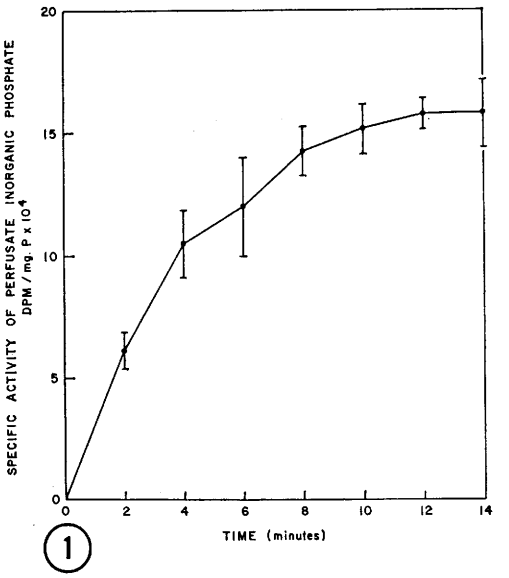
Results. Table I shows the isotope ratio in myocardial ATP after recycled perfusion for 5 and 15 minutes. The ratio of C^{14} specific

TABLE I. Change in Ratio of C^{14}/P^{32} Specific Activity when ATP Is Incorporated into Guinea Pig Myocardium.

ATP isolated from	Ratio: $\frac{C^{14}\text{ specific activity}}{P^{32}\text{ specific activity}}$
Perfusion fluid at 0 time	.67
Myocardium after 5 min perfusion	76.2 ± 6.2
Myocardium after 15 min perfusion	60.1 ± 9.2

Perfusion in 25 ml of buffer with recirculation. P^{32} determinations corrected for decay by counting (β, γ, P^{32})ATP sample with each determination. The values indicate means and standard errors of 3 experiments.

activity to P^{32} specific activity was approximately 100 times greater in the myocardial ATP than in the ATP originally perfused.



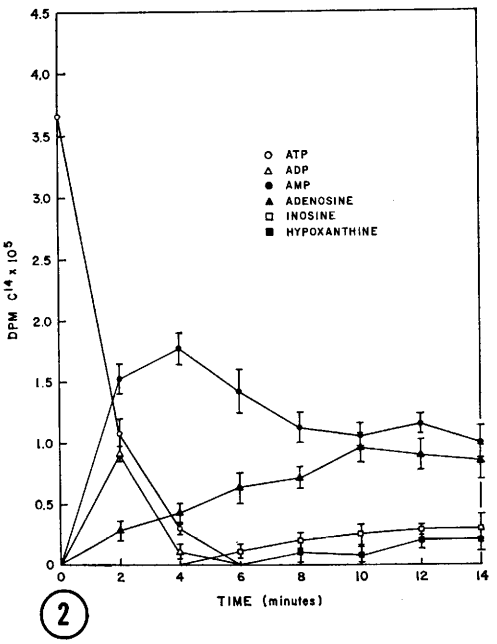


FIG. 1. Change in specific activity of perfusate inorganic phosphate during recycled perfusion of myocardium with ATP-β, γ-P^{32}. Hearts perfused with 50,000 DPM ATP-β, γ-P^{32} in 25 ml of perfusion fluid. Means and standard errors for 4 experiments.

FIG. 2. Appearance of C^{14}-activity in perfusate degradation products of ATP during recycled perfusion of myocardium with ATP-8-C^{14}.

TABLE II. Dephosphorylation of Nucleotide Triphosphates During a Single Perfusion Through the Heart.

Compound	Amt recovered in perfusate (μmoles)*	% of nucleoside triphosphate perfused†
ITP	.16 ± .05	11.0
IDP	.00	—
IMP	.89 ± .10	61.0
Inosine	.37 ± .08	25.3
GTP	.15 ± .01	10.5
GDP	.00	—
GMP	.80 ± .09	55.9
Guanosine	.40 ± .05	28.0
UTP	.16 ± .01	10.7
UDP	.17 ± .08	11.3
UMP	.64 ± .20	42.7
Uridine	.49 ± .13	32.7
CTP	.00	—
CDP	.75 ± .26	62.5
CMP	.26 ± .08	21.7
Cytidine	.14 ± .10	11.6

* Means and standard deviations for 3 exp.

† All perfusions for 15 min. Amounts perfused: ITP, 1.46 ± .11 μmoles; GTP, 1.43 ± .22 μmoles; UTP, 1.50 ± .13 μmoles; CTP, 1.20 ± .09 μmoles. Remainder of nucleoside triphosphate-containing buffer taken through isolation procedure as a control for non-enzymatic hydrolysis.

The specific activity of the inorganic phosphate in the medium during the perfusion of the heart with (β, γ, P^{32})ATP was determined after absorption of the nucleotides on Norite A. As shown in Fig. 1 there was a rapid increase in specific activity of inorganic phosphate indicating its liberation from labeled ATP. Control experiments indicated that the dephosphorylation was not taking place in the perfusate. Analysis of the perfusate during perfusion with (8-C^{14})ATP indicated the appearance of dephosphorylation products of ATP, ADP and AMP, as well as of adenosine, inosine, and hypoxanthine, in the perfusate (Fig. 2). In a separate series of experiments unlabeled UTP, ITP, CTP and GTP were perfused without recirculation in concentrations of 10^{-5} M for 15 minutes to determine whether this dephosphorylation was specific for ATP. Analysis of the perfusates indicated varying degrees of dephosphorylation for all 4 triphosphates (Table II). The dephosphorylation of ATP was not inhibited by the presence of 2×10^{-7} M ouabain in the perfusion fluid.

Discussion. The results indicate that, in common with other tissues studied, the guinea

pig myocardium, while capable of incorporating part of the ATP molecule, does not take it up without chemical modification. If ATP were free to cross the cell wall without chemical reaction, one would expect the ratio of the specific activities of C^{14} to P^{32} to remain equal to that present in the perfusion fluid. Since the ratio is increased almost 100 times in the myocardial ATP, it appears that the purine moiety crosses the barrier much more readily than the whole molecule. From these experiments the passage of AMP cannot be excluded, since the P^{32}-labeled ATP was tagged only in the beta and gamma positions.

The rapid incorporation of (8-C^{14}) adenosine into ATP in the isolated cat heart along with formation of inosine and hypoxanthine in the perfusate has been demonstrated by Jacob and Berne(14). We also observed this in the case of the guinea pig heart, and the most likely explanation for the more rapid incorporation of the C^{14} label of the perfused ATP seems to be that ATP is dephosphorylated somewhere outside the cell with the liberation of inorganic phosphate and adenosine. Adenosine then crosses into the cell where ATP is resynthesized. At the same time, the liberated inorganic P^{32} crosses more slowly into the cell and is reincorporated into the ATP fraction intracellularly.

The exact site of the dephosphorylation is not defined by these studies. It has been demonstrated that dog coronary arterial tissue homogenates are capable of releasing inorganic phosphate from ATP and ADP(15). Coronary tissue has also been shown to contain 5-nucleotidase(16). It has also been suggested by Antoni *et al*(17) that in the isolated frog ventricle, ATP is hydrolyzed on the outer surface of the cell membrane to ADP and AMP. That the Na^+, K^+-stimulated ATPase of isolated myocardial cell membranes(18) is probably not responsible is indicated by its insensitivity to ouabain and by the dephosphorylation of the other nucleotides.

Summary. When the isolated guinea pig heart is perfused with C^{14}- and P^{32}-labeled ATP of known isotope ratio, ATP isolated from the myocardium shows an isotope ratio 100 times greater than the starting material. Inorganic P^{32} is liberated into the perfusion fluid along with C^{14}-labeled dephosphorylation and deamination products of ATP. UTP, GTP, CTP and ITP are also dephosphorylated when perfused. The results indicate that the ATP molecule does not enter the myocardium as such, but first is degraded and later resynthesized intracellularly.

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