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SUBCELLULAR DISTRIBUTION IN SURVIVING RAT HEART SLICES OF PERSANTIN-2,6-^{14}C AND ITS FIXATION TO ISOLATED SUBCELLULAR PARTICLES*

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Abstract—In experiments with surviving rat heart slices it has been shown that Persantin and 2,6-^{14}C-Persantin permeate the myocardial cell. Under our conditions, 50% or more of the drug in the medium was passed to the intracellular space and distributed among the intracellular compartments. The labeled drug was found in all the particles and in the cytoplasm. Fixation studies *in vitro* with sarcosomes or with the microsomal fraction, in amounts comparable to those found in the myocardial cell, showed that about 30% of labeled Persantin in the medium was fixed to the sarcosomes; 60% of this appeared to be tightly bound, whereas the remainder could be washed out with isotonic sucrose. The microsomal fraction *in vitro* fixed about 6% of labeled Persantin in the medium. These differences largely reflect the fact that there is about ten times more nitrogen in the cell attributable to sarcosomes than to the microsomal fraction.

From paper chromatographic studies of the labeled lipid-soluble fraction combined from all intracellular compartments, it can be stated that most of the ^{14}C-Persantin is not chemically altered under the conditions employed for these studies.

PERSANTIN (2,6-*bis*-(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-*d*)pyrimidine) has been shown to act selectively on the coronary arteries, causing a marked increase in coronary blood flow.[1]

Several reports have indicated that the effects of Persantin may be directly observed in the myocardium at the tissue level.[2, 3] If the site of action of this drug is indeed within the myocardial cell, certain requisites should be demonstrable. The permeation of the myocardium by Persantin at the cellular level should be clearly observable and, further, intracellular compartments could be expected to concentrate the drug. Accordingly it was decided to proceed with an investigation of the function of Persantin at the level of surviving tissue.

Experiments are reported in this communication that do indeed provide evidence for permeation of the myocardial cell by Persantin, concentration of the drug by subcellular compartments, and binding of the drug by isolated subcellular particles.

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EXPERIMENTAL

Mature Wistar male rats were stunned by a blow on the head, decapitated and bled for about 3 min. The whole heart was rapidly excised and placed in cracked ice. Each heart was cut longitudinally and tissue slices were prepared in the McIlwain–Buddle tissue chopper[4] at a thickness of 0·5 mm. Usually three to five hearts were combined for each experiment. Five volumes of chilled Krebs-Ringer phosphate buffer[5] were added to the slices, and this solution was equilibrated for 10 min at 37° under 95% O_2–5% CO_2. One tenth ml of an alcoholic solution of radioactive Persantin ($77 \cdot 10^4$ cpm) labeled with ^{14}C in the 2,6-positions* was added to the system to give a final concentration of 1×10^{-4} M with respect to the drug. The slices were incubated at 37° for 2 hr under 95% O_2–5% CO_2 in a gyrotory water-bath shaker. Preliminary fluorometric studies of the disappearance of Persantin from the medium indicated that the absorption of the drug was continuous during this period. The physiological integrity of the heart slice was evaluated manometrically from the respiration with added pyruvate. It was shown that under the conditions of these experiments a linear rate of pyruvate oxidation (ca. 280 μl O_2/hr per 0·5 g heart slices) was maintained over a 2-hr incubation period. After the incubation period, the slices were washed and then homogenized in a Potter–Elvehjem homogenizer with a loose-fitting Teflon pestle in a sucrose–mannitol–triethanolamine–EDTA medium containing the bacterial protease, Nagase.†[6] The slices were washed by transferring them into a flask containing 2 vol. of the incubation medium without Persantin or Nagase; the slices were gently swirled in the flask, and this process was repeated once again. Subcellular fractionation was effected by differential centrifugation as described by Schneider.[7] On microscopic examination of the nuclear fraction, it was found that few, if any, unbroken cells remained, although a slight contamination by red blood cells was unavoidable. Contamination by residual red blood cells could be reduced by permitting them to adhere to the polypropylene centrifuge tube while carefully removing the overlying nuclei. Radioactivity data from extracts of red blood cells showed that the uptake of the drug due to erythrocyte contamination is small. The nuclei, mitochondria, and microsomal fractions were each washed once with the sucrose–mannitol medium without protease, and the washes were combined with the cytoplasmic solution. The total particulate fraction was extracted with ether and then with HCl according to a modification‡ of a previous method.[8] Recovery of ^{14}C-labeled Persantin was 95%. One tenth to 0·5 ml of the final HCl extract was plated on a 2-in. stainless steel planchet with ethanol as the dispersing agent under conditions of infinite thinness. Counting was performed in a windowless gas-flow proportional counter. The radioactivity counts reported have been corrected for background. Efficiency of counting was about 40%.

Paper chromatography was carried out in one dimension on Whatman no. 1 paper and *n*-butanol – acetic acid – water (450 : 50 : 125, v/v).[9]

RESULTS

In Table 1 are shown the results of uptake of Persantin by surviving rat heart slices

* Radioactive Persantin labeled with ^{14}C in the 2,6-positions was graciously provided by the Karl Thomae Laboratories, Biberach, Germany. Unlabeled Persantin and its monoglucuronide derivative were kindly donated by the Geigy Pharmaceutical Co.

† Nagase and Co., Osaka, Japan.

‡ S. Zak, G. T. Quinn and P. Greengard, personal communication.

as measured by fluorescence extracted from the slices after a 2-hr incubation period. On this basis about 50% of Persantin in the medium is taken up by the slices.

TABLE 1. APPARENT UPTAKE OF PERSANTIN IN SURVIVING RAT HEART SLICES (0·5 mm) AS MEASURED BY FLUORESCENCE EXTRACTED FROM SLICES AFTER 2-hr INCUBATION AT 37° UNDER 95% O_2–5% CO_2

	Expt. 1	Expt. 2	Expt. 3
Rat body wt. (g)	245	223	254
Heart fresh wt. (g)	0·86	0·78	0·8
Buffer added (ml)	3·44	3·12	3·2
Persantin in ethanol (3×10^{-4} M; ml)	1·72	1·56	1·6
Apparent uptake of Persantin per total tissue* (μg)	84·8	124	116
Apparent uptake from treating Persantin (%)	57·0	52·5	48

* For these studies the method of Persantin extraction is described in the text. A standard curve and the reported determinations were made with a Farrand fluorometer, activating light at 305 mμ and measuring fluorescence at 505 mμ. Extraction of tissues incubated without Persantin showed little or no fluorescence under these conditions.

When heart slices are processed with sufficient rapidity, the drug disappears from the medium at a linear rate for 2 hr. When larger preparations of tissue slices are utilized, the rate of apparent uptake falls off during the second hour. This deviation could be ascribed to the longer preparatory period for larger amounts of tissue.

After the incubation, one wash of the slices with two system volumes of 0·85% NaCl succeeds in desorbing only about 25% of the fixed drug. The uptake observed, therefore, fulfills the requirements of true intracellular absorption.

TABLE 2. INTRACELLULAR DISTRIBUTION OF 2,6-^{14}C-PERSANTIN IN SURVIVING RAT HEART SLICES

After 2-hr incubation at 37° under 95% O_2–5% CO_2.

Av. body wt. (g)		170·3	(155·2–209·0)
Total heart wt. (g)		2·4	(2·0–2·6)
	No. of Expts.	cpm/total fraction	
Microsomal fraction	4	37,490	(36,860–38,240)*
Mitochondria	4	13,120	(6,980–16,650)
Nuclei†	4	16,652	(14,650–17,170)
Erythrocytes in nuclear fraction	4	3,875	(2,480–5,270)
Cytoplasm	4	185,827	(163,590–214,130)
Treating solution	4	81,652	(71,330–94,380)
Combined wash of slices	4	70,070	(50,830–83,890)
Total counts recovered	4	405,752	(367,380–443,490)

* Range.

† Virtually no unbroken cells are visible microscopically in this fraction. Further washing of the particulate fractions with isotonic sucrose solution reduces total fixation to the particles. The data pertaining to particulate fractions, above, represent the sum of 'loose' plus 'tight' binding of the drug.

In Table 2 are shown the results of the intracellular distribution of the ^{14}C-labeled drug. Measurement of specific incorporation in terms of cpm/mg particulate nitrogen was complicated by the contamination with Nagase which was used to digest the cell membranes. Additional contamination by triethanolamine and Persantin contributed errors to the nitrogen determinations in tissue slice experiments.

It is clear that most of the compound is found in the cytoplasm, and significant amounts are measurable in the microsomal, mitochondrial, and nuclear fractions. Although relatively little is known concerning the subcellular fractionation of heart cells, it would seem that complications, such as removal of red blood cells and the preparation of a discrete microsomal fraction, have been resolved.

Figure 1 shows that little of the Persantin is metabolized to another compound during the incubation period. The sample applied to the paper here was derived by pooling all the fractions. It can be seen that the Persantin spot is significantly labeled, and only a very small percentage of the radioactivity is associated with unlabeled fluorescent compounds appearing on the chromatogram.

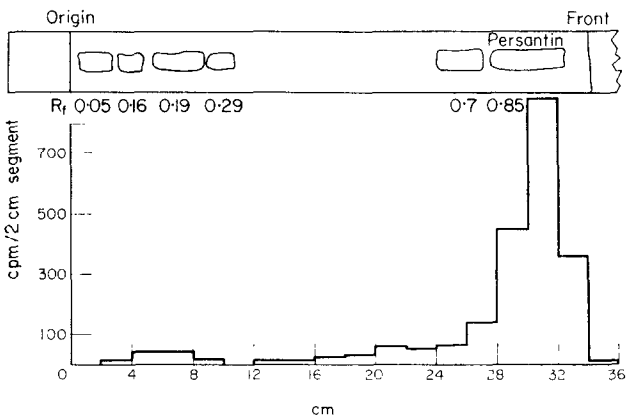

FIG. 1. Paper chromatography of combined lipid extracts from intracellular compartments after a tissue slice experiment, showing the zones of absorption or fluorescence under short wavelength u.v. irradiation and the distribution of radioactivity from ^{14}C-Persantin.

In order to assess, by another criterion, the degree to which ^{14}C-labeled Persantin is bound to subcellular particles, fixation studies with isolated subcellular particles were carried out. The results are shown in Table 3. The same incubation and extraction procedures as applied to surviving slice experiments were employed in these studies. It is obvious that the maximal amount of the drug to be fixed is bound immediately to the particle, and further incubation does not greatly enhance the degree of fixation. In the case of experiments with sarcosomes, about 20% of the labeled Persantin in the medium (10^{-4} M Persantin) was fixed immediately and was resistant to washing with isotonic sucrose. When washing was omitted, about 30% of the labeled drug in the medium was fixed immediately to the particles, and this value was likewise not increased with continued incubation in the medium. Thus it appears that 20% of the labeled drug in the medium was securely bound by sarcosomes; an additional 10% of the drug was loosely bound and could be removed by a mild washing procedure.

In analogous studies with the microsomal fraction, only about 6% of the drug was

TABLE 3. FIXATION OF ^{14}C-PERSANTIN TO ISOLATED RAT HEART SARCOSOMES AND TO ISOLATED RAT HEART MICROSOMAL FRACTION *in vitro**

Fraction	Incubation time (min)	Subsequent treatment	Average ^{14}C-Persantin in medium fixed by isolated particles (%)	Average ^{14}C-Persantin in medium fixed/mg Kjeldahl nitrogen content of particles (%)
Mitochondria	0	None	30·3 (28·3–37·4)†(3)‡	3·1
,,	60	None	32·5 (25·7–37·8) (4)	3·3
,,	0	Washed§	20·3 (18·8–21·6) (3)	2·1
,,	60	Washed	15·9 (11·5–19·7) (4)	1·6
Microsomal fraction	0	None	6·3 (3·8–9·4) (4)	4·4
,,	60	None	7·9 (6·9–8·8) (2)	6·2

* Particle preparations represent same amounts relative to each other as found in slice preparations. Incubation condition, particle washing, and extraction are identical with the procedures used for slice experiments.
† Range.
‡ Number of experiments.
§ Two successive washes were made with isotonic sucrose.

immediately fixed from the medium, and the degree of fixation did not increase with time. Because of the relatively small amount of the labeled drug bound by these particles, washing studies were not carried out.

While the total fraction of mitochondria fixes about three times the amount of the labeled drug compared with the microsomal fraction, the fixation is equivalent or greater in the microsomal fraction when the data are presented as relative specific fixation based on nitrogen content. Meaningful Kjeldahl nitrogen measurements on these fractions prepared prior to incubation can be made when aliquots of such fractions are taken for nitrogen determinations and washed extensively to remove contaminating Nagase (no Persantin will have been added at this stage). Thus nitrogen values for fixation studies *in vitro* are possible, and while these may not be absolute in value, they are of relative value; specific radioactivity measurements are therefore reported, whereas the difficulty in obtaining such estimations in the case of tissue slice experiments has already been described. It should be borne in mind, however, that the data of specific uptake of labeled drug based on milligrams of nitrogen of the particulate fraction do not include information about the greater mass of the mitochondrial fraction within the cell.

DISCUSSION

It is clear that Persantin is well absorbed into the cells of surviving heart slices. These experiments show that the labeled drug is fixed to all subcellular fractions. The high values for the cytoplasm and the microsomal compartments may be a consequence of flooding the cells to the point that the amounts of drug permeating the cell are large enough to saturate the particulate compartments and thereby prevent further accumulation. Since it has been shown by fixation experiments that the mitochondria are capable of tightly binding the drug, the mitochondria pose a provocative problem for further examination of the effects of Persantin upon subcellular metabolism.

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