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On the mechanism of uptake of procarbazine by L5178Y lymphoblasts *in vitro*

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Procarbazine (PCZ) has been shown to have inhibitory effects against a wide variety of transplantable tumors in animals [1]. In combination with other antineoplastic drugs, PCZ has been used successfully in the treatment of human neoplasms, particularly Hodgkin's disease [2, 3]. The disposition of PCZ in man and animals has been investigated [4]. The drug has been shown to undergo auto-oxidation or catalytic oxidation to its azo-derivative. The azo compound undergoes further enzymatic or chemical decomposition to form various metabolites including CO_2, methane, formaldehyde and *N*-isopropyl terephthalamic acid [5–8]. The antineoplastic activity of PCZ has been attributed to methylation [9, 10] and inhibition of DNA, RNA and protein synthesis [11–14]. To our knowledge the mechanism of transport of PCZ has not been reported. In this communication we present calculations which would indicate that PCZ very probably enters L5178Y lymphoblasts by simple diffusion and we present experimental data on PCZ uptake consistent with the predictions of these calculations.

Procarbazine–HCl [carbonyl-^{14}C] (19.59 mCi/m-mole) was generously supplied by Dr. W. E. Scott of Hoffmann-LaRoche Inc., Nutley, NJ. The radiochemical purity was greater than 98 per cent as determined by thin-layer chromatography (t.l.c.) on silica gel using a solvent system of methanol–chloroform (1:3, v/v). Unlabeled PCZ–HCl was obtained from Dr. J. Y. Gareau of Hoffmann-LaRoche Limited, Vaudrevil, Quebec.

L5178Y lymphoblasts in log phase growth were cultured in Fischer medium containing 10% horse serum. In the uptake experiments, cells were suspended at a concentration of 3 to 4×10^6 cells/ml. At intervals after incubation with [^{14}C]PCZ, cells were centrifuged through a layer of 0.25 M sucrose solution in Hopkin's vaccine tubes as described previously [15]. The washed cells were lysed in water at 4° for 15 min. The lysate and supernatant fraction were acidified with 0.1 N HCl and maintained at 4° in an ice bath to minimize decomposition of PCZ. Total radioactivity was obtained by measuring the radioactivity of an aliquot of cell lysate and the supernatant fraction; the results were expressed as a cell/medium distribution ratio.

Aliquots of the lysate and the supernatant fraction were applied to thin-layer chromatography plates (silica gel 60, E. Merck, Darmstadt, Germany) and developed with methanol–chloroform–acetic acid (3:10:1, v/v). Intact PCZ–HCl was found to have an R_f of 0.36. Samples of two main decomposition products, *p*-formyl-*N*-isopropylbenzamide and its methyl hydrazone derivative, were obtained from Hoffmann-LaRoche and were found to have R_f values of 0.86 and 0.84 respectively. After chromatography, the t.l.c. plate was cut into 1 cm strips and the radioactivity of each strip was measured by liquid scintillation spectrometry. Uptake of free intact drug as measured by t.l.c. was expressed as the ratio of radioactivity of intact drug in the cell to that in an equivalent volume of extracellular medium. Cell volume was determined in a Coulter Counter, model Z_{B1} calibrated with giant ragweed pollen (mean cell diameter 19.5 μm) and paper mulberry spores (mean cell diameter 12.5 μm).

Decomposition of [^{14}C]PCZ in Fischer medium was studied at 37° using t.l.c. and was found to follow apparent first-order kinetics with a $T_{(1/2)}$ of 110 min. Since all uptake experiments, other than the time course, were terminated at either 15 sec or 10 min, decomposition of intact drug was assumed to have little effect on the results obtained.

Understanding of the permeability properties of cell membranes has reached the stage where it has become possible to attempt quantitative predictions of the ability of a cell to take up a certain compound of unknown chemical structure by the route of simple diffusion. To make such a prediction, we can use the analysis of Lieb and Stein [16, 17] of the permeability behaviour of a number of different cell membranes, provided we know the molecular weight of the test substance and its partition coefficient between a solvent which mimics the cell membrane and water. Where the partition coefficient is not known, one can even estimate this from the chemical structure using predictive techniques pioneered by Leo *et al.* [18]. The predicted permeability coefficient can then be compared with experimental data. If the predicted number is of much the same order of magnitude as the observed one, there seems little need to postulate a system for uptake more specific than that of simple diffusion, and little biological significance for such a system. It is also important to show, however, that not only is the rate of uptake consistent with that of simple diffusion, but also that the equilibrium level of substrate reached is that expected for simple diffusion, before one can exclude the presence of a mediated uptake system.

Lieb and Stein [16, 17] suggested that the permeability of a membrane toward a particular substance depended on three factors: (1) the molecular weight, M, of the permeant, and the ability of the membrane to discriminate between permeants by virtue of size (the mass selectivity coefficients s_m); (2) the partition coefficient K for the distribution of the permeant between a model solvent and water, and the accuracy with which the model solvent describes the solvent properties of the membrane under consideration (the selectivity index s_k); and (3) the overall tightness of the membrane, the parameter P_0. The permeability coefficient for a given substance, P_s, is indeed given by:

$$P_s = P_0 M^{-s_m} K^{s_k}. \quad (1)$$

The parameters P_0, s_m and s_k can be determined by a multivariate regression analysis of the measured permeabilities of a large number of different substances entering a particular cell. With these parameters available, equation 1 allows the permeability of an unknown substance to be estimated.

For the L5178Y lymphoblasts we are not yet in a position where the required parameters are available. We are forced, then, to adopt the more hazardous approach of taking the parameters from the cells analysed by Lieb and Stein, using these to make an estimate of the permeability properties of our unknown in each of these cell types, and then extrapolating to the L5178Y cell. For this reason the experimental testing of our prediction is doubly necessary.

To calculate the permeability of PCZ, we take the molecular weight (M = 221.3) and the octanol/water partition coefficient as recorded by Leo *et al.* [18], giving $K = 1.148$. For the parameters P_0 and s_m, we use values derived by Lieb and Stein for various cell types as listed in

Table 1. Predicted permeability coefficients for PCZ entering various cell types at 20° and derived half-times of entry for a cell of the size of the L5178Y lymphoblast*

Cell type	P_0 (cm sec^{-1})	s_m	Predicted P_s (cm sec^{-1})	Predicted $T_{(1/2)}$ (sec)
Chara	0.13	2.9	5.3×10^{-4}	0.27
Nitella	0.50	3.7	4.5×10^{-4}	0.32
Phascolosoma	0.040	5.1	2.4×10^{-6}	60
Beef red blood cell	5.0	6.0	5.3×10^{-5}	2.7
Arbacia egg	0.40	4.2	1.4×10^{-4}	1.0

*P_0 and s_m were taken from Ref. 16; K octanol was taken as 1.148[18], and the size of the lymphoma cell as 1000 μ^3.

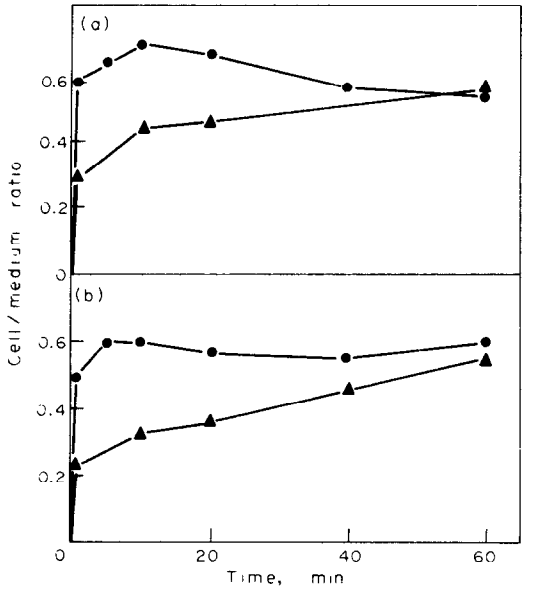

Fig. 1. Panel A: time course of the uptake of total radioactivity by L5178Y lymphoblasts incubated with 0.1 mM [^{14}C]procarbazine at 37° (●) and 4° (▲). Uptake is expressed as cell/medium distribution ratio of total activity. Panel B: a time course of uptake of free intact procarbazine by L5178Y lymphoblasts incubated with 0.1 mM [^{14}C]procarbazine at 37° (●) and 4° (▲). Free intact drug was measured using t.l.c. and uptake is expressed as cell/medium distribution ratio of free drug.

Table 1. The predicted half-time ($T_{(1/2)}$) for entry was calculated by simple integration of Fick's Law of diffusion as described by others[19]. For s_k, we take the value of unity, since recent work has indicated that octanol is an excellent model for the partitioning properties of biological membranes[20]. Indeed, a selectivity index of 0.98 was found using a variety of substances entering egg lecithin bilayer membranes[20]. Using these parameters, we can calculate for each cell type of Table 1 the estimated permeability coefficient for PCZ. In addition, if we take for the cell volume the measured value of some 1000 μ^3, we can calculate half-times ($T_{(1/2)}$) for the entry of PCZ into the L5178Y cell, were it to enter solely by simple diffusion and if the lymphoma cell had the permeability properties of the various cell types listed in Table 1.

The half-times of uptake of PCZ predicted for these various cell types range from less than 1 sec to 1 min, using parameters derived from data at 20°. Were the L5178Y lymphoma cell to behave like the cell types listed in Table 1, one would predict that entry of PCZ would almost certainly be complete by 10 min at 37° and perhaps (if the lymphoma cell behaves somewhat like the beef red blood cell) at 4°.

With such rapid rates of PCZ entry, the presence of a transport system for this substance seems an unlikely postulate. Experimental data bearing on this prediction follow.

A time course of uptake of [^{14}C]procarbazine by L5178Y cells *in vitro* is shown in Fig. 1. The uptake of total radioactivity (Fig. 1A) and of free intact drug (Fig. 1B) shows little difference; in each case uptake reached a plateau within 15 sec, showed little if any temperature dependence and the cell/medium ratio never exceeded 0.7. These findings suggest that drug uptake by an active transport mechanism is unlikely.

One important feature which distinguishes facilitated diffusion from simple passive diffusion is that, in the former, uptake follows saturation kinetics. Cells were incubated simultaneously with 5×10^{-5} M [^{14}C]PCZ and with various concentrations of cold PCZ at 37°, and the uptake of total radioactivity was measured at 15 sec and 10 min. Since the cell/medium ratio of total radioactivity approximated that of intact drug (as shown in Fig. 1), the cell/medium ratio of total radioactivity was used in all subsequent experiments.

The presence of cold PCZ, up to concentrations of 1×10^{-3} M, did not inhibit uptake of 5×10^{-5} M [^{14}C]PCZ, using incubation times of 15 sec and 10 min. This indicated that uptake of PCZ was linear from 5×10^{-5} to 1.05×10^{-3} M. The finding that the kinetics of drug uptake were non-saturable supported the concept that uptake of PCZ is by passive diffusion.

Further evidence for passive diffusion of PCZ came from the study of the effect of metabolic inhibitors. Generally, the concentration of inhibitor used in these experiments was that at which no change in cell viability, as detected by trypan blue dye exclusion, or in cell volume was noted, unless otherwise stated. As shown in Table 2, no significant inhibition of PCZ uptake, either at 15 sec or 10 min, was observed using various metabolic inhibitors. The lack of inhibition by the sulfhydryl reactive reagents iodoacetic acid (IAA), *N*-ethylmaleimide (NEM) and *p*-hydroxymercuribenzoate (POMB) suggested that thiol groups do not play a role in the transport system. The lack of inhibition by the ATP generation inhibitors sodium cyanide (NaCN), dinitrophenol (DNP), oligomycin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) provided evidence that uptake was not energy dependent.

Interestingly, cells preincubated for 15 min in 1 mM DNP and then incubated with [^{14}C]PCZ and DNP for an additional 10 min showed a 19 per cent increase in drug uptake, which was statistically significant (Table 2). However, a 17 per cent increase of mean cell volume was noted after DNP treatment for 25 min, which might explain the apparent stimulation of PCZ uptake. The lack of stimulation of PCZ uptake at 15 sec was not associated with any significant increase in cell volume.

Some carrier-mediated transport systems have been shown to be sodium ion dependent[21]. Uptake of

Table 2. Effect of metabolic inhibitors on uptake of [^{14}C]PCZ by L5178Y lymphoblasts *in vitro**

Metabolic inhibitor	Conc (M)	Per cent of control uptake (mean ± S. E.) 15 sec	10 min
IAA	1×10^{-4}	107.2 ± 1.8	106.4 ± 2.9
NEM	5×10^{-6}	98.8 ± 6.9	102.8 ± 6.6
POMB	5×10^{-5}	100.0 ± 3.6	87.8 ± 1.9
NaCN	1×10^{-4}	87.7 ± 3.6	90.5 ± 5.5
Oligomycin	5×10^{-6}	95.1 ± 1.0	116.4 ± 2.0
CCCP	1×10^{-5}	102.4 ± 4.3	93.4 ± 4.5
DNP	1×10^{-4}	104.4 ± 5.6	99.3 ± 5.1
	1×10^{-3}	102.6 ± 3.9	118.9 ± 3.1†

*Cells were incubated with metabolic inhibitors for 15 min before [^{14}C]PCZ was added and uptake of radioactivity at 15 sec and 10 min was compared in the presence and absence of inhibitors. Results are expressed as a percentage of control cell/medium radioactivity distribution ratio and were statistically evaluated by Student's two-tailed '*t*'-test. Each value represents the mean ±S.E. of four to eight determinations.

†$P < 0.01$;all other results were not statistically significant.

[^{14}C]PCZ by cells incubated in a balanced salt solution described by Martin[22] was compared with uptake in the same solution in which NaCl was replaced by an equivalent amount of either Tris, choline chloride or KCl. Uptake of 5×10^{-5} M [^{14}C]PCZ by L5178Y cells at 10 min in these solutions was essentially identical.

The findings that the rate of PCZ uptake was of the order of magnitude expected for a simple diffusion system and, further, not highly temperature dependent, that the cell/medium distribution ratio of free intact drug was less than unity, that uptake was non-saturable, was unaffected by several metabolic inhibitors, and was sodium-insensitive, all suggest that uptake of PCZ by L5178Y cells *in vitro* occurs by simple diffusion.

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Comparison of isoproterenol, salbutamol and talzolol as lipolytic agents with isolated rodent adipocytes

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Lands *et al.*[1] presented evidence that β-adrenergic receptors could be subdivided into β_1- and β_2-receptors according to their stimulatory activity on the heart and their bronchodilator action respectively. Using a series of agonists, they found a high correlation between stimulation of the heart and of lipolysis in the rat and concluded that the β-adrenergic receptor mediating lipolysis was of the β_1-type. Recent work[2, 3] has challenged this conclusion. Part of the evidence against a β_1-receptor in rat adipose tissue is that salbutamol is an