Concentrative uptake of 6-Diazo-5-Oxo-L-Norleucine by Sarcoma 180, Liver and Muscle *in vivo*.*† (24436)

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The amino acid analogs O-diazo-acetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON) are of interest because of their effects in many biological systems and in particular because of their inhibitory effects on tumors(1-7). Both azaserine and DON block *de novo* synthesis of purines by inhibiting an enzyme involved in conversion of α-N-formylglycinamide ribotide to α-N-formylglycinamidine ribotide(8-10). In short term experiments, incorporation of glycine into purines is markedly inhibited by azaserine but there is no inhibition of glycine incorporation into proteins(8,11). Tumor cells and normal cells compete for essential nutrients in the extracellular space. The cell type which can attain the highest intracellular-extracellular concentration gradients of an essential metabolite may be considered to have a competitive advantage. Christensen and his coworkers have evidence that rapidly growing tissues, foetal, regenerating, and neoplastic, concentrate amino acids to a greater extent than do many adult, non-regenerating tissues (12-14). We have shown(15) that azaserine and DON are concentrated intracellularly in Ehrlich ascites cells by the amino acid transport system. It was suggested that perhaps a relatively greater concentration of these compounds intracellularly in tumors, contributes to tumor inhibition obtained in chemotherapeutic tests. The present study was therefore undertaken to measure intracellular-extracellular concentration gradients of DON in liver, skeletal muscle and sarcoma 180 (S 180) *in vivo*.

Materials and methods. The S 180, obtained from Dr. D. A. Clarke of Sloan-Kettering Institute, and carried subcutaneously in adult Swiss albino ICR mice obtained from Millerton Farms, Millerton, N. Y. Tumor-bearing mice were used for experiments 6-8 days after subcutaneous implantation of S-180. To determine concentration of DON in the intracellular and extracellular water it was necessary to measure DON and water content of a sample of plasma, and total DON, total water, and the fraction of total water in the extracellular space of a sample of each of the tissues. The procedures used follow: Mice were first subjected to a bilateral nephrectomy using ether anesthesia and the dorsal approach to the kidneys, to prevent any loss of large amounts of DON injected. After the mice had recovered from the anesthesia, DON was injected intravenously via the tail vein. The diluent contained sodium chloride and sodium bicarbonate in the molar proportions 5:1 and was diluted to make the final solution approximately isotonic. Heparin was added to a level of 200 units/ml diluent. The animals were sacrificed by decapitation and blood collected in a chilled centrifuge tube. The mice were then chilled in ice water, blotted dry and dissected. Samples of skeletal muscle always consisted of the gastrocnemius and muscles of back of thigh, all freed of any obvious fat, nerve and tendon. Care was taken to dissect the S 180 samples free of necrotic tissue. The fraction of tissue water in the extracellular space was assumed to be closely approximated by the sucrose space. Tissue sucrose spaces were determined on a group of animals bearing 8-day-old S 180. These were handled the same way as those in the DON experiments except that they were not nephrectomized and received 0.5 ml of 0.3 M sucrose containing 1000 units heparin/ml, intravenously. Sucrose was extracted by homogenizing the tissue samples with one ml of 0.5 N NaOH and 8-10 ml of

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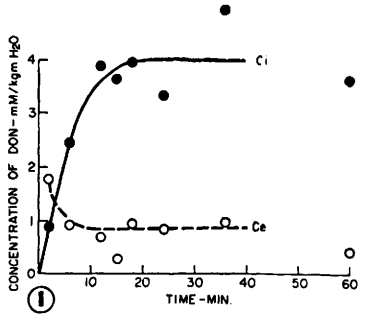

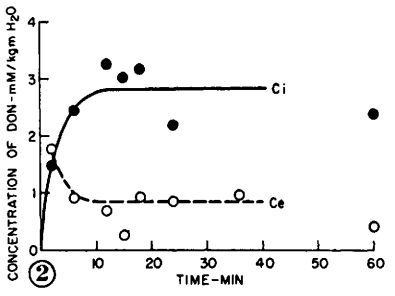

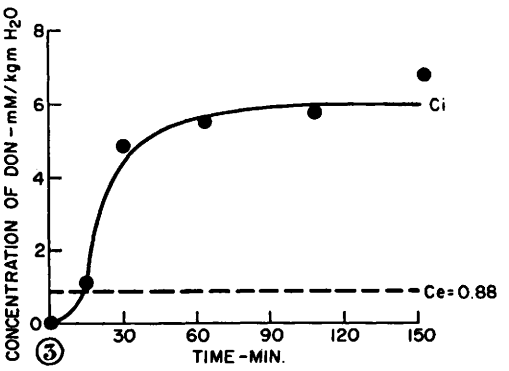

FIG. 1. Concentrative uptake of DON by S 180 *in vivo*. Ci = intracellular concentration; Ce = extracellular concentration.

FIG. 2. Concentrative uptake of DON by mouse liver *in vivo*.

FIG. 3. Concentrative uptake of DON by slices of S 180 suspended in Krebs-Ringer bicarbonate.

water, adding one ml 10% Zn $SO_4 \cdot 7H_2O$, homogenizing and centrifuging. The precipitate was washed once with 10 ml distilled water. The supernatants were combined and diluted to 25 ml. Sucrose was determined by the method of Roe *et al.*(16). Average values for the fraction of tissue water in the extracellular space were, liver—0.16, skeletal muscle—0.14, S 180—0.36. These of course hold only for tissues partly drained of blood by the sampling procedure used. The DON was determined spectrophotometrically on 75% ethanol-barium acetate extracts of tissue homogenates or plasma by method previously described(15). Intracellular concentrations were calculated as though the DON were equally distributed throughout the intracellular water, from the formula,

$$C_i = \frac{A - mh\,We\,Ce}{mh\,(1 - We)}$$

wherein, A is total DON in the tissue sample, m is wet weight of tissue sample, h is water content of the sample in g H_2O/g wet wt., We is sucrose space for the tissue and Ce extracellular concentration of DON in mM/kilo of water. Extracellular concentration of DON was assumed equal to concentration in plasma water.

Results. Fig. 1 and 2 show the time course of DON uptake in S 180 and liver respectively. Although there was considerable variation from animal to animal, intracellular concentrations in S 180 were consistently higher than in the liver of the same animal once the plateau of the uptake curves was reached. Muscle gave no evidence of concentrative uptake, intracellular concentrations being 0.5-1.5 times extracellular.

One experiment was made with S 180 slices incubated in Krebs-Ringer-bicarbonate containing DON, to see if higher intracellular concentrations could be obtained, since it would be expected that competition from normal free amino acids would give somewhat lower values for DON concentration intracellularly *in vivo*. To check this an extracellular concentration close to that obtained in the experiment shown in Figs. 1 and 2, was chosen. The time course of DON uptake is shown in Fig. 3. Intracellular concentration attained *in vitro* was 1.5 times as high as *in vivo*. As expected, the time to reach the steady state in the S 180 slices is much longer than *in vivo*. This is no doubt due to the much slower diffusion into the extracellular space in slices *in vitro*.

Discussion. The present findings in conjunction with previous results on concentrative uptake of DON by Ehrlich ascites cells (15) and data on concentrative uptake in normal tissues(17) readily explain the find-

ings(4) that DON rapidly disappears from blood after intravenous injection and that only minute amounts of it are excreted in the urine. In common with the normal amino acids it would be reabsorbed in the renal tubules and be concentrated intracellularly by the amino acid transport system in many of the visceral tissues. Unfortunately relative concentrations of DON in viscera other than liver were not obtained. Studies on duodenum and kidney would be of particular interest since Noall *et al.*(17) have shown that α-aminoisobutyric acid is concentrated to a greater extent in duodenum and kidney than in liver. It is likely that the general pattern of concentration intracellularly will follow that of the normal amino acids.

The high toxicity of DON is probably explained by inhibition of purine synthesis(8-11) plus its concentration intracellularly by the amino acid transport system. This in itself does not explain the selective inhibition of many transplantable tumors. A somewhat greater concentrative uptake of DON by tumors in conjunction with a relatively greater importance of the *de novo* pathway of purine synthesis in tumors(18,19) may account for the inhibitory effect on transplantable tumors.

Ackerman and Potter(20) have proposed that inhibition of an enzyme which is present in lower concentrations in a tumor than in normal tissue may provide a theoretical basis for cancer chemotherapy. However, the presence of a lower concentration of an enzyme in a tumor may imply that the metabolic pathways in which this enzyme is involved are of less importance in metabolism of the tumor than of normal tissues. We suggest the following alternative generalizations. Active chemotherapeutic compounds are more likely to be compounds which, a) are actively concentrated by tumor cells to a higher intracellular concentration than by normal cells, or b) inhibit enzymes of metabolic pathways which are more active in tumor cells than in normals. Useful compounds are more likely to be found among those which satisfy both of these conditions.

Summary. DON is concentrated intracellularly in S 180 and liver to levels 5-fold and 4-fold higher respectively than the extracellular concentration at a plasma level of 0.8-0.9 mM/kilo of plasma water. Intracellular concentrations in skeletal muscle were the same or less than the extracellular.

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