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The relation between circulating and tissue concentrations of salicylate in the mouse *in vivo*

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The blood and water contents of mouse liver, brain, kidney, heart, spleen and skeletal muscle were measured and used to correct observed values for the salicylate concentrations in these tissues after the intraperitoneal injection of the drug. The binding of salicylate *in vitro* to mouse whole blood, liver, kidney and brain was studied. It was concluded that blood, liver and kidney but not the other tissues, bind the drug *in vivo*.

Salicylate inhibits the activity of several enzyme systems *in vitro* including oxidative phosphorylation reactions, dehydrogenases, aminotransferases (Smith, 1968), nucleic acid polymerases (Janakidevi & Smith, 1969) and aminoacyl-t-RNA synthetases (Burleigh & Smith, 1970). The mechanisms of many of these inhibitory actions of the drug involve competition between the salicylate and either a substrate or coenzyme (Gould, Dawkins & others, 1966; Dawkins, Gould & others, 1967). An important factor is therefore the concentration of salicylate in the reaction mixtures and *in vitro* this is easily controlled and remains constant during the experiment. To explore the possible relevance of *in vitro* inhibitions to *in vivo* effects it is necessary to show that they both occur at equivalent concentrations. The tissue concentrations of salicylate occurring after the administration of the drug must be known to make such comparisons of any value. Sturman, Dawkins & others (1968) attempted to provide suitable data for the mouse by measuring the total salicylate concentrations in several tissues at varying times after the intraperitoneal injection of a range of doses of salicylate. However, their results showed no apparent correlation between the concentrations in the individual tissues. The present work describes certain correction factors concerned with the volumes of blood sequestered in the tissue samples removed for analysis, the water contents of the tissues and the degree of binding of salicylate to blood and tissue proteins.

EXPERIMENTAL

Blood content of tissues

Male mice, 25–30 g, of the albino strain maintained at King's College Hospital on M.R.C. modified cube diet no. 41B were used in this and in the subsequent experiments. Eight mice each received an intravenous (tail-vein) injection (0·2 ml) containing 10 μCi of ^{131}I-labelled human serum albumin (specific activity, 30 μCi/mg albumin, obtained from the Radiochemical Centre, Amersham, Bucks) in 0·9% (w/v) NaCl and were killed after 8 min by cervical fracture. Blood samples were collected, after decapitation, into lithium heparin tubes, and the liver, brain, kidneys, heart, spleen and both quadricep femoralis muscles from each animal were rapidly removed, weighed on a torsion balance, digested in 1 ml of 30% (w/v) NaOH in a

boiling water bath, cooled and made up to 20 ml with distilled water. Aliquots (0·1 ml) were dried on Whatman GF/A (2·1 cm) glass fibre discs (Gallenkamp & Co. Ltd.) and the radioactivity counted in a Beckman LS 200B liquid scintillation system using as phosphor 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene. The results were calculated as percentages of blood (ml) per 100 g wet weight of tissue by dividing the radioactivity (counts/min) per 100 g wet weight of tissue by the radioactivity per ml of blood.

Water content of tissues

The liver, brain, kidneys, heart, spleen and muscles were removed from four mice, weighed in tared containers and heated at 100° to constant weight. The loss in weight was taken as the water content and calculated as the percentage of water (ml) for 100 g wet weight of tissue.

Binding of salicylate to whole blood

Three groups, each of twelve mice, were killed by cervical fracture and the blood, obtained after decapitation, from each group was pooled during collection in lithium heparin tubes. Aliquots (1 ml) from each pooled sample were placed inside dialysis sacs of Visking tubing (8/32 inch inflated diameter, obtained from the Scientific Centre, London) and dialysed against 3 ml of 0·9% (w/v) NaCl solution, containing sufficient sodium salicylate (British Pharmacopoeial grade) to give initial salicylate concentrations ranging from 0 to 5mM, in vessels shaken 100 cycles/min for 24 h either in a water bath at 8° or at room temperature (22°). Salicylate was determined in samples taken from the fluid outside the sacs with an Aminco Bowman spectrophotofluorometer, using an activating wavelength of 294 nm and a detecting wavelength of 413 nm. The salicylate concentration outside the sacs at the end of the dialysis is the unbound concentration. Some salicylate disappears during dialysis due, at least in part, to adsorption onto the Visking tubing. The amount of salicylate added at the beginning of the experiments is therefore not equal to the amount remaining at the end. The amount of salicylate inside the sac containing the homogenate was calculated by subtracting the amount outside the sac at the end of the dialysis from the total amount, i.e. inside and outside the sac, found in similar experiments in which saline had been substituted for the tissue homogenate.

The tissues were removed from 20 mice and individually homogenized in an all-glass Potter homogenizer with an equal volume of 0·9% (w/v) NaCl solution. In some experiments the livers were homogenized without saline. Samples (1 ml) of the homogenates were placed inside Visking dialysis sacs and treated as described above for the whole blood experiments except that additional experiments were made with the liver homogenate in which 0·1 μCi of [^{14}C]carboxyl salicylic acid (specific activity 31·4 mCi/mmol obtained from the Radiochemical Centre) was added to the unlabelled salicylate. Salicylate was determined in samples taken from the fluid outside the sacs and the radioactivity measured in these samples after dialysis.

RESULTS

The percentage contents of blood and water in mouse liver, brain, kidney, heart, spleen and skeletal muscle are given in Table 1. The concentrations of total and

Table 1. *Contents of blood and water in mouse tissues.* Each value represents the mean ± standard deviation and is expressed as ml of either blood or water in 100 g wet weight of tissue.

Tissue	% blood in tissue (8 animals)	% water in tissue (4 animals)
Liver	17·2 ± 2·8	71·6 ± 1·0
Brain	2·6 ± 0·8	79·5 ± 0·8
Kidney	14·8 ± 3·4	76·3 ± 1·0
Heart	22·0 ± 4·0	79·4 ± 1·4
Spleen	13·8 ± 2·0	79·5 ± 1·2
Muscle	2·7 ± 0·8	73·7 ± 1·8

Table 2. *Binding of salicylate to mouse blood.* Each value is given as the mean ± standard deviation of duplicate determinations made on two of the pooled samples of mouse blood.

Salicylate concentration (mM)		
Initially present outside sac before dialysis	Found outside sac at end of dialysis (Unbound concentration)	Calculated as present inside sac at end of dialysis (Total concentration)
0·05	0·03 ± 0·001	0·07 ± 0·002
0·10	0·05 ± 0·001	0·14 ± 0·007
0·50	0·28 ± 0·003	0·60 ± 0·006
1·00	0·60 ± 0·027	1·12 ± 0·065
1·50	0·99 ± 0·008	1·52 ± 0·028
2·00	1·29 ± 0·014	2·06 ± 0·056
5·00	3·11 ± 0·064	5·28 ± 0·153

Table 3. *Unbound and total salicylate in mouse tissues* in vitro. Experimental conditions as in text, each value is the mean of four determinations and is expressed as in Table 2.

Salicylate concentration (mM)					
Brain		Liver		Kidney	
Unbound	Total	Unbound	Total	Unbound	Total
0·04	0·04	0·03	0·07	0·03	0·06
0·09	0·08	0·06	0·12	0·06	0·13
1·29	1·29	0·64	1·09	0·66	1·02
		1·02	1·43	1·06	1·35
		1·36	1·91	1·46	1·62

unbound salicylate in mouse blood exposed at 8° to salicylate concentrations, ranging from 0·1 to 5·0mM, are given in Table 2. Three different samples of mouse blood were used in the experiments, each salicylate concentration being tested against two of these. The percentage of unbound salicylate increased with the total salicylate concentrations in whole blood, being approximately 40% at 0·1 mM and 60% at about 5 mM. The results of similar experiments with homogenates of brain, liver and kidney are given in Table 3. Similar results with the whole blood and the tissues were observed when the dialyses were performed at either 8° or 22°. There

was no difference between the free and total concentrations for brain and this tissue did not therefore bind the drug. However, both liver and kidney showed an apparent binding in that the calculated total salicylate concentrations inside the sacs exceeded the free concentrations measured in the fluid outside the sacs at the end of the dialysis. Similar results were observed with liver homogenates prepared either with or without saline. A further set of experiments was therefore made with mouse liver homogenates using unlabelled salicylate plus a tracer amount of radioactive salicylate. The salicylate concentration and the radioactivity in the fluid outside the sacs were measured at the end of the dialysis. The concentration of salicylate decreased in a similar manner to that shown in Table 3 and this was paralleled by the decrease in radioactivity so that the specific activity remained constant during the dialysis. This result excluded the possibility that salicylate conjugates, which were freely permeable but lacked the characteristic fluorescence of salicylate itself, had been formed. Either salicylate was bound to the liver material or salicylate conjugates were synthesized and subsequently bound to liver proteins.

DISCUSSION

In earlier work (Sturman & others, 1968) the total salicylate concentrations in several mouse tissues, including whole blood, were measured at varying time intervals after the injection of a range of doses of the drug. These data need to be corrected for several reasons. Firstly, the amounts of blood contained in the tissue samples taken for analysis were not taken into account. Secondly, it was assumed that the salicylate was distributed throughout the whole of the tissue sample. Thirdly, the ratios between the protein-bound and unbound drug in the blood were unsatisfactory because insufficient time had been allowed for equilibration in the dialysis method. Finally, no attempt was made to investigate if one or more of the selected tissues could bind salicylate. The present work supplies appropriate correction factors for the blood and water contents of the tissues, allows the percentages of unbound salicylate in the blood to be calculated, and suggests that two of the tissues, liver and kidney, can bind the drug.

The original data of Sturman & others (1968) for the time interval, 30 min after injection, when maximum blood and tissue salicylate concentrations were attained have been recalculated to include the corrected tissue concentrations and free salicylate levels in the blood (Table 4). The recalculated figures represent the concentrations

Table 4. *Distribution of salicylate in mouse tissues.* The values are the data of Sturman & others (1968) for the samples taken 30 min after injection, corrected for blood and water contents of the tissues (see Table 1) and with the concentrations of unbound salicylate in the blood calculated from the results in Table 2.

Dose injected (mg/kg)	Blood		Salicylate concentration (mM)					
	Total	Unbound	Brain	Heart	Spleen	Muscle	Liver	Kidney
50	0·32	0·16	0·06	0·05	0·05	0·07	0·15	0·29
100	0·64	0·35	0·18	0·16	0·16	0·13	0·48	0·46
200	1·09	0·62	0·39	0·40	0·44	0·35	1·20	0·72
400	2·42	1·41	0·92	1·08	1·10	0·98	2·15	1·91
800	3·78	1·94	1·70	1·42	1·58	2·22	2·34	3·10

of salicylate in the combined intracellular and interstitial fluid spaces of the tissues and not the concentrations of the drug in individual cells. The tissues may be divided into two main groups with respect to the relation of their tissue salicylate concentrations and the concentration of unbound salicylate in the blood. The first group comprises brain, skeletal muscle, heart muscle and spleen. At each dose level in each tissue the tissue salicylate concentration is less than the unbound salicylate in the blood suggesting that none of these tissues bind the drug *in vivo*. The results of the *in vitro* binding experiments with mouse brain (Table 3) are consistent with this view. However, as the size of the injected dose is increased so the tissue salicylate concentrations become closer in value to the corresponding free salicylate concentrations in the blood. One explanation of this effect is that with increasing concentration of free salicylate in the blood there is a more rapid equilibration between the blood and the tissues. Thus, during the period at which the blood salicylate concentration is rising after absorption, the drug accumulates in the tissues at a rate proportional to the free salicylate concentration in the blood and hence indirectly to the size of the administered dose. This effect did not occur in the liver and the kidney. In both tissues the salicylate level exceeded the free salicylate concentration in the blood suggesting that either the tissues bound approximately 40% of the drug to intracellular macromolecules or that salicylate conjugates accumulated within the tissues. The results of the *in vitro* experiments (Table 3) show that binding occurs, the figures for bound salicylate again being approximately 50% of the total. Liver and kidney are the two tissues in which salicylate persists for over 18 h after the intraperitoneal injection of the drug in the mouse (Sturman & others, 1968) and this effect is explicable if protein binding of the drug is restricted to them.

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