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The effect of sodium salicylate on the binding of L-tryptophan to serum proteins

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In human serum, L-tryptophan is the only amino-acid bound to protein. Salicylate causes a release of tryptophan from its binding sites on human serum proteins and bovine albumin. Some implications of this finding are discussed.

Salicylate binds to circulating proteins, in particular to the serum albumin, in many species including man (Reynolds & Cluff, 1960; Davison & Smith, 1961). The drug also competes with some other substances for binding sites on proteins. Although the suggestion (Brodie, 1965) that they liberate corticosteroids from transcortin has not been confirmed experimentally (Stenlake, Davidson & others, 1968) it has been established that salicylates displace thyroxine and tri-iodothyronine from their binding sites on serum proteins (Christensen, 1959; Osorio, 1963). There is also some evidence that salicylate releases penicillin (Kunin, 1964) and sulphinpyrazone (Yu, Dayton & others, 1962) from their combination with circulating proteins.

Hormones and drugs comprise only a very small fraction of the small molecules which may be transported by the plasma in bound forms. Any interference by salicylate with such binding could cause a greater availability of a variety of diffusible molecules to enter body cells. The possibility exists that one or more of the metabolic or pharmacological actions of salicylate may be mediated by the increased release of such substances. The separation and measurement of all known plasma constituents is a formidable task but suitable methods are available for certain groups. These include the amino-acids, which may be analysed by automated systems. Only L-tryptophan appears to be bound to serum albumin to an appreciable extent (McMenamy & Orcley, 1958) and this has been confirmed in the present work, which also shows that the bound amino-acid is displaced by salicylate.

EXPERIMENTAL

Materials

Pooled human serum was obtained from the National Transfusion Service, Sutton. Bovine serum albumin (fraction V) and L-tryptophan (Sigma grade) were obtained from the Sigma Chemical Company, St. Louis, and Visking dialysis tubing ($\frac{8}{32}$ inch inflated diameter) from the Scientific Instrument Centre. All other chemicals were of analytical grade and distilled water was used throughout.

Dialysis of serum and measurement of amino-acids

Sacs of the Visking tubing, containing 5 ml of water, were immersed in 50 ml quantities of either the pooled human serum or the serum in which 10 mg of sodium salicylate had previously been dissolved. Each dialysis was allowed to proceed for 24 h at room temperature (22°) and the resulting dialysate stored at −15° for

subsequent analysis. Aliquots (1 ml) of a mixture of 1 ml of the dialysate plus 0·2 ml 0·6 mM norleucine in 0·6N HCl were analysed, using a Technicon amino-acid auto-analyser system. The total amino-acid composition of the undialysed serum was also determined after deproteinization as follows: 6 ml of serum were mixed with 0·5 ml 60% (w/v) trichloroacetic acid and centrifuged. The pellet was washed three times with portions (5 ml) of ice-cold 0·6% (w/v) trichloroacetic acid. The original supernatant and washings were combined with 1 ml of 2·5 mM norleucine in 0·01N HCl and made up to 25 ml with 0·01N HCl. One ml of this solution, after filtration, was analysed as described above. Amino-acids and other ninhydrin-positive substances were identified by comparing the elution times with those of authentic compounds and by co-chromatographing standards with the test sample in the standard Technicon system.

Tryptophan binding experiments

All dialysis tubing was presoaked in two changes of distilled water for 20 min before use. Bovine albumin was made up as a 3% (w/v) solution in 0·1M phosphate buffer, pH 7·4, and exhaustively dialysed against five changes of the same buffer to remove any diffusible ninhydrin-positive substances. Two ml of this solution was dialysed against 4 ml of the phosphate buffer containing all combinations of L-tryptophan (0·075, 0·25, 0·375, 0·75 and 1·13 mM) with salicylate (0·0, 0·45, 1·9 and 4·5 mM). The levels of salicylate were chosen to approximate the blood levels observed in patients receiving salicylates either as analgesics or as therapy for rheumatism and those found in acute intoxication with the drug. The dialysis vessels were shaken at 100 cycles/min on a Luckman rotary shaker at room temperature (22°) for 20 h. The final concentration of L-tryptophan remaining outside the dialysis tubing in the buffer was determined by reaction with ninhydrin, using a Technicon auto-analyser. The free concentration is determined by the concentration of L-tryptophan in the dialysate outside the dialysis tubing. The total amount of the amino-acid inside the dialysis tubing with the protein was calculated by subtracting the total amount outside the dialysis tubing from the amount originally added. The concentration of bound tryptophan is then the total concentration inside the dialysis sac minus the free concentration. The results of preliminary experiments showed that the presence of bovine albumin did not affect the determination of tryptophan, the equilibrium was reached in the experimental time and that the tryptophan was not absorbed on either the dialysis tubing or on the glassware. Extra analyses of the final tryptophan concentrations, in the absence of salicylate, were made on an Aminco Bowman spectrophotofluorimeter at an excitation wavelength of 278 nm and an emission wavelength of 366 nm, and the results were in good agreement with those obtained with the auto-analyser system.

RESULTS

Effect of sodium salicylate on the levels of free amino-acids in human serum

Table 1 gives the concentrations of amino-acids in the undialysed sera and in the dialysates. In this, and subsequent tables, the results have been analysed by the *t*-test; the minimal acceptable level of significance being taken as $P = 0{\cdot}05$. With the exception of tryptophan, the values for the levels of each amino-acid obtained by either procedure did not differ significantly. However, the level of tryptophan determined by the protein precipitation method was approximately five times higher

than that by equilibrium dialysis. These results show that, whereas most amino-acids are present in the serum as free molecules not bound to non-dialysable macromolecules, tryptophan occurs normally both as the free amino-acid and bound to protein.

In the presence of salicylate, the concentration of tryptophan in the experimental dialysate rose from 0·01 to 0·046 mM, showing a considerable release of tryptophan from its binding sites. Sodium salicylate had no significant effect on the free concentrations of any other amino-acids. When L-tryptophan was added to serum before dialysis, a proportion became bound, but when salicylate was present a far greater proportion remained free. For example, when the free tryptophan concentration of a control solution was increased to 0·045 mM, 20 mg sodium salicylate per 100 ml serum caused this level to rise to 0·132 mM.

Table 1. *Concentrations of amino-acids in pooled human serum.* The concentrations of amino-acids measured in the control dialysates (free amino-acids) have been multiplied by 1·1 to correct for the dilution during dialysis and to allow a comparison to be made with the total amino-acid concentrations determined in the deproteinized serum supernatants. Each value represents the mean of four determinations, ± standard deviation

Amino-acid	Total amino-acid concn (mM)	Free amino-acid concn (mM)	*P* value
Aspartate	0·062 ± 0·002	0·075 ± 0·025	N.S.*
Threonine	0·184 ± 0·005	0·181 ± 0·009	"
Serine	0·253 ± 0·009	0·246 ± 0·009	"
Glutamine	0·330 ± 0·003	0·380 ± 0·028	"
Proline	0·314 ± 0·027	0·325 ± 0·020	"
Glutamate	0·445 ± 0·023	0·457 ± 0·025	"
Citrulline	0·040 ± 0·006	0·042 ± 0·003	"
Glycine	0·529 ± 0·019	0·508 ± 0·061	"
Alanine	0·591 ± 0·032	0·581 ± 0·039	"
Valine	0·243 ± 0·014	0·259 ± 0·026	"
Methionine	0·039 ± 0·002	0·040 ± 0·001	"
Isoleucine	0·090 ± 0·003	0·097 ± 0·008	"
Leucine	0·218 ± 0·011	0·240 ± 0·017	"
Tyrosine	0·092 ± 0·002	0·087 ± 0·007	"
Phenylalanine	0·142 ± 0·004	0·145 ± 0·008	"
Ethanolamine	0·025 ± 0·007	0·022 ± 0·002	"
Tryptophan	0·059 ± 0·003	0·011 ± 0·002	<0·001
Ornithine	0·287 ± 0·011	0·266 ± 0·020	N.S.
Lysine	0·297 ± 0·008	0·281 ± 0·013	"
Histidine	0·163 ± 0·011	0·162 ± 0·010	"
Arginine	0·083 ± 0·010	0·088 ± 0·004	"

* Not significant.

Albumin binding of tryptophan and effect of salicylate

Fig. 1 shows that bovine albumin (0·5 mM) binds about 80% of L-tryptophan when the free concentration is low and that saturation of the binding sites occurs when 0·4 mM L-tryptophan is bound. As the salicylate concentration is increased from 0 through to 4·5 mM, the amount of tryptophan bound for any given level of free tryptophan is decreased progressively. For example, the amount bound when 0·1 mM is free is reduced from 0·3 mM to 0·07 mM by 1·9 mM salicylate.

McMenamy & Orcley (1958) have shown that the ratio of moles of bound tryptophan to moles of protein is approximately 1. This allows the calculation of the

association constant K_A. Table 2 shows that the association constant for tryptophan and albumin decreases significantly as the level of salicylate increases relative to the albumin concentration.

Table 2. *Variation in the association constant* K_A *for* L-*tryptophan and* 3% *bovine albumin with increasing concentrations of salicylate*

$$K_A = \frac{[\text{Bound tryptophan}]}{[\text{Free tryptophan}]\,[\text{Free binding sites on albumin}]}.$$

The value of 60,000 has been used for the molecular weight of bovine serum albumin

Salicylate Concentration (mM)	0·0	0·45	1·9	4·5
K_A (M^{-1})	0·0143	0·0054	0·0017	0·0007
s.d.	±0·0019	±0·0004	±0·0002	±0·0002
P		<0·001	<0·001	<0·001

DISCUSSION

The results presented confirm the findings of McMenamy & Orcley (1958) that the only circulating amino-acid to be significantly bound to protein is L-tryptophan and that less than 20% is in a freely diffusable form. However, in the presence of sodium salicylate at a concentration of 20 mg per 100 ml serum, the proportion of free tryptophan rose to 85%. A proportion of L-tryptophan added to pooled human serum was partly bound to protein in a manner readily reversed by the presence of salicylate.

The dialysis experiments (Fig. 1) show that L-tryptophan is reversibly bound to purified bovine serum albumin and in the presence of increasing concentrations of sodium salicylate proportionally more of the amino-acid remains in a free unbound form.

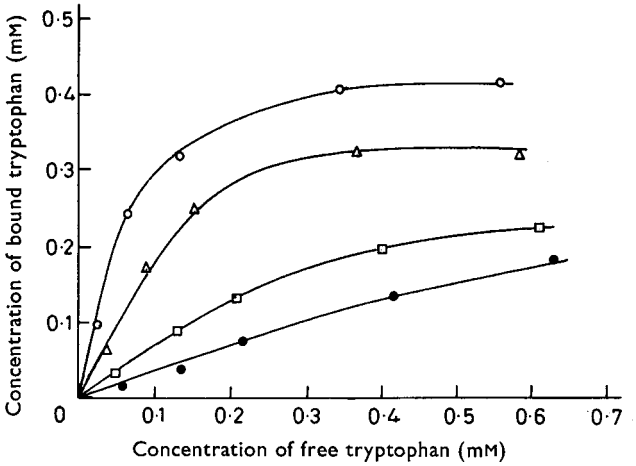

FIG. 1. The effect of salicylate on the relative concentrations of free and bound L-tryptophan in the presence of 3% bovine serum albumin. The experimental conditions were as described in the experimental section. Two ml portions of 3% bovine serum albumin were dialysed against 4 ml of buffer containing various concentrations of L-tryptophan and the following concentrations of sodium salicylate: ○ none. △ 0·45 M. □ 1·9 mM. ● 4·5 mM.

The results suggest that after ingestion of a therapeutic dose of aspirin the resulting serum concentration of salicylate ion could cause an increased level of free tryptophan in the blood. An increased rate of entry of L-tryptophan into the tissues, followed by an increased rate of its metabolism, would tend to restore the free level of L-tryptophan in the blood to that present before ingestion of the salicylate. However, after elimination of the salicylate by conjugation and excretion, more L-tryptophan would bind again to protein and the level of free L-tryptophan would drop. In cases of prolonged salicylate therapy, the concentration of bound L-tryptophan would be much lower and the free concentration would become established at a level similar to that present before salicylate therapy. The overall results would be a lowered reserve of bound L-tryptophan and a reduced capacity to bind free L-tryptophan when the amino-acid was added to the circulation by any route.

It has been shown that the urine of patients suffering from rheumatoid arthritis contains abnormally high concentrations of kynurenine and 3-hydroxyanthranilic acid (McMillan, 1960; Bett, 1962; Spiera, 1966). A tryptophan load given to these patients caused a rise in the urinary kynurenine levels much greater than that produced in normal individuals, whereas the urinary excretion of 3-hydroxyanthranilic acid was increased by similar amounts in both groups. Bett (1962) stated that previous salicylate therapy or continued therapy during a tryptophan loading test did not affect the quantitative pattern of tryptophan metabolites in the urine. Spiera (1966) studied the levels of various intermediates and end products of the metabolism of tryptophan *via* the kynurenine and 5-hydroxytryptamine (5-HT) pathways, in a group of control patients suffering from a variety of diseases and in a group of patients suffering from rheumatoid arthritis. He found that there were significant increases in the levels of kynurenine, 3-hydroxyanthranilic acid and xanthurenic acid, but not of *N*-methyl nicotinamide, total indoles, 5-hydroxyindole acetic acid, indole acetic acid or tryptamine. However, twelve of the thirty-one patients in the control group were receiving aspirin. If the control group is subdivided into two sub-groups with respect to aspirin administration (Table 3), it can be calculated, using the data of Spiera, that there is a significant difference in the urinary excretions of xanthurenic acid and of this metabolite plus kynurenine and 3-hydroxyanthranilic acid between these two sub-groups. There is also a significant difference in the values between

Table 3. *Excretion of tryptophan metabolites* (*kynurenine plus 3-hydroxyanthranilic acid plus xanthurenic acid*) *expressed as mg/24 h.* The original results of Spiera (1966) and the regrouped controls show that there is a significant difference due to aspirin and not rheumatism. Each value is the mean ± the standard deviation

Control				Rheumatoid
26·4 ± 19·6				63·4 ± 42·6
		$P < 0{\cdot}001$		
Control		Control		
No aspirin		Aspirin		Rheumatoid
20·4 ± 10·0		35·8 ± 26·9		63·4 ± 42·6
	$P < 0{\cdot}05$		$P < 0{\cdot}1$	

the non-aspirin treated controls and the rheumatoid patients, but there is no significant difference between the control sub-group receiving aspirin and the rheumatoid group, all of whom were treated with aspirin.

The levels of tryptamine, indoleacetic acid and 5-hydroxyindoleacetic acid in the three groups of patients were not found to be significantly different, and since aspirin was being prescribed to two of these groups, there is no indication that the tryptamine and 5-HT pathways are being affected by long-term salicylate therapy. However, these metabolites have not been measured in individuals following an acute dose of aspirin, and it may well be that the levels of tryptophan and its metabolites in blood and urine are raised following such a treatment.

The liver enzyme, tryptophan pyrrolase, which converts tryptophan to *N*-formyl kynurenine is an inducible enzyme. The activity of this enzyme has been increased as much as tenfold by feeding tryptophan (Knox & Mehler, 1950). The release of more free tryptophan into the tissues as a consequence of the presence of salicylate in the blood may result in an increased activity of this enzyme and may be a factor directing the metabolism of more tryptophan *via* the kynurenine pathway, thereby restoring the level of free tryptophan to normal. The activity of tryptophan pyrrolase and other enzymes involved in the metabolism of tryptophan has not been studied during salicylate therapy but it is known that 3-hydroxyanthranilic acid oxidase is strongly inhibited by salicylate *in vitro* (Vescia & di Prisco, 1962).

A high proportion of tryptophan is metabolized *via* acetate to carbon dioxide and water, and only a small proportion of ingested tryptophan can be accounted for in the urinary end-products of the kynurenine and 5-HT pathways (Jepson, 1966). If the oxidation of tryptophan to carbon dioxide and water is operating maximally at a stage after 3-hydroxyanthranilic acid formation, then increased levels of tryptophan would either accumulate as such or as one of its intermediates before the rate-limiting step of the metabolic sequence. If fluctuations in the level of tryptophan occur normally, such as would be expected following a protein meal, then in the presence of salicylate there will be fewer binding sites available to accommodate much of the tryptophan and there will be even greater fluctuations in the free level. This may cause at various times much more tryptophan to be metabolized *via* the kynurenine pathway, giving rise to higher levels of the intermediates in the tissues and blood, and if the renal threshold for these compounds is low, much more will appear in the urine.

It has been observed that, whereas most aminotransferases are inhibited by salicylate, the tryptophan-α-oxoglutarate aminotransferases from the liver, kidney and heart of the rat are activated by salicylate (Gould & Smith, 1965). A possible explanation for this activation could be envisaged if tryptophan were bound either to the enzyme protein itself at a site not involved in its activity, or to protein associated with the enzyme. In the presence of salicylate, more of the tryptophan might be released to give a higher effective concentration of free substrate which could have been responsible for the observed increased activity. If the enzyme were inhibited by salicylate, then the stimulation caused by the released substrate might have been sufficiently great to mask this.

Davis, Fisher & McGowan (1968) have shown that 0·1 ml of 0·01% solution of tryptophan inhibits the infiltration of leucocytes into an area of local inflammation of rats. There is, therefore, the possibility that part of the anti-inflammatory action of salicylate is mediated *via* tryptophan.

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