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The effect of sodium salicylate on the binding of long-chain fatty acids to plasma proteins

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Salicylate causes a release of palmitic, stearic, oleic and linolenic acids from their binding sites on human plasma proteins and bovine albumin. The implications of this finding with respect to other effects of salicylate on fatty acid metabolism are discussed.

A large variety of small molecules are transported in the circulation bound to plasma albumin. Any interference by drugs with such binding could increase the availability of diffusible molecules, these would then be able to enter body cells to either change metabolic rates or initiate pharmacological actions. Salicylate can release some normal metabolites, including L-tryptophan (McArthur & Dawkins, 1969) and uric acid (Bluestone, Kippen & Klinenberg, 1969) from their binding sites on plasma proteins and thus affect their rate of entry into the tissues. The present work shows that the drug can also displace long-chain fatty acids from human plasma proteins and bovine albumin *in vitro*.

EXPERIMENTAL

Materials

Pooled human plasma was obtained from the National Transfusion Service, Sutton. Bovine albumin (fraction V) and palmitic, stearic, oleic and linolenic acids were obtained from the Sigma Chemical Company, St. Louis, palmitic acid-1-C^{14} (specific activity 55·2 mCi/mmol), stearic acid-1-C^{14} (specific activity 48·4 mCi/mmol), oleic acid-1-C^{14} (specific activity 57·8 mCi/mmol) and linolenic acid-1-C^{14} (specific activity 41·5 mCi/mmol) from the Radiochemical Centre, Amersham and 2,5-diphenyloxazole (PPO) from the Packard Instrument Company, London. All other chemicals were of analytical grade and distilled water was used throughout.

Measurement of binding of fatty acids to protein

The technique of partition analysis (Goodman, 1958a) was used to measure the concentrations of unbound fatty acids in solutions of bovine albumin and in human plasma. The method depends on the determination of the distribution of varying quantities of the fatty acids between n-heptane and either an aqueous buffer, a solution of bovine albumin in the buffer or human plasma. The use of trace amounts of labelled fatty acids mixed with the non-radioactive fatty acids enabled accurate measurements to be made at low concentrations. The presence of salicylate did not alter the partition of the fatty acids between the n-heptane and aqueous phases in the absence of albumin nor did the salicylate enter the heptane layer.

In the experiments using bovine albumin, a series of mixtures, each of 20 ml volume, were prepared using 0·1M phosphate buffer, pH 7·4, to contain the unlabelled

fatty acid; 0·001 to 0·5 mM, the corresponding radioactive fatty acid, 0·5 μCi; bovine serum albumin, 0·2 mM (1·36% w/v) and sodium salicylate, 0 to 5 mM. The individual components were added in the order given above. In the experiments with human plasma 0·4 ml of a solution, containing 0·5 μCi of each of the four radioactive fatty acids plus sufficient sodium salicylate to give final salicylate concentrations ranging from 0 to 5 mM dissolved in the 0·1M phosphate buffer, pH 7·4, was added to 19·6 ml of pooled human plasma.

Each mixture was placed in a 25 ml round bottom glass-stoppered flask, 2 ml of n-heptane were added, and the flask and contents shaken for 48 h at 125 rev/min in a Luckman rotary shaker at room temperature (22°). The results of preliminary experiments using incubation periods up to 96 h showed that equilibrium was reached within 48 h. The flasks were inverted to prevent evaporation of the heptane. At the end of the equilibration period the contents of each flask were centrifuged at 2000*g* for 20 min and duplicate aliquots (0·25 ml) of the heptane layers were mixed with 5 ml of 0·5% (w/v) PPO in toluene. After complete removal of the remaining heptane, duplicate samples (0·1 ml) of the aqueous layer in the bovine albumin experiments were transferred by pipetting on to 2·1 cm diameter glass fibre discs. The discs were allowed to dry in air for 12 h and then added to 5 ml of the PPO–toluene phosphor. In the experiments with human plasma the material remaining after removal of the heptane was diluted 1 to 5 with distilled water before the 0·1 ml aliquots were transferred to the glass fibre discs. This was done to avoid subsequent quenching of the phosphor by a high concentration of protein. The radioactivity in the final toluene–phosphor mixtures was counted in a Beckman LS 200B liquid scintillation system, at least 10000 counts being recorded for each sample.

The total concentration of fatty acid in the heptane divided by the total concentration of fatty acid in the phosphate buffer gives the distribution ratio of each fatty acid within the system after equilibration (Goodman, 1958a). In the experiments with bovine albumin and human plasma the concentrations of unbound fatty acid in the aqueous protein phases were calculated by dividing the total concentration of fatty acid in the corresponding heptane phase by the appropriate distribution ratio for the fatty acid.

RESULTS

The effects of salicylate, in concentrations ranging from 0·5 to 5 mM, on the concentrations of unbound palmitic, stearic, oleic or linolenic acids present in solutions containing 0·2 mM bovine albumin are given in Table 1. In every experiment the presence of increasing concentrations of salicylate increased the concentrations of each fatty acid in free solution. Similar results were observed in the experiments with human plasma in which the four radioactive fatty acids were added as a mixture to the pooled plasma. For concentrations of salicylate of 0, 3·0 and 5·0 mM, the per cent unbound radioactive fatty acid (as a percentage of the total of the labelled acid added) was: 0·035 $\pm$ 0·007 (s.d., 8 det.), 0·078 $\pm$ 0·008 (s.d., 8 det.) and 0·114 $\pm$ 0·007 (s.d., 8 det.) respectively. In solutions of bovine albumin the concentration of an individual fatty acid in free solution depends on the presence or absence of other fatty acids. This is illustrated by the results in Table 2 which show that the final unbound concentration of a radioactive fatty acid in a bovine albumin solution is increased if a different non-radioactive fatty acid is added simultaneously. Presumably this effect is due to the various fatty acids competing for the same binding

Table 1. *The effect of salicylate on the concentrations of unbound fatty acids in the presence of bovine albumin.* Each value represents the mean of three separate observations

Salicylate concn (mM)	Fatty acid concn (μ-equiv/litre) added to albumin solution	Unbound fatty acid concn (n-equiv/litre)			
		Palmitic	Stearic	Oleic	Linolenic
0·0	1·0	0·09	0·09	0·44	0·11
0·5		0·12	0·15	0·57	0·17
2·0		0·19	0·32	1·35	0·33
5·0		0·32	0·66	2·70	0·59
0·0	10·0	1·00	0·90	7·20	1·20
0·5		1·50	1·40	6·90	1·50
2·0		2·40	3·20	17·10	2·80
5·0		4·30	6·90	29·50	6·30
0·0	100·0	11·0	15·0	58·0	22·0
0·5		15·0	20·0	97·0	25·0
2·0		29·0	55·0	215·0	39·0
5·0		59·0	130·0	425·0	63·0
0·0	500·0	140·0	245·0	695·0	145·0
0·5		250·0	590·0	1160·0	215·0
2·0		640·0	1495·0	2465·0	320·0
5·0		1365·0	3280·0	6110·0	575·0

Table 2. *Effect of salicylate on the concentration of unbound radioactive fatty acid in a bovine albumin solution to which was added either a radioactive fatty acid or a mixture of one radioactive and one non-radioactive fatty acid.* In each experiment the bovine albumin concentration was 0·2 mM, radioactive fatty acid was added to give a final concentration of 0·5 μ-equiv/litre, non-radioactive fatty acid to give a final concentration of 500 μ-equiv/litre and salicylate, when present, to produce a final concentration of 5 mM. Each experimental value represents the mean of three separate determinations and is expressed as n-equiv/litre of radioactive fatty acid in free solution

Radioactive fatty acid	Non-radioactive fatty acid	Unbound radioactive fatty acid concn	Increase in unbound radioactive fatty acid concn with salicylate
Palmitic	None	0·045	0·115
	Linolenic	0·105	0·455
Linolenic	None	0·055	0·240
	Palmitic	0·110	0·650
Palmitic	None	0·045	0·115
	Stearic	0·145	1·255
Stearic	None	0·220	1·130
	Palmitic	0·170	1·675

sites on the protein. Table 2 also shows that the effect of salicylate in displacing the albumin-bound radioactive fatty acid is substantially enhanced if a second non-radioactive fatty acid is added to the albumin solution at the same time as the labelled fatty acid.

DISCUSSION

The greatest net transport of fat in the plasma from the adipose to other tissues occurs as long-chain fatty acid anions although these represent only a few per cent

of the total plasma lipids (Fredrickson & Lees, 1966). The normal level of these fatty acids in human plasma is of the order of 0·5 m-equiv per litre (Thorp, 1963) and oleic, palmitic, stearic and linolenic acids comprise over 80% of the fatty acid fraction (Robertson, Sprecher & Wilcox, 1968). In the circulation these fatty acids are largely complexed with albumin and *in vitro* determinations (Goodman, 1958b) suggest that this binding amounts to over 99% of the fatty acid fraction in normal human plasma. Only a very small fraction of the plasma fatty acids are therefore available to enter body cells at any one moment of time. Steinberg (1966) has proposed a series of equilibria representing the transfer of fatty acids from albumin via unbound fatty acid anions in free solution to the cell surface, followed by transfer into metabolic pathways. With ascites tumour cells his experimental data suggest that the concentrations of the fatty acids at or near the cell surface governs their subsequent rate of utilization inside the cell. The present results show that salicylate displaces several long-chain fatty acids from their combination with purified bovine albumin and human plasma proteins *in vitro*. The magnitude of the effect is small in relation to the amount of protein-bound fatty acid but represents a ten-fold rise in the concentrations of fatty acids which are free in solution. If this effect occurs *in vivo* it may explain, at least in part, several reported actions of salicylate on fatty acid metabolism in man and in experimental animals.

The first of these concerns the changes in the plasma non-esterified fatty acid concentrations. It is generally agreed that in the rat the acute administration of sodium salicylate causes a marked, if transient, decrease in this component in the plasma (Bizzi, Garattini & Veneroni, 1965; Torsti & Mattila, 1966; Wooles, Borzelleca & Branham, 1967). Salicylate has been differentiated from other drugs, such as 3,5-dimethylpyrazole and nicotinic acid, that lower plasma fatty acid concentrations in the rat in that it shows activity in fed and noradrenaline-treated animals but not rats fasted up to 48 h (Garattini & Bizzi, 1966). The published work on the effects of salicylate on plasma fatty acids in man is more confusing. Gilgore, Drew & Rupp (1963) claimed that the infusion of sodium salicylate caused a 20% rise in the plasma fatty acids in normal subjects and a 50% rise in diabetic patients. These results have been criticized (Bizzi & others, 1965) on the basis that the presence of salicylate in the plasma interfered with the measurement of the fatty acids. Field, Boyle & Remer (1967) observed no significant effect on plasma fatty acids in normal subjects and diabetic patients who received an infusion of sodium salicylate. However, all the subjects showed a significant increase in plasma-insulin levels and the authors themselves comment that this combination of results appears difficult to explain in view of the well-documented ability of insulin to decrease the plasma fatty acid concentrations. In contrast, Carlson & Ostman (1961) reported that the oral administration of acetylsalicylate to normal man and to mildly diabetic subjects decreased the plasma fatty acid concentrations. In all the human experiments described above the subjects were fasted either overnight or for 12 h before the salicylate was given. The influence of more prolonged fasting was studied by DeFelice & Gilgore (1968) who found that infusion of sodium salicylate decreased the plasma fatty acids in obese non-diabetic subjects after a 72 h fast but not before the fasting period.

Some consideration has been given to the mechanism by which salicylate could induce decreased levels of plasma fatty acids. An increased transfer of the acids from the circulation to the tissues was apparently excluded by the results of Carlson & Ostman (1961). These workers infused diabetic patients with a solution containing

human albumin and C^{14}-labelled palmitate at such a rate that a constant amount of radioactivity/ml of circulating plasma was maintained over several hours. The subsequent intravenous administration of calcium acetylsalicylate did not affect the concentration of radioactivity in the plasma although the level of circulating fatty acids was markedly decreased. Carlson & Ostman used only trace amounts of radioactive palmitate, approximately 1 μmol per 200 μmol of protein, to label the human albumin before infusion. It has been shown by Goodman (1958b) that there are several different types of binding sites for fatty acids on human albumin. These sites differ in their affinities for palmitate. A trace (radioactive) dose of the acid would become attached to the sites possessing the greatest affinity and would therefore be less likely to become dissociated than the non-radioactive fatty acid molecules which bind to the albumin after its introduction into the circulation. Thus the subsequent administration of salicylate may have preferentially displaced fatty acids other than the labelled palmitate. An expected result of this displacement would be increased concentrations of the fatty acids in free solution in the plasma which in turn would cause decreased mobilization of fatty acids from depot fat in adipose tissue. Thus the lipolytic effect of salicylate in the rat and in man may be caused by a diminished rate of entry of fatty acids into the plasma and mediated by the drug displacing a small, but important, percentage of the fatty acids from their binding sites on circulating albumin.

Some support for this view is provided by the results of experiments with adipose tissue preparations *in vitro*. Carlson & Ostman (1961), using rat epididymal fat bodies, and Stone, Brown & Steele (1969), using isolated fat cells, found that salicylate possessed antilipolytic effects *in vitro* if the incubation mixtures contained either human or bovine albumin. It has been shown (Campbell, Martucci & Green, 1964) that fatty acids are only released from adipose tissue if a substance with special properties as an acceptor of fatty acids is present and that plasma albumin performs this function most effectively. The lower degree of binding of the fatty acids to the albumin in the presence of salicylate would in turn decrease their release from the triglycerides of the adipose tissue.

An increased rate of entry of fatty acid molecules into body tissues from the plasma as a response to the acute administration of salicylate may also be related to the development of metabolic acidosis in salicylate intoxication in children (Winters, 1963). The principal cause of the acidosis seems to be an accumulation of anions of organic acids, including acetoacetate. It has been suggested (Smith, 1968) that a combination of the uncoupling action of salicylate together with its inhibitory effects on aminotransferase and dehydrogenase enzymes would lead to an increased formation and a restricted metabolism of acetyl-CoA so that there is an increased conversion of this intermediate to acetoacetyl-CoA and hence acetoacetate.

The formation of acetoacetate in the liver would be exacerbated by the increased rates of entry and catabolism of the fatty acids in the presence of salicylate. It has been shown by Ontko & Zilversmit (1966) in the rat and Steinberg (1966) in man that there appears to be a direct relation between the concentrations of fatty acids and ketone bodies in the plasma. These results indicate that an increased level of plasma fatty acids and hence an increased entry of fatty acid anions into the liver can accelerate hepatic ketogenesis. The results of Numa, Bortz & Lynen (1965) suggested that the metabolic pathways for the utilization of acetyl-CoA may be further restricted if fatty acids accumulate in the tissues. These workers discovered that long-chain

fatty acids and their acyl-CoA derivations inhibit acetyl-CoA carboxylase [acetyl-CoA: carbon dioxide ligase (ADP) EC 6.4.1.2] activity *in vitro* and thus decrease fat synthesis. However, Pande & Mead (1968) observed that fatty acids inhibit several enzyme activities *in vitro* by an unspecific mechanism depending on their detergent properties and consider that the extrapolation to *in vivo* situations must be viewed with caution. Salicylate itself inhibits acetyl-CoA carboxylase activity in cell-free systems (Goldman, 1967) and if this effect occurs *in vivo* it may reinforce the development of ketosis in acute salicylate intoxication in children.

A further interaction of salicylate and fatty acid metabolism is the fatty infiltration and degeneration of the liver observed either in acute salicylate poisoning in man (Gross & Greenberg, 1948) or after the chronic administration of the drug to experimental animals (Janota, Wincey & others, 1960; Niederland, 1963). Such effects may result from an increased entry of fatty acids into the liver as a consequence of increased concentrations of fatty acids in free solution in the plasma.

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