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BIOCHEMICAL AND STRUCTURAL CHANGES IN RAT LIVER RESULTING FROM THE PARENTERAL ADMINISTRATION OF A LARGE DOSE OF SODIUM SALICYLATE

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Abstract—The effects of a large parenteral dose of sodium salicylate on rat liver and plasma enzymes have been studied. A dose of 400 mg/kg by i.p. injection did not result in significant elevation of three plasma enzymes investigated: β-glucuronidase, acid phosphatase and alanine aminotransferase. There was, however, a rapid decrease in the liver's concentration of $NADPH_2$ and a similarly rapid choleresis. Although the total concentration of ATP in the liver was not altered by salicylate dosing the distribution between the large particle fraction and the cell sap was changed in favour of there being relatively more ATP in the cell sap fraction. Sodium salicylate produced only a small change in the microsomal cytochrome P_{450} spectrum *in vitro* in comparison with the effects found with the drug SKF 525A that is known to bind strongly to P_{450}. Liver sections obtained from rats dosed 1–3 hr previously with salicylate appeared relatively normal when examined by light microscopy. Electron microscopy, however, demonstrated several early effects of salicylate on liver parenchyma: there appeared to be an early increase in the number of peroxisomes, and multivesicular bodies were in evidence around the Golgi zone. The endoplasmic reticulum and attached ribosomes exhibited a normal appearance. These effects of salicylate on rat liver are discussed and contrasted with the results found previously with known hepatotoxic agents.

A HIGH dose of sodium salicylate has been used to alleviate the pharmacological effects resulting from the liberation of kinin-like substances.[1] For example, it was found that such a dose of sodium salicylate administered together with an antihistamine drug decreased the inflammatory effects resulting from the intradermal injection of the fungal toxin Sporidesmin into rats.[2]

The dose used in the above-mentioned experiments (400 mg/kg body wt.) was very high and it was considered important to check whether such a dose produced any significant degree of liver cell damage as evidenced, for example, by a leakage of liver enzymes into the plasma or by morphological changes detectable by light microscopy. The relevant data are reported in this paper.

Further, in the course of our investigations concerning the effects of a number of

hepatotoxic agents and other substances on bile flow rate and liver ATP content, it was observed that sodium salicylate caused a pronounced choleresis together with changes in the concentration of $NADPH_2$ in the liver,[3] and in the intracellular distribution of ATP. In view of these findings, which are described in this paper, it was decided to study the effects of sodium salicylate on liver cell morphology in some detail using electron microscopy so that the changes in bile flow, in ATP distribution and in liver cell morphology may be correlated with each other. A preliminary account of some of this work has previously appeared.[4]

METHODS

Adult female albino rats, body weight 120–150 g were used; they were fed water and standard diet (M.R.C. Diet 41B) *ad lib.*

A solution of sodium salicylate (pH 7·0; 100 mg/ml) in 0·9% NaCl was injected i.p.; the dose was 0·4 ml/100 g body weight (equivalent to 400 mg/kg). Control rats received an equivalent volume of 0·9% NaCl.

A solution of sodium malonate (pH 7·4, 1 M) was injected i.p.; the dose was 1·2 ml/100 g body weight (equivalent to 178 mg/kg). Control rats received an equivalent volume of 1 M NaCl.

Carbon tetrachloride was mixed with liquid paraffin (1 + 3 v/v) and 0·5 ml of this mixture (equivalent to 1·25 ml CCl_4/kg body wt.) was given by stomach tube with the rats under light ether anaesthesia. Rats were killed 2 hr after dosing.

A microsomal fraction was prepared from rat liver homogenates by the following procedure. Liver was homogenised in 0·25 M sucrose (approx. 1:5 w/v) and this suspension was centrifuged at +4° for 10 min at 15,000 *g*. The supernatant suspension was carefully removed from the pellet using a pipette with a bent tip and the centrifuging step was repeated on this supernatant suspension. The supernatant obtained from this second centrifuging was then centrifuged at 200,000 *g* for 40 min at +4° using an M.S.E. Superspeed 50 centrifuge. The microsomal pellet so obtained was suspended in 0·15 M KCl so that the microsomes obtained from 1 g of original liver were suspended in 1 ml of KCl.

Liver measurements. ATP was estimated in samples of liver essentially by the method of Lamprecht and Trautschold.[5] To prevent the loss of ATP that occurs in whole liver *in situ* very rapidly after death, small pieces of the liver were frozen in liquid nitrogen prior to weighing and extraction.

The compartmentation of ATP between the soluble fraction and the particulate fraction of rat liver homogenates was determined using the micro-filtration method of Delaney and Slater.[6] The strength of the homogenate used for the compartmentation studies was estimated by comparing its DNAP content relative to the DNAP content of an homogenate of known strength prepared from the same liver. DNAP was estimated by the method of Slater.[7]

Uricase activity in liver homogenates was measured by the method of Schneider and Hogeboom.[8] Catalase in liver homogenates was measured by the method of Rørth and Jenson.[9]

The effects of sodium salicylate on the difference spectrum of cytochrome P_{450} in rat liver microsomes was determined by the method of Schenkman, Remmer and Estabrook.[10]

Protein concentration in tissue suspensions was determined by the method of Lowry *et al.*[11]

Serum measurements. β-Glucuronidase activity in rat serum was estimated by the method of Gianetto and De Duve.[12] Acid phosphatase activity was measured by the method of King[13] using sodium phenyl phosphate as substrate. Alanine amino transferase was measured by the standard kit procedure supplied by Boehringer (UK) Ltd.

Bile flow. With the rat under Nembutal anaesthesia (6 mg/100 g body wt.) a mid-line incision was made from a point just below the level of the left kidney to the xiphisternum. The bile duct was ligated above the junctions with the pancreatic ducts and a flexible nylon cannula (outside diameter 0·62 mm and bore 0·5 mm) was tied into the bile duct making sure that lower tributaries from the caudal lobes were not obstructed. The abdomen was closed and bile collected in pre-weighed tubes that were cooled in ice. Analyses, other than bile salt estimations, were performed immediately due to rapid loss of enzyme activity in bile.

The effect of sodium salicylate on bile flow and composition in unanaesthetised unrestrained rats was performed after taking the cannula through subdermal connective tissue to the back of the neck[14] where bile was collected in glass saddle-vessels essentially as described by Van Zyl.[15] In this case the rats were allowed three days to recover before administering the drug; they were fed standard diet and 0·9% NaCl *ad lib.*

The estimation of β-glucuronidase and acid phosphatase in bile was performed using the same procedures as described for serum. Bilirubin was estimated by the method of Malloy and Evelyn.[16] Total bile salt was estimated using 3α-hydroxysteroid dehydrogenase[17] (Worthington Biochemicals Ltd.).

Microscopy. For standard light microscopy, liver sections were fixed in formol-saline embedded in paraffin wax and stained with haematoxylin-eosin.

For electron microscopy, representative samples from the median lobe were put into 4% glutaraldehyde–cacodylate buffer (pH 7·2, 0·1 M) for 4 hr at 4°. After 10 min fixation, 2 mm blocks were cut into tiny fragments. The fixed blocks of tissue were washed overnight in 0·1 M cacodylate buffer, containing 0·25 M sucrose. They were then post fixed for 1 hr in 1% OsO_4 in Millonig's buffer, dehydrated through a graded series of ethanol solutions and embedded in Epon (Shell Epikote 812). Thin sections (silver/grey) were cut on a Porter Blum MT1 ultratome and viewed in an AEI EM6B electron microscope, usually at 60 Kv.

RESULTS

Liver estimations. Sodium salicylate had no appreciable effect on the total ATP content of rat liver 1 hr after parenteral dosing (Table 1). There was, however, a considerable alteration in the distribution of ATP between the mitochondrial and supernatant fractions (Table 1). Treatment with salicylate increased the proportion of ATP in the supernatant fraction; the opposite effect was found after dosing i.p. with sodium malonate (Table 1). It is interesting that i.p. dosing with NaCl (Table 1) or various types of anaesthesia (V. B. Delaney and T. F. Slater, unpublished data) increase the percentage of ATP in the cell sap fraction from about 45% in untreated controls to about 60%. The mechanism underlying this apparent stress effect is unknown.

No changes in the activities of liver urate oxidase and catalase were found 1–3 hr after i.p. dosing with sodium salicylate (Table 2).

A very small change in the spectrum of cytochrome P_{450} was observed after the addition of 5 mM sodium salicylate to a microsomal suspension *in vitro*. In contrast diethylaminoethyl diphenyl propyl acetic acid (SKF 525A, 0·21 mM) produced a much larger change in the spectrum (Fig. 1).

Serum enzymes. No significant changes in the activities of the serum enzymes studied were observed following dosing with sodium salicylate (Table 3).

TABLE 1. EFFECTS OF SODIUM SALICYLATE, SODIUM MALONATE AND SODIUM CHLORIDE ON THE CONCENTRATION OF ATP IN RAT LIVER AND ON THE PERCENTAGE OF ATP IN THE SUPERNATANT FRACTION S (SEE METHODS)

Treatment	Time (min)	ATP in whole liver μmoles/g wet wt.	Time (min)	% ATP in fraction-S
Untreated	—	2·54 ± 0·14 (7)	—	47 ± 3 (7)
NaCl	60	2·24 (2)	30	60 ± 3 (4)
Sodium salicylate	60	2·20 ± 0·04 (4)	30	74 ± 3 (4)*
NaCl	30	2·57 (2)	30	63 ± 2 (4)
Sodium malonate	30	2·56 (2)	30	40 ± 1 (4)†

The numbers of rats used are in parentheses. Mean values are given ± S.E.M.
* $P = 0·01$ from NaCl-treated group.
† $P < 0·001$ from NaCl-treated group.

TABLE 2. THE EFFECTS OF DOSING WITH SODIUM SALICYLATE ON THE ACTIVITIES OF LIVER URICASE AND CATALASE

Treatment	Time (hr)	Uricase μmoles/g/min	Catalase mμmoles oxygen/μg protein/min
Control	1	1·13 ± 0·05 (3)	16·3 (2)
Salicylate	1	1·06 ± 0·14 (3)	15·1 (2)
Control	2	1·61 (2)	—
Salicylate	2	1·62 (2)	—
Control	3	—	19·3 ± 4·9 (3)
Salicylate	3	—	17·9 ± 3·9 (4)

Mean values are given ± S.E.M.; the numbers of rats used are in parentheses.

TABLE 3. EFFECT OF SODIUM SALICYLATE ON THE ACTIVITIES OF β-GLUCURONIDASE ACID PHOSPHATASE AND ALANINE AMINO-TRANSFERASE IN SERUM

Treatment	Time (hr)	β-Glucuronidase*	Acid Phosphatase*	AAT*
Control	1	116 ± 24	189 ± 45	9 ± 1
Sodium salicylate	1	136 ± 24	158 ± 26	8 ± 1
Control	3	98 ± 5	118 ± 18	9 ± 1
Sodium salicylate	3	124 ± 30	159 ± 18	11 ± 4

Four rats were used in each group and the mean values ± S.E.M. are given.
* Units: β-glucuronidase, μg phenolphthalein liberated/hr/ml serum; acid phosphatase, μg phenol liberated/hr/ml serum; alanine aminotransferase (AAT), I.U.B. milli-units/ml serum.

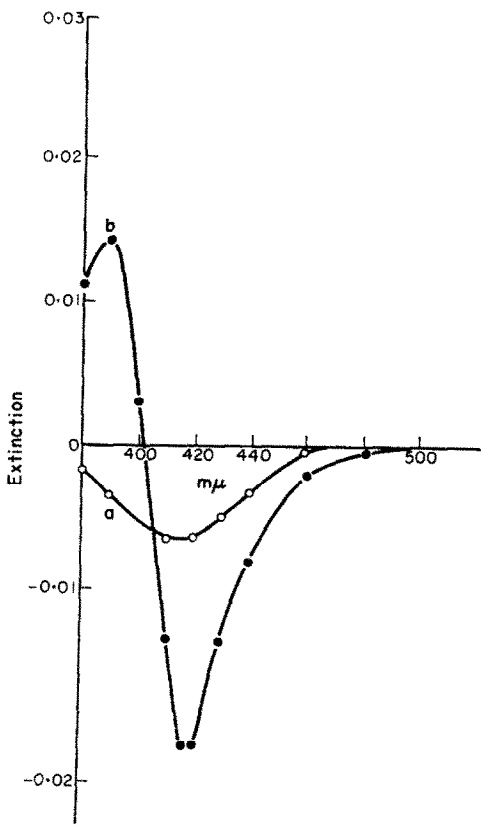

FIG. 1. Difference spectra obtained by the interaction of rat liver microsomes with (a) 5·6 mM sodium salicylate, (b) 0·21 mM SKF 525A. The cuvettes contained approx. 5 mg microsomal protein/3 ml phosphate buffer, 0·1 M, pH 7·4. For other details see Methods.

Bile flow. Sodium salicylate markedly increased bile flow rate in anaesthetised rats. In rats that were unanaesthetised and unrestrained a similar effect was observed (Fig. 2).

Microscopy. No gross morphological changes were observed in liver sections by light microscopy after staining with haematoxylin and eosin.

Examination of sections by electron microscopy revealed the following changes (Figs. 3–5). In the liver samples obtained from rats dosed with sodium salicylate larger numbers of peroxisomes were observed compared with control sections; these intracellular organelles (for general background information on peroxisomes and the relationship to microbodies, see reference 32) were identified by the distinctive density of the matrix and by the crystalloid inclusion body (Fig. 3). In an attempt to place this observation on a semiquantitative basis the number of microbodies in twenty representative areas of both control and salicylate treated liver sections were counted and the figures were corrected for the area of cytoplasm covered in the section. This calculation gave a ratio of 2·3:1 for the sodium salicylate-treated rats compared with the control group.

A further finding of interest was the presence of large numbers of multivesicular bodies in the region of the Golgi apparatus (Fig. 4). These vesicles were rarely observed in the control tissue.

Examination of the endoplasmic reticulum showed that the ribosomes remained firmly attached following sodium salicylate treatment (Fig. 5). The material obtained from the rats dosed with sodium salicylate contains rough endoplasmic reticulum in regular array which compared favourably with the control material. In contrast, sections obtained from rats dosed with carbon tetrachloride showed considerable dissociation of the ribosomes from the rough endoplasmic reticulum and swelling of the cisternae.

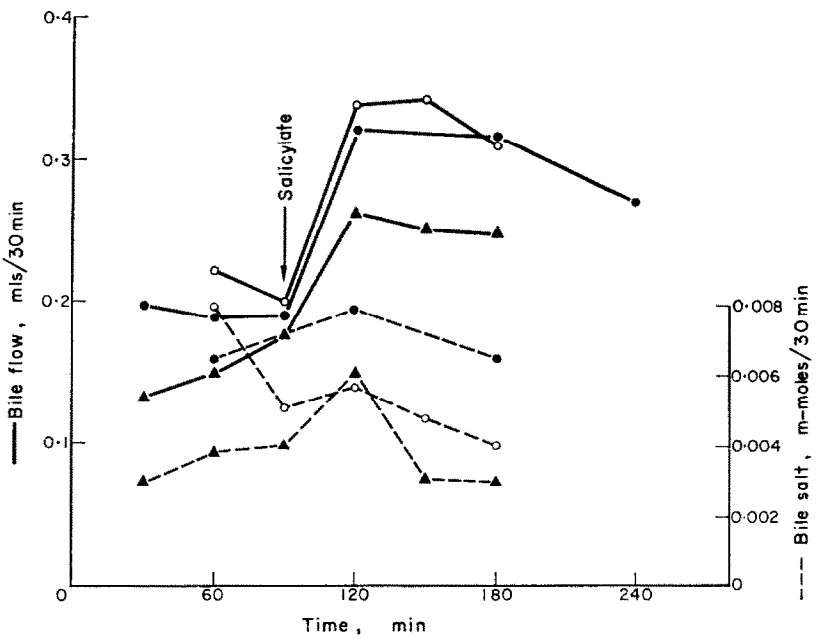

FIG. 2. Bile flow rate in three unanaesthetised, unrestrained rats before and after i.p. dosing with sodium salicylate (400 mg/kg body wt.). The corresponding bile salt outputs in bile are also shown as dashed lines. For other details see Methods. The continuous lines give results obtained for bile flow in three individual expts; the dashed lines give the corresponding changes in bile salt excretion rate.

DISCUSSION

The dose of sodium salicylate used in this study was very large and is considerably in excess of that ingested in normal clinical practice. It is the dose, however, that has been frequently used in studies on the inflammatory process in the rat and it has been found to produce several interesting effects on liver structure and function. It has previously been reported[3] that a large parenteral dose of sodium salicylate causes a decrease in the total liver content of NADP + $NADPH_2$ that is rapid and similar in extent to that produced by CCl_4.[18] The decrease in the sum of NADP + $NADPH_2$ that follows the administration of salicylate does not appear to be due to a shortage of the substrates in the NAD-kinase biosynthetic route, for there is no change in the total liver content of NAD or of ATP in the liver during the period studied (ref. 3 and

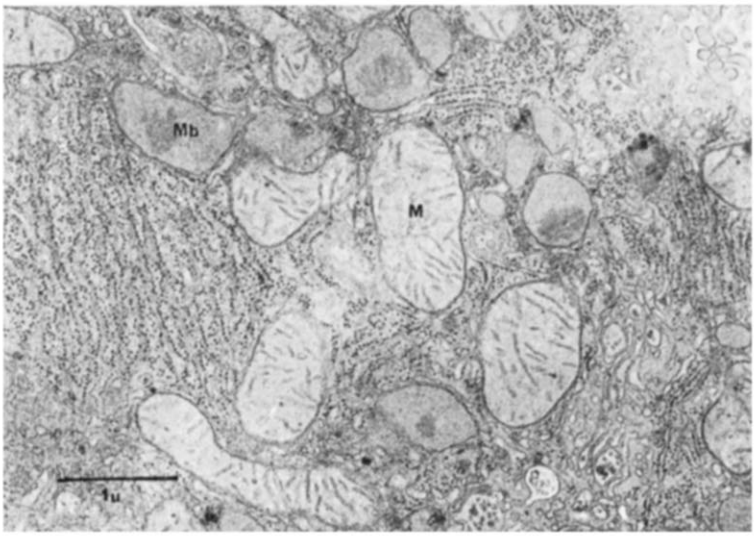

FIG. 3. Development of an unusually large number of microbodies (Mb) in a liver cell treated with sodium salicylate. M = mitochondrion. (Mag. ×15,000).

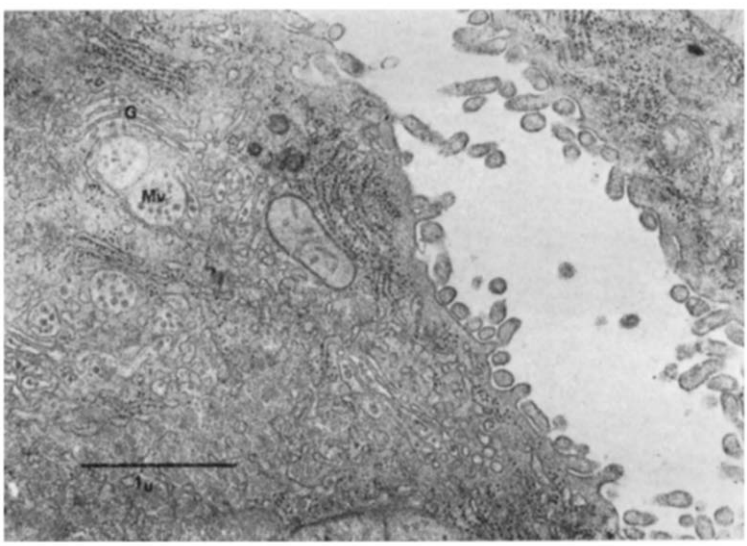

FIG. 4. Salicylate-treated liver, biliary pole of a cell showing proliferation of the Golgi apparatus (G) and associated multivesicular bodies (Mv). (Mag. ×10,000).

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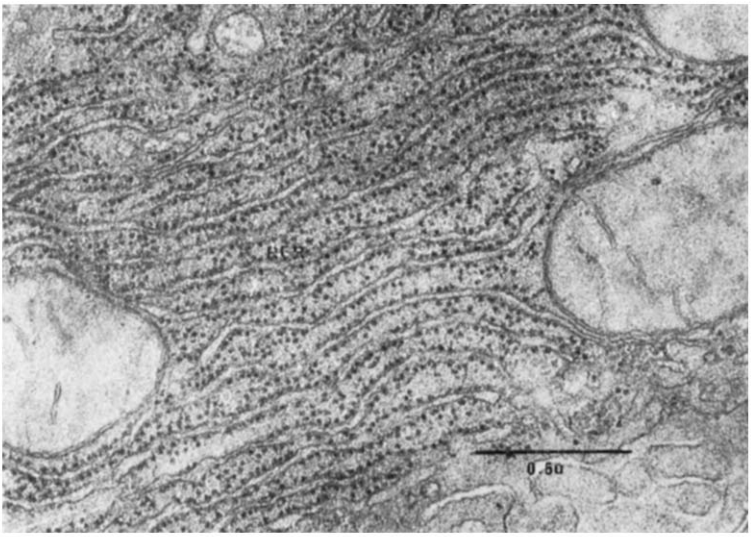

FIG. 5. Rough endoplasmic reticulum (RER) in a liver parenchymal cell after treatment with sodium salicylate. The ribosomes remain firmly attached to the membranes. (Mag. $\times$ 20,000).

Table 1). Changes in the intracellular distribution of these substrates are not likely to be responsible for the decreases in the level of NADP + $NADPH_2$ since the majority of liver cell NAD is in the cytoplasm (ref. 19; Delaney and Slater, unpublished data) and salicylate treatment increases the percentage of ATP in the supernatant fraction where NAD kinase is located. The mechanism responsible for the decrease in NADP + $NADPH_2$ in liver following salicylate dosing cannot be determined with present data.

The failure to observe any appreciable decrease in total liver ATP after dosing with sodium salicylate suggests that significant uncoupling of oxidative phosphorylation does not occur under these *in vivo* conditions. Salicylate is known to uncouple oxidative phosphorylation *in vitro* in mitochondrial suspensions.[20]

A similar lack of effect *in vivo* by an agent known to be very active *in vitro* in uncoupling oxidative phosphorylation has been found[21] with 2·4-dinitrophenol. It is possible that in the latter case the lack of effect *in vivo* is due to the strong binding between 2·4-dinitrophenol and plasma albumin[22] (the work by Parker,[36] on dinitro-ortho-cresol is relevant to these findings). Salicylate also binds to albumin but presumably some of the administered material enters the parenchymal cells since salicylate appears in bile[23] and has effects on bile flow rate, on NADP + $NADPH_2$ content and on parenchymal cell morphology (see later). It may be argued that these changes result from the action of a substance, normally bound to albumin, that is displaced by the high dose of salicylate used and is subsequently taken up by the liver. However, a large dose of malonate, which is also anionic at body pH, causes opposite changes to those found with salicylate (decreased bile flow, decreased ATP in cell sap) which suggests that the displacement mechanism may not be relevant. This suggestion is supported to some extent by our findings[21] that ethyl α-(4-chlorophenoxy)-α-methyl-propionate, an agent known to displace anions like thyroxine from albumin,[24] does not affect bile flow and, as shown by Platt and Cockrill[25] increases the concentration of NADP + $NADPH_2$ in liver. From such indirect evidence it may be argued that at least some of the administered salicylate enters the liver cells but that the concentration reaching the sites of oxidative phosphorylation within the mitochondria is not sufficient to cause significant uncoupling.

Although the total ATP content of liver is not changed after dosing with sodium salicylate there is a redistribution of ATP between the particulate and supernatant fractions leading to a relative increase of ATP in the supernatant fraction of the tissue homogenate. A large parenteral dose of sodium malonate, which decreases bile flow, caused the opposite effect on the distribution of ATP: a decrease in the supernatant content and an increase in the ATP content of the mitochondrial fraction (Table 1). In these two cases, therefore, the changes in the percentage distribution of ATP and the changes in bile flow rate show corresponding variations. This correlation will be discussed in detail in connection with the effects produced on bile flow and liver ATP by a number of other agents.[21]

The high dose of sodium salicylate used here produced no significant changes in the activities of the serum enzymes studied. In contrast a hepatotoxic agent like carbon tetrachloride, which produces centrilobular necrosis, produced a rapid rise in the serum of all three enzymes studied here. For example, Slater and Greenbaum[26] found that 3 hr after dosing rats with CCl_4 the activities of acid phosphatase and of β-glucuronidase in the plasma had increased by factors of 2 and 20 respectively. It

may be concluded from the serum enzyme analyses that no appreciable damage to the liver had occurred after salicylate dosing and examination of sections by light microscopy confirmed the absence of gross morphological lesions. However, as has been mentioned already in the Results section, considerable fine structural changes occur within the cell and which are demonstrable by electron microscopy.

Sodium salicylate is excreted to a small extent unchanged in bile.[23] In both the anaesthetised and in the unanaesthetised rat, sodium salicylate increased bile flow (Fig. 2), accompanied by a transient release of acid hydrolases and of bilirubin in the bile, but no comparable increase in bile salt excretion (Fig. 2).

Electron microscopic examination of material obtained from rats dosed with sodium salicylate indicated that the Golgi zone had increased in extent and that associated with this were numerous multivesicular bodies. In view of the many associations of the Golgi zone with secretory processes[27] it may be that the effect of sodium salicylate on the Golgi apparatus is related to the increased flow of bile observed.

An increase in the number of peroxisomes similar to that mentioned here in the Results section has also been observed to occur after treatment with 'Atromid' (ethyl chlorophenoxy isobutyrate + androsterone) or ethyl chlorophenoxyisobutyrate.[28, 29] Prolonged ingestion of acetyl salicylic acid, thioacetamide, tetracycline or azaserine has been reported to increase the crystalloid content of the microbodies.[30] Peroxisomes frequently appear in the cytoplasm in clumps and Essner[31] has demonstrated that they may bud off from the endoplasmic reticulum. However, direct analysis of liver samples after dosing rats with sodium salicylate failed to give any increase in liver uricase or catalase activities, known components of peroxisomes.[32] Following prolonged ingestion of ethyl chlorophenoxy isobutyrate ('Atromid-S'), Svoboda *et al.*[29] found no correlation between the increased number of peroxisomes and liver uricase activity but observed a distinct relationship between the number of peroxisomes and catalase activity. Perhaps the failure to observe an increased liver catalase in these studies following administration of sodium salicylate is due to the short periods of time involved between dosing and sampling (1–2 hr). If the increase in the number of peroxisomes that is suggested by electron microscopical examination is a genuine event, then it would appear that uricase and catalase activities appear somewhat slowly after initial particle formation.

The high dose of sodium salicylate used, although known to cause a rapid decrease in the liver's content of $NADPH_2$, did not cause significant loss of membrane-bound ribosomes from the endoplasmic reticulum over the same interval (Fig. 6). This data contrasts with the results found with the hepatotoxins CCl_4 and dimethylnitrosamine, which not only rapidly decrease the liver's concentration of $NADPH_2$[18, 33] but also produce extensive ribosomal dissociation from the endoplasmic reticulum at approximately the same phase of the injury.[34, 35]

Finally, it is evident from Fig. 1 that salicylate shows only a weak interaction with microsomal cytochrome P_{450} in comparison with SKF 525A that is known to bind strongly with the P_{450} site. It is unlikely therefore that the high dose of salicylate used in this and other experiments interferes in a competitive manner with the P_{450}-linked metabolism of other drugs administered concomitantly.

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