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INHIBITION OF PROTEIN BIOSYNTHESIS IN MOUSE LIVER BY SALICYLATE

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Abstract—The incorporation of L-[U-^{14}C]leucine and L-[ring-2-^{14}C]histidine into the protein of a post-mitochondrial supernatant fraction from mouse liver is inhibited by salicylate concentrations of 1 mM and above. The intraperitoneal injection of 500–600 mg/kg of salicylate in the mouse causes the following: an increase in the liver concentration of leucine but not that of histidine; a decrease in the incorporation of radioactive leucine and histidine into liver protein *in vitro;* an inhibition of the incorporation of radioactivity from intraperitoneally injected leucine into liver protein *in vivo;* a decrease in the transfer of α-aminoisobutyrate from the peritoneum to the blood but not from the circulation to the tissues and an inhibition of protein biosynthesis in the liver after the intravenous administration of radioactive leucine and histidine. It is concluded that salicylate interferes with protein synthesis in mouse liver both *in vitro* and *in vivo*.

HIGH concentrations of salicylate (10–15 mM) inhibit the *in vitro* incorporation of radioactive glutamate and proline into the protein of rat costal cartilage[1] and that of labelled threonine into the epithelial proteins of sheep mucosal scrapings[2] and of pig and human gastric mucosa.[3] Protein biosynthesis in suspensions of human lymphocytes and in the isolated rat diaphragm is more sensitive to the drug and salicylate concentrations from 0·5 to 5 mM have been shown to interfere with the incorporation of glutamate, glycine, lysine and leucine.[4–6] The effect also occurs in cell-free preparations from rat liver[6,7] and it has been shown[8] that salicylate interferes with the biosynthesis of proteins *in vitro* by preferentially inhibiting the formation of certain aminoacyl-RNAs. This paper describes experiments designed to determine if the drug inhibits protein biosynthesis *in vivo* in the mouse.

EXPERIMENTAL

Chemicals. Pyruvate kinase, potassium phosphoenolpyruvate and the disodium salt of ATP were obtained from the Sigma Chemical Company Ltd., London, L-lactic dehydrogenase, $NADH_2$ and the sodium salt of ADP were from the Boehringer Corporation (London) Ltd., 2,5-diphenyloxazole (PPO) from the Packard Instrument Company, Inc., and all other non-radioactive chemicals were from either BDH Chemicals Ltd., or Hopkin & Williams Ltd. L-[ring-2-^{14}C]histidine (44 mc/m-mole), L-[U-^{14}C]leucine (312, 344 mc/m-mole) and [1-^{14}C]α-aminoisobutyrate (45 mc/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks.

Animals. Male albino mice (body weight 25–30 g), maintained on MRC cube diet no. 41, were allowed food and water *ad lib.* throughout all the experiments and were killed by stunning and cervical fracture.

Post-mitochondrial supernatant (PMS) fraction from liver. Immediately after death, the livers were removed from the animals, blotted with filter paper, weighed and homogenized in 2·5 vol. of 0·25 M sucrose containing 75 mM KCl, 10 mM $MgCl_2$ and 35 mM tris–HCl, pH 7·8, in a loose-fitting, all glass homogeniser for 25 sec. The PMS fraction was prepared by centrifuging the homogenate at 15,000 *g* for 15 min at 4° in the 40 head of a Spinco model L preparative ultracentrifuge.

The mouse liver PMS preparations (0·4 ml) were incubated for 25 min at 37° with gentle shaking in pyrex glass test tubes, 1·5 × 12 cm, in a final volume of 0·8 ml in the presence of the following: K phosphoenolpyruvate 10 mM, ATP 1 mM, pyruvate kinase (20 γ/ml), radioactive amino acid (approx. 0·5 μc/ml) and varying concentrations of K salicylate and KCl to bring the final K^+ ion concentration to 80 mM. The enzyme reactions were started by adding the PMS and stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid.

Treatment of animals. Groups of mice were each given an intraperitoneal injection of 0·5 ml of a solution containing sodium salicylate in dose levels ranging from 50 to 600 mg/kg body weight. Control animals were given a similar injection of sodium chloride. The Na^+ ion concentration of the injections for the control and salicylate-treated animals were adjusted to 225 mM. Trace amounts of the radioactive amino acid were given in 0·2 ml isotonic saline (0·9%, w/v, NaCl) either intraperitoneally or intravenously via the tail vein. After injection, the animals were placed in separate cages and allowed food and water *ad lib.*

Extraction of protein and measurement of radioactivity. In experiments involving the *in vivo* incorporation of radioactive amino acids into tissue proteins, the mice were killed 6 min after the injection of tracer. The excised tissues were blotted, weighed and immediately homogenised in 5% (w/v) trichloroacetic acid. After centrifugation the acid supernatants were decanted and the radioactivity in aliquots (1 ml) counted in a Beckman LS 200B Liquid Scintillation Counter, using 10 ml dioxan phosphor containing 0·4% (w/v) PPO and 6% (w/v) naphthalene. The precipitated protein both from the *in vitro* incubations and from the *in vivo* experiments were extracted as described previously.[6] The final protein precipitate was dissolved in 0·2 N NaOH and aliquots (0·1 ml) were dried on GF/A (2·1 cm dia.) glass fibre discs, and counted in 5 ml toluene phosphor containing 0·5% (w/v) PPO. Each sample was counted in duplicate until at least 10,000 counts had been recorded. Protein was determined by the Biuret method for the *in vivo* experiments and by the method of Lowry[9] for the *in vitro* experiments.

Distribution of α-amino isobutyric acid in mouse tissues. Mice were injected intraperitoneally with solutions of either sodium salicylate or sodium chloride and after 30 min were given an injection of radioactive α-amino isobutyrate in normal saline either intraperitoneally or intravenously. After a further 6 min the mice were killed and samples of blood, liver, kidney and brain were collected and heated with 0·5 ml 30% (w/v) KOH in a boiling water bath for 1 hr. After cooling, 2·5 ml water was added to each tube, followed by 2 ml 25% (w/v) trichloroacetic acid and after centrifugation at 2000 *g* for 15 min, 1 ml aliquots of the acid-soluble supernatant containing the free amino acid were counted in 10 ml dioxan phosphor.

Amino acid analyses. The concentrations of leucine and histidine in the blood and livers of mice injected with sodium salicylate were measured as described previously.[10]

RESULTS

In vitro *experiments*

The results in Table 1 show that salicylate, in concentrations ranging from 2·5 to 20 mM inhibits the activity of pyruvate kinase. Since pyruvate kinase is employed in the *in vitro* protein synthesis system for the regeneration of ATP, its concentration was varied to check that any observed effects were not due to the action of salicylate on this enzyme. Table 2 shows that the percentage decrease in the incorporation of the labelled leucine into protein was not altered by increasing the pyruvate kinase concentration from 20 to 40 μg/ml. The decrease in both the control and experimental values with the higher pyruvate kinase concentration may have been due to the ammonium sulphate added with the enzyme.

TABLE 1. EFFECT OF SALICYLATE ON PYRUVATE KINASE

Salicylate conc. (mM)	E/min
0	0·204 ± 0·002 (3)
2·5	0·182 ± 0·003 (3)*
5	0·161 ± 0·001 (3)*
7·5	0·146 ± 0·003 (3)*
10	0·125 ± 0·001 (3)*
15	0·109 ± 0·002 (3)*
20	0·089 ± 0·002 (3)*

Incubation mixtures were set up in silica cells of 1 cm light path and optical density measurements were carried out at 365 mμ. Each cuvette contained $NADH_2$, 0·42 μmole; ADP, 3 μmoles; K phosphoenolpyruvate, 0·75 μmole; $MgCl_2$, 12 μmoles; KCl, 300 μmoles; lactic dehydrogenase, 40 μg and K salicylate 0–60 μmoles in a total volume of 2·8 ml of 50 mM tris–HCl buffer, pH 7·5. The reaction was started by the addition of 0·2 ml pyruvate kinase (0·5 μg). The values given are means ± standard deviations, the number of estimations being in brackets. *Denotes a significant difference ($P < 0·05$) between experimental and control.

A time course of the incorporation of leucine into protein under the standard experimental incubation conditions is shown in Table 3. Although the rate of incorporation decreased rapidly, the degree of inhibition by salicylate over the period 5–30 min remained constant. The effect of increasing the salicylate concentration on the *in vitro* synthesis of protein is shown in Table 4. Increasing concentrations of salicylate of 1 mM and above caused a progressive decrease in the incorporation of both leucine and histidine into protein.

TABLE 2. EFFECT OF VARYING THE CONCENTRATION OF PYRUVATE KINASE ON THE SALICYLATE-INDUCED INHIBITION OF PROTEIN SYNTHESIS *in vitro*

Conc. of enzyme (γ/ml)	Incorporation of ^{14}C		Inhibition (%)
	Control	20 mM Salicylate	
20	3840 ± 60	1338 ± 9	65
40	2472 ± 63	860 ± 27	65

Mouse liver post-mitochondrial supernatant was incubated as described in the experimental section with [^{14}C]leucine as the labelled amino acid. The results are expressed as counts per minute per milligram protein and are the means ± S.D. of four separate determinations.

The incorporation of radioactive amino acids in vitro *by liver preparations from salicylate-treated mice*

Groups of control and experimental animals were injected with either sodium chloride or sodium salicylate (600 mg/kg body weight) and killed at 0·5, 2 and 4 hr. The ability of the post-mitochondrial supernatant fractions to incorporate [^{14}C]-leucine into protein was assessed in the standard *in vitro* assay system and the results are shown in Table 5. The liver preparations from the experimental animals killed at 0·5 and 2 hr after injection of salicylate incorporated less [^{14}C]leucine than the controls.

The concentration of leucine, but not of histidine, was increased in the liver of mice given salicylate (Table 6). The increased leucine concentration roughly paralleled the dose of salicylate and persisted over a 4 hr time period.

The experiments were therefore repeated with three groups of animals injected with either saline or sodium salicylate, at dose levels of 300 mg/kg and 600 mg/kg body weight, and killed 1 hr after injection. The post-mitochondrial supernatant fractions were

TABLE 3. PROGRESS OF THE INCORPORATION OF [^{14}C]LEUCINE INTO THE PROTEIN OF MOUSE LIVER *in vitro* IN THE ABSENCE AND PRESENCE OF SALICYLATE

Times of incubation (min)	Control	20 mM Salicylate	Inhibition (%)
5	2084 ± 170	692 ± 77	66·8
10	2895 ± 55	1006 ± 32	65·3
15	3104 ± 76	1086 ± 42	65·0
20	3538 ± 136	1255 ± 70	64·5
25	3517 ± 98	1323 ± 66	62·4
30	3682 ± 148	1333 ± 75	63·8

The incubation conditions were as described in the experimental section except that the reactions were stopped at time intervals ranging from 5 to 30 min. The results are the means ± S.D. of four separate determinations.

TABLE 4. EFFECT OF SALICYLATE ON THE INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS INTO MOUSE LIVER PROTEIN *in vitro*

Salicylate conc. (mM)	[^{14}C]Leucine	[^{14}C]Histidine
0	3648 ± 35 (6)	805 ± 34 (4)
1	3637 ± 61 (6)	712 ± 46 (4)*
2	3447 ± 172 (6)*	656 ± 37 (4)*
5	2983 ± 83 (6)*	577 ± 28 (4)*
10	2663 ± 128(6)*	447 ± 81 (4)*
20	1271 ± 9 (4)*	233 ± 15 (4)*

Mouse liver post-mitochondrial supernatant was incubated as described in the experimental section with either [^{14}C]leucine or [^{14}C]histidine and a range of salicylate concentrations for 25 min. The results are expressed as counts per minute per milligram protein and are given as means ± S.D., the number of incubations being given in brackets. *Denotes a significant difference ($P < 0.05$) between the results obtained either in the absence or in the presence of salicylate.

assayed using [^{14}C]histidine. The results (Table 7) show that the lower dose produced no effect but the higher dose caused a 30 per cent decrease in the ability of the liver preparation to incorporate histidine into protein.

The effect of salicylate on the incorporation of radioactive amino acids into the tissue proteins of the mouse in vivo. In a preliminary experiment the time course for the incorporation of intraperitoneally injected [^{14}C]leucine into the liver proteins was

TABLE 5. INCORPORATION OF [^{14}C]LEUCINE INTO PROTEIN BY THE POST-MITOCHONDRIAL SUPERNATANT FRACTION FROM THE LIVERS OF CONTROL AND SALICYLATE-TREATED MICE

Time after injection (hr)	Control	Salicylate
0·5	3159 ± 899	1882 ± 755*
2	3866 ± 1193	2088 ± 926*
4	3056 ± 1164	2688 ± 1319

Animals were injected with either NaCl or Na salicylate at a dose level of 600 mg/kg body weight, and killed at 0·5, 2 and 4 hr after injection. The post-mitochondrial supernatant fractions were incubated as described in the experimental section. Each value is expressed as counts per minute per milligram protein and represents the means ± S.D. of eight determinations. *Denotes a significant difference ($P < 0.05$) between experimental and control.

TABLE 6. CONCENTRATIONS OF LEUCINE AND HISTIDINE IN THE LIVERS OF MICE TREATED WITH SALICYLATE

Salicylate (mg/kg body wt.)	Leucine	Histidine
0	0·292 ± 0·044 (7)	0·425 ± 0·131 (7)
75	0·318 ± 0·019 (3)	0·474 ± 0·135 (3)
150	0·318 ± 0·011 (3)	0·426 ± 0·048 (3)
300	0·348 ± 0·021 (3)	0·395 ± 0·093 (3)
600	0·422 ± 0·027 (5)*	0·411 ± 0·041 (5)

Animals were injected with sodium salicylate and killed 1 hr later. Each value is expressed as μmoles amino acid per gram wet weight liver and represents the mean ± S.D., the number of observations being in brackets. *Denotes a significant difference ($P < 0·05$) between experimental and control.

established. Figure 1 shows that the incorporation is very rapid and by 30 min the specific activity of the isolated protein had reached a maximum. In subsequent experiments the animals were killed 6 min after injection of the labelled amino acids. The results in Table 8 show the effect of injecting sodium salicylate at a dose level of 500 mg/kg body weight 1 hr prior to giving the radioactive amino acid. The incorporation of ^{14}C-labelled leucine and histidine into liver proteins were significantly inhibited. The injection of lower dose levels of salicylate produced no significant effects on the incorporation of these labelled amino acids into tissue proteins. The results in Table 9

TABLE 7. INCORPORATION OF [^{14}C]HISTIDINE INTO PROTEIN BY THE POST-MITOCHONDRIAL SUPERNATANT FRACTION FROM THE LIVERS OF CONTROL AND SALICYLATE-TREATED MICE

Salicylate dose (mg/kg body weight)	Incorporation (counts/min/mg protein)
0	327·6 ± 64·2
300	327·8 ± 63·5
600	238·5 ± 34·4*

Animals were injected with either NaCl or Na salicylate at dose levels of either 300 or 600 mg/kg body weight, and killed 1 hr later. The post-mitochondrial supernatant fractions were incubated as described in the experimental section. Each value is expressed as counts per minute per milligram protein and represents the mean ± S.D. of eight determinations. *Denotes a significant difference ($P < 0·05$) between experimental and control.

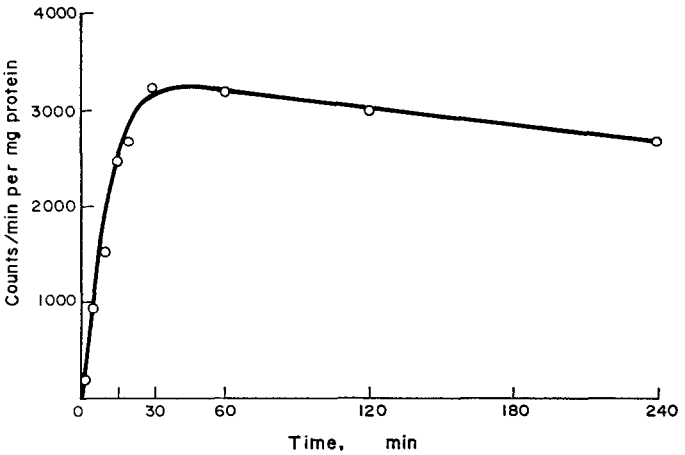

FIG. 1. Time course of the incorporation of [^{14}C]leucine into liver proteins. Groups of mice were injected intraperitoneally with 0·2 ml normal saline containing 5 μc [^{14}C]leucine and killed at time intervals ranging from 2 to 240 min after the injection and the specific activity of the total liver protein determined.

shows the effect of intraperitoneal salicylate injections on the levels of α-amino isobutyrate acid in the circulation and in the tissues 6 min after either the intraperitoneal or the intravenous injection of the ^{14}C-labelled amino acid. After intraperitoneal injection of α-amino isobutyric acid the blood of the salicylate-treated animals contained significantly lower concentrations of this amino acid than did the controls, whereas after intravenous injection the levels in the two groups were approximately the same. In addition, the ratios of α-amino isobutyric concentration in liver to that in blood were higher in the salicylate-treated animals. Similar results had been noticed in earlier experiments using the radioactive leucine.

TABLE 8. EFFECT OF SALICYLATE ON THE INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS INTO MOUSE LIVER PROTEIN *in vivo*

Mode of injection of ^{14}C-labelled amino acid	Control	Salicylate
Intraperitoneal leucine	1301 ± 314 (5)	756 ± 284 (8)*
Intravenous leucine	1573 ± 202 (4)	1103 ± 229 (8)*
Intravenous histidine	1149 ± 124 (10)	1007 ± 189 (14)*

Animals were injected with NaCl or Na salicylate at a dose level of 500 mg/kg body weight. One hour later the animals were injected with 5 μc of [^{14}C]-leucine either intraperitoneally or intravenously or with 5 μc of [^{14}C]histidine intravenously. Six min after the second injection the animals were killed and the radioactivity in the total liver protein was determined as described in the experimental section. The results are expressed as mean counts per minute per milligram ± S.D., with the number of animals in brackets. *Denotes a significant difference ($P < 0·05$) between experimental and control.

TABLE 9. EFFECT OF SALICYLATE ON THE DISTRIBUTION OF [^{14}C]α-AMINO ISOBUTYRIC ACID IN MOUSE TISSUES *in vivo*

Mode of injection of [^{14}C]α-amino-isobutyric acid	^{14}C present in acid-soluble fraction of tissue			
	Blood	Liver	Kidney	Brain
Intravenous control	1124 ± 134	1573 ± 509	9689 ± 3140	179 ± 24
Intravenous salicylate	986 ± 144	2355 ± 913	11037 ± 3753	202 ± 45
Intraperitoneal control	922 ± 149	1391 ± 443	6450 ± 2313	86 ± 24
Intraperitoneal salicylate	551 ± 119*	910 ± 222	2560 ± 458*	50 ± 17*

Animals were injected with either NaCl or Na salicylate at a dose level of 600 mg/kg body weight. After 30 min the animals received a second injection of 2 μc of [^{14}C]α-aminoisobutyric acid either intraperitoneally or intravenously and were killed after a further 6 min. The radioactivity in the blood, liver, kidney and brain was determined and the results are expressed as mean counts per minute × 10^2 per gram tissue ± S.D. of five determinations. *Denotes significant difference ($P < 0.05$) between experimental and control.

DISCUSSION

It has been established that salicylate, in concentration of 0·5–5 mM, inhibits the *in vitro* incorporation of a variety of labelled amino acids into the protein of preparations from rat tissues.[4–6] The rat is not an ideal species to study protein biosynthesis *in vivo* because of the relatively large amounts of radioactive precursors which have to be injected in each animal. The mouse was therefore used in the present work. The results in Table 4 show that the sensitivity of a cell-free preparation from mouse liver to the inhibitory effect of salicylate on protein biosynthesis *in vitro* closely resembles that of the corresponding preparation from the rat.

The next series of experiments were designed to study the effects of pretreatment with the drug on the ability of the post-mitochondrial supernatant of mouse liver to incorporate labelled amino acids into protein. Radioactive leucine was used as a labelled precursor because it is incorporated into protein *in vitro* to a reasonable extent and is not metabolised via alternate pathways to an appreciable degree. The initial results (Table 5) appeared to show that if salicylate were given to the animals, up to 2 hr before killing, then the subsequent incorporation of the leucine into the protein of a PMS fraction from mouse liver was significantly inhibited. Previous work[11,12] had shown that the salicylate concentration in the liver after injection of the chosen dose of salicylate reached a maximum of 2·2 mM 30 min after injection, was 1·5 mM at 2 hr and fell to 0·8 mM at 4 hr. However, it had also been shown that the injection of salicylate in the mouse alters the sizes of amino acid pools in several tissues including the liver.[10] In the present work (Table 6) it was observed that the injection of salicylate increased the concentration of leucine in mouse liver, that this effect roughly paralleled the dose of salicylate and persisted over the time period of the incorporation experiments. The increased pool size of leucine would be expected to cause a dilution of the radioactive leucine in the liver preparation *in vitro* and hence could explain the decreased incorporation of radiocarbon which occurred in the salicylate-treated animals. Similar experiments were therefore carried out using labelled histidine because injection of salicylate did not alter the concentrations of this amino

acid in mouse liver. The results (Table 7) show that, although the incorporation of histidine was considerably less than that of leucine, pretreatment of the mouse with 600 mg/kg salicylate before killing caused a significant decrease in the incorporation of isotope into the protein of the PMS fraction from the liver.

Further experiments were performed in which mice were injected with the labelled amino acid. Preliminary work showed that the incorporation of the labelled precursors into the isolated protein was rapid, e.g. with [U-^{14}C]leucine it could be detected in the first few minutes after injection and by 30 min the specific activity of the isolated protein had reached a maximum (Fig. 1). In the subsequent experiments the mice were each given an intraperitoneal injection of varying doses of salicylate, followed after 1 hr by an intraperitoneal injection of a trace dose of the labelled leucine, killed after a further 6 min and the liver protein isolated. The results (Table 8) show that 500 mg/kg body weight salicylate significantly inhibited the incorporation of ^{14}C from the labelled amino acid into the liver proteins. The results observed with the leucine could have been caused, at least in part, by the increased pools of amino acids in the liver resulting from the administration of salicylate. The radioactivity in the blood was also measured during the experiments and was found to be decreased in the salicylate-treated animals. This observation suggested that pretreatment of the mice with salicylate may have interfered with the transport of the amino acids from the peritoneum to the circulation. Accordingly the effects of the previous intraperitoneal injection of salicylate on the blood and tissue levels of the non-metabolisable amino acid, α-aminoisobutyrate, given either intraperitoneally or intravenously, were performed. The results (Table 9) showed that the circulating concentrations of α-aminobutyrate were significantly decreased when the substance was administered intraperitoneally but not when it was given intravenously to the salicylate-treated mice. The decreased incorporation of radioactive leucine into the liver protein of mice given a previous intraperitoneal injection of salicylate therefore could also have been due to the drug decreasing the absorption of the amino acid from the injection site.

The possibility that the drug could be decreasing the uptake of the labelled amino acids from the circulation to the liver was considered. However, the ratio of radioactive α-aminoisobutyrate concentrations between liver and blood was higher in the salicylate-treated mice than in the corresponding control (Table 9) and a similar effect was observed with the labelled leucine suggesting that the drug actually increased the penetration of amino acids into the liver. A final series of experiments were therefore carried out in which the radioactive amino acids were given by intravenous injection. The results (Table 8) show that the intraperitoneal administration of 500 mg/kg salicylate 1 hr before the amino acids were given, caused a significant decrease in the incorporation of radioactivity from both leucine and histidine into the isolated protein. It must therefore be concluded that salicylate inhibits protein biosynthesis in mouse liver both *in vitro* and *in vivo*. The effect became evident in a PMS fraction of mouse liver incubated with salicylate concentrations of 1 mM and above and in intact animals injected with a single dose of 500 mg/kg body weight. This latter amount is equivalent to a single dose of 35 g of salicylate in a 70 kg man and even when allowance is made for species difference, this quantity of salicylate must represent a toxic dose for man. An interference with the general biosynthesis of protein in the liver cannot be of much significance in relation to either the analgesic or clinical anti-inflammatory actions of salicylate. However, the possibility that therapeutic doses

of the drug may preferentially inhibit the biosynthesis of individual proteins, such as the immunoglobulins, is not excluded by the present work. It has been shown[13] that high doses of salicylate inhibit the biosynthesis of collagen but not of non-collagen protein of carrageenin granuloma in the rat. In addition, protein synthesis in rapidly dividing tissues and in growing animals may be more susceptible to salicylate than that in adult mouse liver. Thus the daily administration of 250–300 mg/kg body weight of salicylate caused lower rates of weight gain and impaired skeletal growth in immature rats[14] and in growing chicks.[15] The observation that exposure to 0·4–10 mM salicylate inhibits the growth of wheat coleoptiles[16] may be explained, at least in part, by the drug interfering with protein synthesis.

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