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Research Article

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Since salicylate uncouples oxidative phosphorylation, it is postulated that high energy phosphate in the brain is maintained near normal levels by a compensatory increase in cerebral glycolysis. Apparently the brain glucose level falls because the rate of utilization exceeds the rate at which glucose can be supplied from the blood. Concurrent administration of glucose with salicylate elevated brain glucose concentration and was associated with striking improvement in the condition and the increased survival of the animals. These findings stress the fact that in salicylate poisoning the supply of glucose to the brain may be inadequate even when blood glucose levels are normal.

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Reduced Brain Glucose with Normal Plasma Glucose in Salicylate Poisoning

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ABSTRACT After the intraperitoneal injection into young mice of 700–800 mg/kg of salicylate, brain glucose fell to one-third or less of control values despite normal plasma glucose levels; brain lactate was nearly doubled and there were small decreases in phosphocreatine (18%) and in glycogen (17%). ATP, pyruvate, α -ketoglutarate, and glutamate were unchanged. In liver, glycogen was reduced 79% and lactate was five times higher than in control animals; glucose, glucose-6-phosphate, and ATP were unchanged.

Since salicylate uncouples oxidative phosphorylation, it is postulated that high energy phosphate in the brain is maintained near normal levels by a compensatory increase in cerebral glycolysis. Apparently the brain glucose level falls because the rate of utilization exceeds the rate at which glucose can be supplied from the blood. Concurrent administration of glucose with salicylate elevated brain glucose concentration and was associated with striking improvement in the condition and the increased survival of the animals. These findings stress the fact that in salicylate poisoning the supply of glucose to the brain may be inadequate even when blood glucose levels are normal.

INTRODUCTION

It is well known that salicylate uncouples oxidative phosphorylation, increases glycogen breakdown, decreases glycogen synthesis, and inhibits many enzyme systems (see Smith and Smith [1] for a critical review). As a result one would expect a decrease in the production of energy-rich compounds with secondary im-

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pairment of biosynthesis, active transport, and adverse effects upon other energy-dependent cellular functions. And in fact, Smith and Jeffrey (2) found a large drop in phosphocreatine and ATP levels in the isolated rat diaphragm incubated with salicylate. Today, techniques of rapid freezing of small animals and methods of tissue extraction designed to prevent the loss of labile metabolites permit the evaluation of the *in vivo* effects of drugs in experimental animals. The present report is a study of the *in vivo* effects of salicylate on the components of the energy reserve and other selected metabolites of the brain, liver, and plasma of young mice.

METHODS

Preparation of animals. The *in vivo* effects of salicylate were investigated in 15 litters of Swiss Webster albino mice. Mice 11–21 days of age and weighing 4.8–11 g were used. Young animals and this dose range were employed to simulate salicylate poisoning in infants and young children. A dose of 700–800 mg of sodium salicylate per kg body weight was given by intraperitoneal injection in 0.1–0.3 ml of water. In each litter one-half of the animals served as controls. Controls received an equal volume of normal saline. Mice were killed at intervals from 1 to 30 min after injection. For the investigation of the metabolic effects of salicylate on brain and liver, the animals were killed by quick freezing in liquid nitrogen. When the blood and brain of the same animal were needed, the head was amputated directly into liquid N₂ and blood was collected from the neck vessels in chilled heparinized tubes. To obtain samples for salicylate levels in plasma, brain, and liver, the animals were decapitated and blood was collected from the severed neck vessels. Liver and brain were dissected without freezing the animals.

Repeated attempts to obtain sufficient blood free of small clots and air bubbles for a determination of pH and partial pressure of blood gases were unsuccessful.

Preparation of blood, tissue extracts, and homogenates. After freezing in liquid N₂, the whole animal or the decapitated head was stored at -75°C or dissected immediately. Dissection of forebrain and liver was performed in a room at -20°C or in a cryostat at -35°C . In the cold room tissue

was ground with pestle and mortar previously chilled with liquid N₂. In the cryostat mortar and pestle were surrounded by dry ice (-80°C). No differences attributed to these two methods were recognized. The powdered brain or liver samples were weighed in the same environment. The tissue powders were then extracted according to the procedure of Lowry, Passonneau, Hasselberger, and Schulz (3). Until the time of assay, extracts were stored at -75°C.

For the determination of plasma salicylate, glucose, and lactic acid levels, the heparinized blood was promptly centrifuged at 4°C and aliquots of plasma were assayed immediately.

Reagents. All enzymes except phosphorylase-a (2.4.1.1, -1,4-Glucan-orthophosphate glucosyltransferase, Sigma Chemical Co., St. Louis, Mo.) and ox heart lactate dehydrogenase (1.1.1.27, L-lactate: NAD oxidoreductase, Worthington Biochemical Corp., Freehold, N. J.) were obtained from Boehringer and Sons through Calbiochem., Los Angeles, Calif.

Methods of assay for metabolites. All metabolites were measured fluorometrically by specific enzymatic methods. ATP, ADP, AMP, phosphocreatine, glucose, glucose-6-phosphate, and fructose-1, 6-diphosphate were assayed by the methods of Lowry et al. (3). Lactate and glycogen were measured by the methods of Gatfield, Lowry, Schulz, and Passonneau (4) and glutamate by a modification of the method of Young and Lowry (5). α -Ketoglutarate was measured following the procedure of Goldberg, Passonneau, and Lowry (6). Plasma glucose was determined enzymatically by the same method that was used for the assay of brain and liver glucose. Since no preliminary extraction was necessary, 1 μ l of plasma was added directly to the assay reagent.

To rule out the possibility of some substance in the tissues of salicylate-treated animals interfering with the enzymatic measurements, internal standards were included in each set of analyses. Recoveries were 95-103% of the expected values.

Method of assay for salicylate. Salicylate levels in plasma, brain, and liver were assayed by the method of Trinder (7). Tissue samples were weighed at 25°C and homogenized by hand at 4°C in color reagent. Brain and liver samples weighed 100-150 mg and the volume of plasma and sodium salicylate standards was 100 μ l. The volume of color reagent (ferric nitrate, mercuric chloride, and hydrochloric acid) added was 1.5 ml. The homogenate was centrifuged at 2500 rpm for 5 min at 4°C and the supernatant fluid was assayed for salicylate. Recovery of sodium salicylate standards in water or plasma was linear up to and including 100 mg/100 ml. The recovery of sodium salicylate added to brain and liver was 97-104%.

Comment on clinical aspects. The LD₅₀ of sodium salicylate by intraperitoneal injection in mice weighing about 20 g is 560 mg/kg (8). Soon after injection, animals receiving 700-800 mg/kg of salicylate were either very excited, trying desperately to get out of the cage, or unusually quiet, remaining immobile in one spot. Within a few minutes, most of the mice exhibited peripheral weakness particularly of the posterior extremities and assumed a "splayed" position. The tail was often held stiff and erect and appeared engorged. In animals that failed to survive the dose of salicylate, death was always preceded by generalized seizures. However, it was possible for an animal to have recurrent seizures and survive without other therapy. Though susceptibility to early death varied from litter to litter, there was little variation in this response among

animals of any particular litter. Early death after salicylate was clearly not related to age. Weanling mice could have seizures and die within 5 min after injection whereas a litter of 11 day old mice might survive the entire interval of the experimental design or longer without either seizures or death. Since seizure activity per se is known to have profound effects on cerebral metabolism (9), no animal exhibiting convulsive activity was included in this study.

RESULTS

Salicylate levels at intervals after injection are shown in Fig. 1. By 3 min plasma concentration was in the range reported by Done (10) in children and adults with moderate, severe, or fatal salicylate intoxication. In the liver salicylate levels, which in vitro are associated with complete inhibition of oxidative phosphorylation (2-5 mM) (11), were reached within 1-3 min. In homogenates and mitochondrial preparations of brain, 1-2 mM salicylate causes 50-100% uncoupling (11, 12). In this study the salicylate concentration in the brain was 1.2 mM at 5 min.

There is a dramatic effect of salicylate on hepatic glycogen content (Table I). 30 min after intraperitoneal injection of salicylate, liver glycogen was reduced 80%. Despite the profound drop in glycogen content, liver glucose, glucose-6-phosphate, and ATP were unchanged.

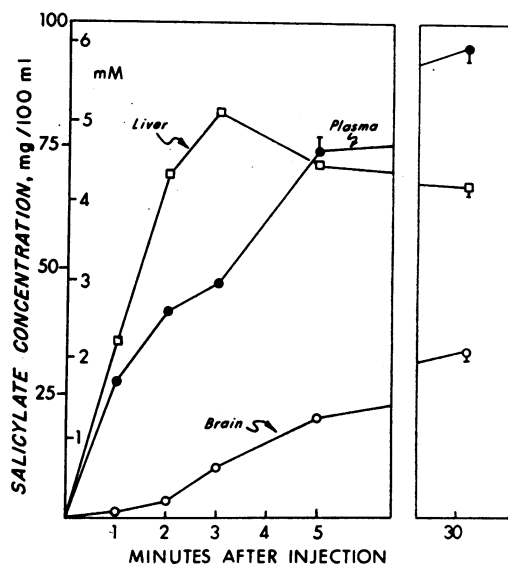


FIGURE 1 Plasma, liver, and brain salicylate concentrations after intraperitoneal injection of 750 mg/kg of salicylate. Salicylate levels have been corrected for "blank" values from control animals which received normal saline. Each point represents the average value from at least three animals except for the 3-min samples and the 2 min value for brain, in these instances only two animals were used. Where values are from five or more animals (5-min plasma values and all 30-min samples), the standard error of the mean (\pm) is represented by a vertical line.

TABLE I
Effect of Salicylate on Selected Metabolites in Brain and Liver

Metabolite	Brain			Liver		
	Control	Salicylate	P value	Control	Salicylate	P value
Phosphocreatine	3.15 ±0.19	2.54 ±0.07	0.009			
ATP	2.81 ±0.06	2.70 ±0.06	0.177	2.24 ±0.11	2.10 ±0.11	—
Glucose	1.27 ±0.08	0.44 ±0.04	<0.001	5.60 ±0.36	5.58 ±0.51	—
Glucose-6-phosphate	0.116 ±0.10	0.099 ±0.006	0.172	0.188 ±0.01	0.190 ±0.03	—
Glycogen (as glucose)	1.50 ±0.008	1.24 ±0.06	0.009	62.8 ±5.4	13.0 ±3.1	<0.001
Lactate	1.64 ±0.15	2.67 ±0.16	<0.001	0.78 ±0.10	4.37 ±1.07	0.003
Pyruvate	0.336 ±0.031	0.382 ±0.049	—			
α-Ketoglutarate	0.075 ±0.006	0.082 ±0.012	—			

30 animals (three litters) were used in this study. The dose of salicylate in each litter was 700, 750, and 800 mg/kg respectively. Control animals were given normal saline. Brain and liver samples were obtained 30 min after injection. In this dosage range the biochemical findings were similar and results were pooled. Metabolite values expressed in millimoles per kilogram wet weight are given as the mean ±SEM. Brain pyruvate levels are from 9 samples; α-ketoglutarate from 21.

Lactate values, on the other hand, were five to six times higher than in control animals.

In the brains of these same animals the most striking finding was the extreme decrease in brain glucose concentration (Table I). 30 min after salicylate injection, brain glucose values were reduced to one-third of control values. Glycogen and phosphocreatine were reduced slightly but there was no change in ATP. Pyruvate levels were unchanged, but the lactate concentration was increased 63% in the salicylate-treated animals. Levels of α-ketoglutarate were similar in control and salicylate-treated animals.

Brain glutamate levels were 8.0 ±1.0 and 7.9±0.6 mmoles/kg in the control and salicylate-treated animals used for Table II. These data do not support an interpretation of significant inhibition of brain α-ketoglutarate or glutamic dehydrogenase activity in vivo. In pyriethamine-induced thiamine deficiency, inhibition of α-ketoglutarate dehydrogenase produces a dramatic rise in brain α-ketoglutarate (13).

TABLE II
A Correlation of Brain and Plasma Glucose and Lactic Acid Levels after Salicylate

Metabolite	Brain		Plasma	
	Control	Salicylate	Control	Salicylate
	mmoles/kg		mmoles/liter	
Glucose	2.01 ±0.12	0.46 ±0.08	6.28 ±0.46	5.69 ±0.47
	P < 0.001		P > 0.37	
Lactic acid	1.18 ±0.15	2.26 ±0.33	3.12 ±1.93	4.68 ±1.28
	P = 0.017		P > 0.37	

Six 11 day old mice were injected with 700 mg/kg of salicylate (i.p.). Five littermates received normal saline. The interval before sacrifice was 30 min. Metabolite values are given as the mean ±SEM.

In an attempt to discover whether the changes in brain glucose and lactate reflected changes in the blood levels of these substances, another group of animals was used. As before, there was a profound decrease in brain glucose (75%) and a doubling of the brain lactate concentration in the salicylate-treated animals (Table II). However, the blood glucose and lactate levels do not differ significantly in the two groups.

The effect of salicylate on brain glucose concentration is evident as early as 1 min after injection (Fig. 2). At this time the mean brain glucose level in salicylate-treated mice (0.57 ±0.07 mmoles/kg) was one-third lower than the control value (0.86 ±0.04 mmoles/kg, P = 0.005). The brain salicylate concentration at this time was less than 5 mg/100 ml (0.3 mM) (Fig. 1). This is not too surprising since Fishgold, Field, and Hall (14) found a significant increase in oxygen consumption of brain slices (a correlate of uncoupling) with salicylate levels as low as 1 mg/100 ml (0.06 mM).

Levels of fructose-1,6-diphosphate (FDP) and glucose-6-phosphate (G6P) were also determined at intervals after salicylate injection (Fig. 2). There was a significant increase in fructose-1,6-diphosphate (51%) 2 min after injection with a return to control levels by 5 min. As fructose-1,6-diphosphate rose, glucose-6-phosphate fell. In contrast to fructose-1,6-diphosphate levels of glucose-6-phosphate do not return to normal but remain low after the initial drop. The increasing ratio of the concentrations of the two metabolites, FDP/G6P, suggests activation or deinhibition of phosphofructokinase with a consequent increase in flux through the Embden-Meyerhof pathway. 30 min after injection when the brain glucose concentration was very low (animals of Table II), fructose-1,6-diphosphate was significantly reduced, 0.039 ±0.007 mmoles/kg as compared to 0.064 ±0.007 for controls (P = 0.03).

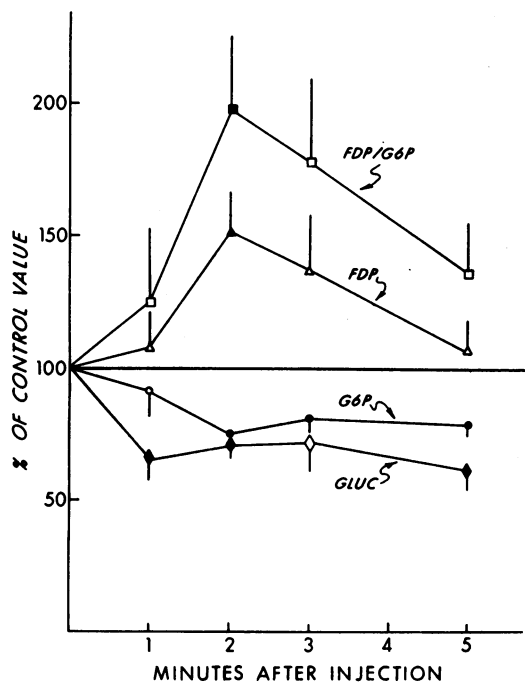


FIGURE 2 Concentrations of selected metabolites in brain at intervals after the injection of 750 mg/kg of salicylate. Values in controls injected with normal saline did not change with time and results were pooled. There were nine mice in the control group and four to six in each of the other groups (total 29 animals). Values are expressed as the percentage of control levels. In millimoles per kilogram \pm the standard error, these values were, glucose 0.86 ± 0.04 , G6P 0.124 ± 0.005 , and F6P 0.076 ± 0.008 . FDP/G6P in control animals was 0.64 ± 0.09 . The vertical line through each symbol is equal to the SEM. Values marked with a filled symbol are significantly different from the control values at $P \leq 0.016$. The 3 min glucose value differs from the control at $P = 0.05$; the remainder of the values marked with an open symbol are not significantly changed ($P > 0.05$). Abbreviations used are Gluc, glucose; G6P, glucose-6-phosphate; FDP, fructose-1, 6-diphosphate.

Early effects of salicylate on other metabolites were also determined. In the brains of the animals used for Fig. 2, levels of phosphocreatine, ATP, ADP, AMP, and lactate were found to be unchanged.

The effect on the brain glucose content of the administration of glucose (30 mmoles/kg) together with salicylate (750 mg/kg) is shown in Table III. The mean brain glucose concentration is more than four times that of mice receiving salicylate without glucose, twice that of saline controls, but only one-half of that seen in mice receiving glucose only. At the time of sacrifice (15 min) animals given glucose showed only the mildest signs of salicylism. In some the tail was erect and appeared much pinker than normal whereas those without added glucose exhibited profound splaying

of the extremities and appeared to be on the verge of seizures. This effect of glucose administration on the clinical behavior of animals injected with toxic doses of salicylate was first discovered by Lutwak-Mann (15). In 1942 she reported the following observation. "Salicylate: the dose 1.0 mg/g was tolerated only if the animals were given an excess of glucose (5-10% solution substituted for water) in addition to their normal diet during one or more days preceding the experiment; otherwise this dose caused a 50% mortality." In further experiments we have confirmed her findings. Nine 14 day old mice (littermates) were injected with 750 mg/kg of salicylate. In five mice the diluent was hypertonic glucose (18%, 30 ml/kg i.p.). In the remaining four mice the diluent was saline of equal osmolality. 3½ hr after injection the four mice who received salicylate without glucose were dead. At this time all five mice who received glucose were alive. Three out of five glucose-treated mice exhibited intermittent seizures during this interval; by contrast mice given salicylate alone remained in epileptic status from the onset of seizures till the time of death. 6 hr after injection all glucose-treated mice were still alive and appeared seizure free.

DISCUSSION

Liver. The dramatic effect of salicylate on hepatic glycogen content confirms previous in vivo studies in normally fed animals (15, 16). Lutwak-Mann (15) found almost complete disappearance of glycogen from the liver with doses of salicylate as low as 200 mg/kg. Though a similar effect of salicylate is seen in the isolated diaphragm (2), the evidence for an equal effect of salicylate in liver slices in vitro is less clear. With salicylate concentrations as high as 20 mM in a high sodium medium, Smith (17) found no reduction in the glycogen content of liver slices. Even in a high potas-

TABLE III
The Effect of Glucose Administration on the Decrease in Brain Glucose after Salicylate

Metabolite	Saline control	Glucose control	Salicylate plus saline	Salicylate plus glucose
Glucose	1.09 ± 0.07	3.37 ± 0.19	0.41 ± 0.05	1.84 ± 0.22

The study comprised 17 2 wk old mice (two litters). The dose of salicylate was 750 mg/kg i.p. In four animals salicylate was dissolved in 0.5 M NaCl, in five the diluent was 1.0 M glucose (18%). Two other controls were included. Four mice received 0.5 M NaCl and an equal number were injected with 1.0 M glucose. The volume of injections was 30 ml/kg. The dose of glucose administered was thus 30 mmoles/kg. Animals were frozen whole 15 min after injection. Values are given in millimoles per kilogram wet weight as the mean \pm SEM. All glucose values differ significantly from each other with a P value of <0.001 .

sium medium, 5 mM salicylate caused only a 25% decrease in the glycogen content at the end of 1 hr. These findings suggest that much of the change seen in the liver of intact animals is secondary to the effect of salicylate in other parts of the body. Other data in our study support this suggestion. 5 min after injection of salicylate when brain glucose values were reduced 38% (Fig. 2), there was no difference in the glycogen content of the liver of these animals (34.8 ± 5.5 mmoles/kg for controls, and 32.9 ± 6.6 mmoles/kg for salicylate-treated animals).

In earlier experiments blood glucose levels were found to be either unchanged after salicylate (8, 15) or elevated (18). *In vivo* a major and essential role of the liver is the maintenance of normal blood glucose levels. Early changes in blood glucose values would reflect the balance between the increased demand for glucose due to increased peripheral utilization secondary to the generalized uncoupling effect and the supply available from the hepatic (and renal) glycogen reserves. Once glycogen stores were depleted, blood glucose concentration could only be maintained by newly formed glucose via gluconeogenesis from nonglucose precursors. The role of the adrenal gland in these functions is well known. Smith (18) has shown that adrenal-demedullation markedly reduced the hyperglycemic response to salicylate and that in adrenalectomized animals salicylate produced a rapid fall in blood glucose to levels which often resulted in death of the animals with hypoglycemic convulsions. The extent of these various physiologic responses to increased glucose demand after salicylate would determine the blood glucose levels observed.

The failure of liver ATP to be affected after salicylate *in vivo* is quite surprising considering the pronounced uncoupling effect of salicylate in liver mitochondrial preparations (11, 19, 20). Studies of the effect of salicylate on ATP levels in isolated liver tissue have not been reported.

Liver lactate values in the salicylate-treated mice were five to six times higher than in control animals. Without further investigation explanations for this increase can only be speculative. First, one could consider a decrease in oxygen consumption secondary to an inhibition of oxidative enzymes of the tricarboxylic acid cycle. Normal liver glucose and glucose-6-phosphate values argue against significant hepatic anaerobiosis (21). Though *in vitro* many enzymes are inhibited by salicylate (see Smith and Smith [1] for a critical review), inhibition was unusual with concentrations of salicylate less than 5 mM. The levels found in the present experiments did not exceed this level. Tentatively it may be proposed that the increase in liver lactate values in the salicylate-treated animals is a release and diversion of lactate from

peripheral tissues to the liver for glucose production. This explanation would appear to be most logical in view of the drastic reduction of brain glucose concentration and the nearly complete depletion of liver glycogen stores.

Brain. *In vitro* with 2 mM salicylate oxidative phosphorylation in rat brain mitochondria is reduced 50% (11) and in brain homogenates the inhibition is complete (12). Therefore, the profound drop in brain glucose levels after salicylate may well result from a stimulation of cerebral glycolysis as a result of this phenomenon. However, it is not possible with the methods used to assess directly the extent of uncoupling. It appears that as long as the supply of glucose to the brain is adequate, nearly normal levels of phosphocreatine and ATP can be maintained despite the inhibition of oxidative phosphorylation. A major control point of glycolysis in the brain is at the site of phosphorylation of fructose-6-phosphate by phosphofructokinase. In an attempt to establish the hypothesis of a stimulation of cerebral glycolysis due to the uncoupling effect of salicylate, fructose-1, 6-diphosphate was measured at intervals after salicylate injection. Lowry et al. (3) have shown that within 6 sec after decapitation, in mice this age, brain fructose-1, 6-diphosphate levels were increased 56% and glucose-6-phosphate fell. The increase in fructose-1, 6-diphosphate was quite evanescent with a return to normal values or less by 30 sec. A similar phenomenon appears to obtain here. The increase in fructose-1, 6-diphosphate seen in this study was of equal magnitude to that reported by Lowry et al. (3) but the fall in glucose-6-phosphate was less, and the changes in both metabolites were much slower. Since the rate of entry of salicylate into the brain after intraperitoneal injection is relatively very slow (Fig. 1) (compared to the suddenness of the changes produced by decapitation), these differences are probably to be expected.

Other explanations for the decrease in brain glucose concentration have been considered but seem less likely. The finding of high cerebral ATP and phosphocreatine values with very low glucose values is highly characteristic of the early stages of insulin hypoglycemia (9). However, there are important differences; after salicylate, brain lactate is increased not decreased as it is after insulin injection. Most importantly, plasma and liver glucose levels in the salicylate-treated animals were not depressed. Inhibition of transport of glucose from the blood into the brain also seems unlikely because of the high level of brain lactate. It is even possible that glucose transport into the brain is actually increased after salicylate as is the case for muscle (22).

No changes in brain lactate were seen before 30 min. The explanation for the increase in lactate at this time is not clear. Though the increase in brain lactate on a basis of tissue hypoxia is considerably less than that which would be expected from the observed decreases in glucose, glucose-6-phosphate, and glycogen levels, a degree of functional hypoxia due to direct depression of the mitochondrial electron transport system by salicylate cannot be ruled out. In this case, hypoxia would be an added stimulus to glycolysis. The increase in brain lactate concentration cannot be attributed to an increase in blood lactate levels. In both control and salicylate-treated animals, the level of lactate in the brain was considerably less than that found in plasma (Table II). Furthermore the ratio of plasma lactate to brain lactate was higher in control animals. If salicylate increased cerebral glycolysis as we have postulated, then the production of lactate could conceivably exceed the oxidative capacity of the citric acid cycle. The recent study of Alexander and Smith (23) supports this mechanism. In artificially ventilated goats with blood salicylate concentration constant at 70 mg/100 ml for 2 hr, there was indirect evidence of increased brain lactate production (increased cisternal cerebrospinal fluid lactate concentration). In none of these animals was there evidence of increased cerebral anaerobiosis. Salicylate intoxication increased brain oxygen consumption about 58% and increases in cerebral venous P_{O_2} substantiated adequate cerebral oxygen delivery. These studies are of further interest in relation to our findings. Alexander and Smith found that salicylate had a pronounced effect on cerebral blood flow. Even in hypocarbic animals (end-tidal carbon dioxide tension about 20 torr), cerebral blood flow was increased 26%. In normocarbic salicylate intoxicated animals (P_{CO_2} 38 torr), cerebral blood flow was increased 81%. The increase of blood flow, with the concomitant increase in the amount of glucose arriving at the brain, would help explain maintenance of cerebral high energy phosphate at near normal levels.

In summary, large doses of salicylate produce a profound decrease in glucose concentration in the brains of young mice. From the data the hypothesis of an increase in glycolysis in compensation for uncoupling of oxidative phosphorylation by salicylate seems most likely. Though hypoglycemia is well recognized as a complication of severe salicylate poisoning, decrease of brain glucose concentration with normal plasma glucose levels has not previously been reported. The biochemical and clinical responses to glucose suggest the possibility that in salicylate poisoning the supply of glucose to the brain may be inadequate even when blood glucose levels are normal.

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