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Effect of Salicylic Acid on Adrenal-Pituitary System. III. Studies on Mechanism of This Effect.* (21116)

GEORG CRONHEIM† AND NELTA HYDER.

From the Research Department, S. E. Massengill Co., Bristol, Tenn.

Salicylic acid (SAL) has in normal animals a specific effect on the adrenal-pituitary system which can be demonstrated by a depletion of adrenal ascorbic acid (AAA)(1-5) and adrenal cholesterol(3). Since the presence of an intact pituitary is necessary(3,4), it has been suggested that SAL stimulates directly or indirectly the production or release of increased amounts of ACTH. The present study was undertaken to investigate in some detail: 1. The duration of the SAL action; 2, The effect of repeated doses; 3. The effect of pretreatment with cortisone; 4. Attempts to determine the mechanism of action.

Methods and evaluation. The experiments were made in Wistar rats as described previously(4). All AAA values are given in per cent of that found in *untreated* controls. This eliminates daily variations(4). An injection of physiological saline solution produces a small, but significant depletion of AAA. In previous publications(4,5) saline controls were arbitrarily assigned a value of 100%.

TABLE I. Adrenal Ascorbic Acid of Rats, 2 Hours after Single Subcutaneous Injection of Saline Solution or Salicylic Acid.

	Adrenal ascorbic acid in % of	
	Saline controls (previous papers)	Untreated animals (present paper)
Untreated animals	135	100
Saline	100	75
SAL, 300 mg/kg	59	44

The present experiments can be described more clearly if AAA of untreated animals is considered to be 100%. Table I presents a comparison of the two forms of presentation.

Results. The results of time-effect experiments (Table II) permit 2 conclusions: 1. The maximum depletion of AAA required more than one hour. 2. The level of AAA had not returned to normal within 16 hours after 300 or 500 mg/kg of SAL. The SAL blood levels at the end of the 16-hour experiment were 6.7 and 11.2 mg %, respectively.

The question of adrenal response to repeated doses of SAL was investigated in 2 ways. First, it was necessary to establish the effect of a second dose of SAL given to animals in which the adrenals had not yet recovered

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† Present address: Riker Laboratories, Los Angeles, Calif.

TABLE II. Adrenal Ascorbic Acid of Rats after Single Subcutaneous Injection of Saline Solution or Salicylic Acid.

Time (hr)	1	2	3	16
	Adrenal ascorbic acid in % ± S.D. of untreated controls			
Saline	86 ± 9 (13)	75 ± 9* (87)	87 ± 11 (8)	97 ± 10 (20)
SAL, 300 mg/kg	52 ± 7† (10)	44 ± 6† (76)		74 ± 9† (8)
" , 500 "		38 ± 8† (6)		60 ± 10† (5)

No. in parentheses refers to No. of animals.
* Significantly lower ($p = 0.05$ or less) than untreated animals.
† Significantly lower than untreated animals and the corresponding saline controls.

completely from a first dose of the drug. Twelve animals were injected with 500 mg/kg each of SAL. After 16 hours, 6 of the animals were killed, while the other 6 received a second dose of SAL (300 mg/kg) and were sacrificed 2 hours later. AAA values in the 2 groups were 365 ± 27 mg % and 201 ± 9 mg %, respectively.‡ The responsiveness of the adrenals was not impaired by the first dose of SAL. The per cent depletion from the second dose was of almost the same magnitude as that found after a single injection of 300 mg/kg of SAL and greatly exceeded the effects of repeated saline injections (Table III).

The next step was an experiment with repeated doses extending over 96 hours. Three groups of 8 animals each received on 4 successive days 2 injections daily of either 10% SAL (200 mg/kg at 8 A.M. and 300 mg/kg at 4 P.M.) or saline solution (2 and 3 ml/kg, respectively), or a 4.23% solution of sodium chloride (2 and 3 ml/kg) which is equimolar to a 10% SAL solution. All animals were killed 16 hours after the last injection. Repeated injections of sodium chloride, either as isotonic or as hypertonic solution, did not deplete AAA. On the contrary, a slight, but significant increase was observed, suggesting overcompensation (rebound) due to the repeated, non-specific stress of sodium chloride injections. The effect of SAL was the same after the last as after the first injection (compare with Table II). In both cases, AAA had not returned to normal values within 16 hours. Thus, repeated doses of SAL did not exhaust the adrenals or impair their responsiveness, as indicated by AAA. The adrenal weights in the 3 groups were not different from each other or from that of untreated animals (30.0 ± 3.2 mg).

The effect of pretreatment with cortisone on the activity of SAL is summarized in Table IV. Cortisone acetate§ was injected intramuscularly in doses from 0.16 to 80.0 mg/kg, followed 16 hours later by a standard subcutaneous dose of SAL (300 mg/kg), or saline. The animals were killed 2 hours after the second injection. Under these conditions doses from 0.16 to 0.64 mg/kg of cortisone had no influence on the SAL action. In the range from 0.90 to approximately 8.0 mg/kg of cortisone, the effect of SAL was gradually reduced, while above 10 mg/kg even an 8-fold increase of cortisone had no measurable additional influence. The saline controls were affected in the same manner as the SAL animals, while cortisone alone did not produce changes in AAA. Although the experimental data do not exclude the possibility that still larger doses of cortisone would have a greater inhibitory effect, the observed dose-response relationship makes this rather unlikely. The results agree with observations by Hetzel and Hine(2) which, however, covered a much narrower dose range of cortisone.

All previously described experiments had extended the original observation that SAL acts on the adrenal-pituitary system by an increased production or release of ACTH. To obtain some information on the mechanism of this effect, animals under pentobarbital anesthesia were investigated. Pentobarbital sodium (PEN) in a dose of 40 mg/kg was injected intraperitoneally. As soon as the animals were in complete anesthesia (usually after 1-2 minutes), either SAL or physiologic saline was given subcutaneously. Duration

‡ This and other values with standard deviation.

§ Generously supplied by Merck and Co.

TABLE III. Adrenal Ascorbic Acid of Rats after 4 Days of Subcutaneous Injections of Salicylic Acid or Physiologic or Hypertonic Sodium Chloride Solution.

Drug	Daily dose	No. of animals	Adrenal wt in mg (± S.D.)	AAA in % of untreated animals (± S.D.)
SAL	AM: 200 mg/kg PM: 300 "	8	28.8 ± 3.8	82 ± 14†
Saline	AM: 2 ml/kg PM: 3 "	8	31.4 ± 3.5	110 ± 17
4.23% NaCl*	AM: 2 " PM: 3 "	8	30.9 ± 4.1	114 ± 8†

* Equimolar to a 10% solution of Na salicylate.
† Significant difference ($p = 0.05$ or less) from untreated controls.

of the experiments was 2 hours, following SAL injection (Table V). AAA was reduced to 82% by PEN alone and to 70% by PEN plus saline. When 300 mg/kg of SAL was administered to 16 anesthetized animals, AAA depletion reached 58%. However, the actual data centered around a low and a high value and this classification coincided exactly with gross observations on depth and length of anesthesia. All animals which had shown signs of incomplete anesthesia or had started to wake up before the end of the experiment, had low AAA (258 ± 47 mg %), while the opposite was the case with all animals which stayed completely anesthetized during the experiment (352 ± 61 mg %). Therefore, this group of 16 animals is divided into 2 subgroups as indicated in the table. When anesthesia with PEN was complete, SAL (300 mg/kg) did not cause a significant depletion of AAA when compared to the corresponding control (PEN plus saline). When anesthesia was incomplete, the effect of SAL was the same as in non-anesthetized animals. All 4 animals receiving PEN + 600 mg/kg of SAL had shown signs of incomplete anesthesia, and did not differ from non-anesthetized animals.

TABLE IV. Effect of Salicylic Acid on Adrenal Ascorbic Acid of Rats following Pretreatment with Cortisone Acetate.

No. of animals	Cortisone, mg/kg	2nd drug	AAA in % ± S.D. of untreated controls
2	80	Sal, 300 mg/kg	56 *
4	40	"	55 ± 8*
4	20	"	54 ± 10*
6	10	"	54 ± 10*
6	5	"	51 ± 7*
6	2.5	"	49 ± 6*
6	1.25	"	47 ± 10
4	.62	"	44 ± 8
4	.31	"	43 ± 9
4	.16	"	44 ± 10
†	—	"	44 ± 6
8	40	Saline	88 ± 12
12	—	"	76 ± 8
8	40	—	101 ± 12

* Significantly different ($p = 0.05$ or less) from effect of SAL without pretreatment with cortisone.
† Previous data(4).

Discussion. The previously established specific effect of SAL on the adrenal-pituitary system lasts much longer than the non-specific stress of a saline injection, and it can be maintained by repeated administration of the drug. If one accepts depletion of AAA as an index of circulating ACTH, and since SAL acts only in the presence of an intact pituitary, the experimental data imply that the SAL induced stimulation of the pituitary continues as long as a minimum concentration of SAL is present in the circulation. This distinguishes the specific action of SAL (and similarly acting drugs) from the non-specific, short-lasting effect of an injection of saline or other inert materials.

It has been postulated by Sayers(6) that an autoregulatory mechanism governs the secretion of ACTH by the pituitary and of corticoids by the adrenal cortex. According to this concept, an increase in circulating ACTH results in an increased secretion of corticoids which in turn suppress further secretion of ACTH by the anterior pituitary until equilibrium is reestablished. Thus, injections of cortisone should suppress the normal "endogenous" secretion of ACTH. The observed partial inhibition of AAA depletion

TABLE V. Effect of Pentobarbital Anaesthesia on Adrenal Ascorbic Acid Depletion by Salicylic Acid.

Drug administered			No. of animals	SAL blood level, mg %	Adrenal ascorbic acid, % ± S.D.	Comment
PEN, mg/kg	Saline, ml/kg	SAL, mg/kg				
40	—	—	14	—	82 ± 16*	
40	3	—	11	—	70 ± 7*	
40	—	300	16	41	58 ± 23†	All animals
40	—	300	10	39	64 ± 17*	Fully anaesth.
40	—	300	6	45	47 ± 18†	Not fully anaesth.
40	—	600	4		39 ± 12†	" " "

* Significantly lower ($p = 0.05$ or less) than untreated animals.
† " " than untreated animals and the corresponding controls without SAL.

by pretreatment with cortisone may possibly be an index of this endogenous ACTH. However, the direct stimulatory effect of SAL is independent of the amount of circulating cortisone. This leads to the suggestion that the response of the pituitary to circulating corticoids and circulating SAL is mediated through different mechanisms. This explains also why the response of the adrenal to repeated injections of SAL is practically the same as to one injection.

This observation differs from that of Sayers and Sayers(7) that ascorbic acid depletion from exposure to cold or injection of epinephrine or histamine may be completely blocked by pretreatment with adrenal cortical extract. This discrepancy again suggests a difference between the mechanism of action of SAL and certain other "non-specific" stress agents.

The results in PEN anesthetized animals are taken as an indication that SAL acts directly or indirectly on the hypothalamus which in turn relays a stimulatory effect to the anterior pituitary. The following observations are in support of this assumption:

1. PEN is known to act on the hypothalamus, blocking the temperature regulating center. The antipyretic action of SAL is assumed to be mediated through the hypothalamus(8). 2. Barbiturates do not interfere with the ability of injected ACTH to deplete AAA(9,10). Thus, the blockade of the SAL effect by PEN indicates that in this case the release of ACTH is inhibited in the completely anesthetized animals. 3. The place of primary action of SAL must be different from that of injected histamine or epinephrine since the adrenal response to these agents is not inhibited by PEN(9). 4. Rats under PEN anesthesia do not respond to the stress of low temperature (3°C for one hour) which ordinarily results in depletion of adrenal ascorbic acid (9). 5. Evidence for a direct hypothalamic influence on the anterior pituitary has been presented by Harris(11), Greer(12), and Porter(13). 6. Hume(14) has proposed a hormonal mechanism in the hypothalamus capable of stimulating an increased secretion of ACTH.

The experimental data obtained with SAL under varying conditions indicate certain differences from other stress-producing agents such as histamine or epinephrine. To the observations following premedication with cortisone or pentobarbital may be added that the effect of SAL is not inhibited by adrenergic blockade(15). It is suggested that the SAL effect is mediated through the hypothalamus and that it differs from that of epinephrine and histamine and all those stressing conditions which involve one of the last named agents.

The absence of hypertrophy of the adrenals is contrary to most observations on the effect of ACTH and other stress producing agents. However, similar negative results have been reported(16). It has also been shown that ascorbic acid in large doses will prevent adrenal weight increases due to exposure to cold(17). Since SAL is known to have considerable influence on ascorbic acid metabolism, the explanation for the anomalous adrenal weights may perhaps be found in a study of ascorbic acid mobilization and synthesis under the influence of SAL.

The observation that the SAL effect was blocked by PEN only in 10/16 animals may be explained by the central stimulating effect of salicylates. Such an effect has been reported in salicylate poisoning(8). It was also indirectly observed in the present experiments, in that 6 of the SAL treated animals, but none of 25 controls showed signs of incomplete or shortened anesthesia.

Summary. The previously established specific action of salicylic acid on the adrenal-pituitary system has been investigated as to duration, effect of repeated doses of salicylic acid, effect of pretreatment with cortisone and possible mechanism of action. The effect of a single injection of salicylic acid (300 mg/kg) lasted more than 16 hours, at which time still appreciable bloodlevels of salicylic acid were present. Repeated injections of salicylic acid did not impair the responsiveness of the adrenals. Pretreatment with cortisone reduced, but did not abolish the effect of salicylic acid. Complete anesthesia with pentobarbital blocked the effect of salicylic acid. The results are interpreted as an effect of salicylic acid on the hypothalamus with subsequent stimulation of the pituitary.

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Intracellular Multiplication of *Toxoplasma gondii* in Adult Mammalian Macrophages Cultivated *in vitro*.* (21117)

WOLFGANG A. VISCHER AND EMANUEL SUTER. (Introduced by J. K. Frenkel.)
From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

Toxoplasma gondii, an obligate intracellular parasite, can easily be propagated in cultures of different types of tissues of either embryonic or adult origin and the parasites have been maintained in serial passages in tissue cultures without detectable loss of virulence for mice(1,2). The organisms invade and probably multiply in cells derived from all 3 embryonic layers and there is no evidence for any cellular specificity. In experimental toxoplasmosis, especially after intraperitoneal infection, the parasites are mainly located in macrophages. These cells can be maintained in tissue cultures and provide a system for *in vitro* study of intracellular parasites(3). After preliminary experiments had shown that Toxoplasma do multiply in macrophage cultures, the ability of Toxoplasma to multiply *in vitro* within macrophages derived from susceptible and resistant animals was studied.

Materials and methods. Animals. Swiss white mice (Schwentker strain) were raised and supplied by the Department of Bacteriology and Immunology, Harvard Medical School. Rats, guinea pigs and rabbits were obtained from outside sources. The animals

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