Effect of Salicylic Acid and Similar Compounds on the Adrenal-Pituitary System.* (19519)

GEORG CRONHEIM,† J. STANTON KING, JR., AND NELTA HYDER.
(Introduced by George L. Maison.)
From the Research Department of S. E. Massengill Co., Bristol, Tenn.

The extensive use of salicylic acid (SAL) in the treatment of rheumatoid arthritis and rheumatic fever left the impression that this drug has some specific effect in addition to its analgesic and antipyretic action. However, no satisfactory explanation for these good clinical results has been offered. The beneficial effect of adrenocorticotropic hormone (ACTH) in arthritis provided a new hypothesis. This made it possible to reinvestigate the pharmacology of SAL with special emphasis on the effect on the adrenal-pituitary system. An indication that such a mechanism might be involved was found in a report by Blanchard *et al.*(1) that a new "anti-arthritic" drug, 3-hydroxyphenylcinchoninic acid, may produce a depletion of adrenal ascorbic acid. A few exploratory experiments suggested a similar action of SAL, aminopyrine and cinchophen.

In the present study, the effect of SAL and related compounds on the adrenal-pituitary system was investigated in detail. The main purpose was to determine the specificity of any SAL effect and thereby to obtain a better understanding of the mechanism of the action of this drug. The ascorbic acid content of the adrenals was used as indicator since, according to Sayers and co-workers(2), this component can serve as a reliable measure of cortical activity. It is known that "stress" of various types may affect adrenal ascorbic acid and the latter apparently varies also with the weight and age of the experimental animals and seems also to be subject to seasonal fluctuations. Therefore, it was necessary to devise a procedure which would permit a comparison of experiments made at different times and under different conditions.

Methods. Normal female Wistar rats weighing 80 to 150 g were used. In each experiment the weight range was kept within ±20 g. The diet of Rockland pellets was available at all times *ad libitum*. Hypophysectomized female rats of the Sprague-Dawley strain‡ were used within 48 hours after the operation. The drugs were injected subcutaneously as sodium salts in aqueous solution at 37°C. All dosage figures refer to the free acid. The volume of the injection was kept constant at 0.3 ml per 100 g of body weight while the concentration was varied. It is essential that a sufficient number of control animals, receiving an equal volume of isotonic saline solution, is included in each experiment. Two hours after the injection, the animals were anesthetized by intraperitoneal injection of a 6.5% solution of pentobarbital sodium and the adrenals were quickly removed and weighed. The pH of the solutions used varied from about 5 to 9. As a rule, no attempt was made to adjust the pH since exploratory experiments with SAL had indicated that this factor is of no particular importance. The analytical procedures were: adrenal ascorbic acid, Roe and Kuether(3); salicylic acid, Brodie *et al.*(4), and Saltzman (5); salicyluric acid, Smith *et al.*(6); gentisic acid, Gerald and Kagan(7).

Results. The basic observation showing the action of SAL (300 mg/kg) is indicated in Table I, which contains also the adrenal ascorbic acid values of the corresponding saline controls. These data which were obtained over a period of 14 months, proved conclusively that SAL causes a depletion of adrenal ascorbic acid. They also showed that the values for both groups may vary considerably and in an unpredictable manner. Since such variations make it difficult, if not impossible, to evaluate the experimental data obtained at different times and under different conditions,

* Preliminary report presented at meeting and *J. Pharm. Exp. Ther.*, December, 1951, v103.

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

‡ These animals were obtained from Hormone Research Laboratory, Chicago, Ill.

TABLE I. Adrenal Ascorbic Acid of Normal Rats (Average Values with Stand. Error) 2 Hr after a Subcut. Inj. of 300 mg/kg of Salicylic Acid (10% Sol. as Sodium Salt) or Saline Sol. (Control Group).

Control group		Salicylate group		
			Adrenal ascorbic acid	
No. of animals	Adrenal ascorbic acid in mg %	No. of animals	In mg %	% of control group
6	363 ± 28	6	225 ± 15	62
5	385 ± 21	5	214 ± 16	55.6
8	353 ± 19	8	209 ± 14	59.2
7	381 ± 17	4	220 ± 12	57.8
4	438 ± 25	4	266 ± 24	60.8
7	434 ± 9	8	273 ± 22	62.9
8	469 ± 18	7	271 ± 26	57.8
12	389 ± 28	7	216 ± 14	55.6
11	445 ± 34	8	256 ± 15	57.6
9	396 ± 23	10	248 ± 23	62.6
3	415 ± 21	3	244 ± 17	58.8
13	459 ± 13	6	281 ± 10	61.2
			Avg	59.3 ± .9

TABLE II. Adrenal Ascorbic Acid of Normal and Hypophysectomized Rats 2 Hr after a Subcutaneous Injection of Varying Doses of Salicylic Acid (As the Sodium Salts).

Drug	Dose	No. of animals	Adren. ascorb. acid in % of saline animals	Salicylate blood levels in mg %
		Normal animals		
0		24	135	
Isotonic saline	3 ml/kg	87	100	
Salicylic acid	75 mg/kg	12	89	11
	150	36	73	26
	300	76	59	42
	600	12	49	72
		Hypophysectomized animals		
Isotonic saline	3 ml/kg	12	100	
Salicylic acid	300 ml/kg	12	101	45

a simple method of comparison was desirable. This variability can be eliminated almost completely by expressing the results for the SAL animals in per cent of those of the control animals (Column 5). Therefore, all results presented in this paper will be expressed in per cent of the corresponding saline controls. This does not represent the "normal" concentration, since saline injections alone will cause within 2 hours a loss of about 25% of the ascorbic acid. If one assigns to the saline controls a value of 100%, untreated "normal" animals show accordingly a value of 135%.

The question of specificity of SAL action was approached in a 3-fold manner: 1) the dose-effect relationship; 2) the effect of hypophysectomy, and 3) the effect of various compounds, structurally related to or different from SAL. The results with varying doses of SAL (Table II) indicated that the depletion of adrenal ascorbic acid is in proportion to the amount of drug administered. The SAL blood level showed almost exactly the same dose relationship. The latter observation explains the previously made statement that the pH of the drug solution is of no particular importance. Obviously, one would not expect that variations in pH would greatly affect the absorption of a water soluble drug from the site of a subcutaneous injection.

Hypophysectomy completely abolished the effect of SAL (Table II), thus indicating the essential role of the pituitary and, specifically ACTH in the mechanism of the action of this drug. This observation excluded any direct action of SAL on the adrenals, since removal of the pituitary does not prevent the response of these glands to ACTH(2).

TABLE III. Aliphatic Acids Which Did Not Affect the Adrenal Ascorbic Acid of Normal Rats. Dose and No. of animals ( ) as indicated.

Acid	mg/kg	
Acetic	150	(4)
Adipic	150	(4)
Ascorbic	300	(11)
Citric	300	(12)
Dehydroascorbic	300	(4)
Fumaric	300	(7)
Gluconic	300	(4)
Glucuronic	300	(8)
Lactic	150	(7)
Pantothenic*	300	(9)
Pyruvic	300	(6)
Succinic	300	(8)

* Used as calcium salt.

The requirement of an intact pituitary and the good dose response relationship is only partial proof for the specificity of the SAL action. It is known that chemical, as well as physical, physiological, and even psychological stimuli, may influence directly or indirectly the adrenal function in the normal animal. As a more direct experimental approach to prove the specificity of action, the influence of varying the structure of the administered drug was studied. A total of 32 aliphatic and aromatic acids were used as sodium salts.

The results with 12 aliphatic acids were uniformly negative. With the exception of adipic acid, all substances investigated are involved in normal metabolic processes and 3 of the compounds (glucuronic, succinic and ascorbic acid) have been reported as being beneficial in the treatment of arthritis, although the opinion on this point is divided. The experiments with aliphatic acids also indicated that neither hypertonicity nor sodium content, which in several instances were considerably greater than those of saline solution, are of importance in this test.

The series of 20 aromatic acids is best divided into substituted benzoic and substituted salicylic acid (Table IV). In the first group only 2 compounds (benzoic and p-methoxybenzoic acid) produced a significant reduction in ascorbic acid. The observation of Blanchard that p-hydroxybenzoic acid was ineffective, was confirmed and extended to another salicylic acid isomer, m-hydroxybenzoic acid.

In the group of 11 substituted salicylic acids, 9 produced, like SAL, a depletion of ascorbic acid. The 2 exceptions were p-hydroxysalicylic and p-aminosalicylic acid, which on repeated tests gave negative results.

The figures in the fourth column of Table IV estimate the relative activity of substituted salicylic acids. The main variants were salicyluric and gentisic acid, both of which were much weaker than SAL, while benzoic and γ-resorcylic acid occupied an intermediate position. The 2 chlorosalicylic acids were more toxic than the other drugs because on a dose of 300 mg/kg all animals died before the end of the experiment. Whether or not this higher toxicity is the reason for the relatively great reduction of ascorbic acid, particularly after 4-chlorosalicylic acid, or whether it is a case of greater activity will require additional experiments.

Salicylaldehyde and salicylamide have a different structure and, strictly speaking, do not belong in a group of substituted salicylic acids. Qualitatively, they acted like SAL although their potency seemed to be less. This may be due to lower solubility which made it necessary to inject both compounds as aqueous suspensions.

The reason for the relatively low activity of salicyluric and gentisic acid was investigated in some detail, because both are normal metabolites of SAL in man and the latter is also used clinically. Qualitative tests for SAL in both blood and urine were negative, so that the effect could not be ascribed to partial hydrolysis or reduction of the administered drug. However, it was found that both salicyluric and gentisic acid were excreted very rapidly. The former was barely detectable 105 minutes after the injection and the latter was found in a concentration of only about 15 mg % 2 hours after a dose of 300 mg/kg. This rapid excretion of both salicyluric and gentisic acid may explain why considerably larger doses of these drugs were required to produce significant depletion of ascorbic acid.

Discussion. The experiments furnish adequate evidence that SAL reduces the adrenal ascorbic acid and that an intact pituitary is required to produce this effect. Furthermore, the results with the 32 different aliphatic and aromatic acids demonstrate that this ascorbic acid depletion is not due to general "stress,"

TABLE IV. Effect of Various Aromatic Acids§ on the Adrenal Ascorbic Acid of Normal Rats.

Drug	No. of animals	Dose, mg/kg	Ascorbic acid, % of control	Disassociation constant
Benzoic acid	11		72*	6.16 × 10^{-4}
m-HO-Benzoic acid	5		91	7.61
p-HO	5		115	2.80
o-CH_3O	13	300	101	8.3
p-CH_3O	23		79*	3.1 × 10^{-5}
o-C_2H_5O	5		106	7
p-C_2H_5O	9		90	5
p-NH_2	17		105	1.15
Salicylic acid	60	300	59*	1.06 × 10^{-3}
4-HO-Salicylic acid	11	300	106	5.05 × 10^{-4}
5-HO-Salicylic acid (Gentisic acid)	10	600	75*	1.06 × 10^{-3}
6-HO-Salicylic acid (γ-Resorcylic acid)‖	14		72*	5 × 10^{-2}
5-CH_3O-Salicylic acid	6	300	69*	
3-CH_3	6		65*	1 × 10^{-3}
4-CH_3	6		69*	
4-Cl	10	150	63*	
5-Cl	10	150	69*	1.95 × 10^{-3}
5-HO_3S	6	300	64*	
4-NH_2	12	300	109	3.5 × 10^{-7}
Salicyluric acid‖	17	426‡	82†	
Salicylaldehyde	6	300	71*	
Salicylamide	6	300	73*	

* Statistically significant difference ($p = .01$ or less).

† $p = .02$.

‡ This quantity of salicyluric acid is equimolar to 300 mg of salicylic acid.

§ The authors are indebted to Dr. T. R. Aalto of Heyden Chemical Corp. for samples of many of the substituted benzoic and salicylic acids.

‖ Salicyluric acid and γ-resorcylic acid were prepared by N. H. Leake and M. L. Fielden of the Research Department of S. E. Massengill Co.

because many of the compounds tested did not produce a greater change than saline solution. The results with molecular variants are interpreted as indicating a specific effect of substances with a characteristic chemical structure. On the basis of the available evidence, the salicylic acid configuration represents one example of such structure.

Since the ascorbic acid content of the adrenals is considered a reliable indicator of cortical activity, which in turn is governed by ACTH, all the experimental observations fit into the assumption that SAL stimulates through some specific mechanism the output of ACTH by the pituitary. At present, it is impossible to answer the following questions: 1) Is the observed effect the result of increased production or of mobilization of a stored reserve of ACTH? 2) Is the pituitary the primary target of the action of SAL or is it only secondarily stimulated following a primary effect on some other receptor such as the hypothalamus? It may be mentioned that more recent experiments with animals under complete pentobarbital anesthesia indicate quite conclusively that the hypothalamus is directly involved in the SAL action. The details of this work will be presented elsewhere.

As far as specificity is concerned, it is not restricted to the SAL configuration, but includes, at the present time, benzoic acid and, as shown by Blanchard *et al.*(1), also aminopyrine, cinchophen and certain cinchoninic acid derivatives. It is of interest that all cinchoninic acid derivatives found to produce ascorbic acid depletion have an hydroxylgroup in ortho position to a carboxylgroup. It is noteworthy that all the above mentioned drugs have been or are used in the treatment of arthritic conditions.

In a recent publication, Reid *et al.*(8) have suggested that the action of SAL is connected with its ability to form chelate compounds. In support of this theory, these authors claim

that γ-resorcylic acid (2,6-dihydroxybenzoic acid) which has a stronger chelation effect than SAL, is more effective in the treatment of rheumatic disease. In the ascorbic acid depletion tests, reported in this study, γ-resorcylic acid is definitely less active than SAL. This agrees with a short statement in Reid's paper. Since the disassociation constant of organic acids is considered to reflect their chelation ability, the pK values, as reported in the literature, are listed in Table IV. It is apparent that the available figures do not indicate a relationship between the disassociation constant of these drugs and their effect on adrenal ascorbic acid.

Summary. 1. The effect of salicylic acid and similar compounds on the adrenal-pituitary system has been investigated. 2. Salicylic acid and a number of substituted salicylic acids cause a significant depletion of adrenal ascorbic acid. The only exceptions found so far are p-aminosalicylic and p-hydroxysalicylic acid. 3. The SAL blood levels parallel very closely the ascorbic acid depletion of the adrenal. 4. Hypophysectomy prevents the effect of salicylic acid on adrenal ascorbic acid. 5. Benzoic acid has an effect similar to that of salicylic acid, while several substituted benzoic acids, including p-aminobenzoic acid, are ineffective. 6. Twelve aliphatic acids of various types did not produce a change in adrenal ascorbic acid. 7. The experimental results are interpreted as indicating a specific direct or indirect action of salicylic acid on the anterior pituitary which results in the production or release of increased amounts of adrenocorticotropic hormone.

1. Blanchard, K. C., Dearborn, E. H., Maren, T. H., and Marshall, E. K., Jr., *Bull. Johns Hopkins Hosp.*, 1950, v86, 83.

2. Sayers, G., Sayers, M. A., Tsan-ying Ling and Long, C. N. H., *Endocrinol.*, 1946, v38, 1.

3. Roe, J. A., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.

4. Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Ther.*, 1944, v80, 114.

5. Saltzman, A., *J. Biol. Chem.*, 1948, v174, 399.

6. Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalok, S., *J. Pharm. Exp. Ther.*, 1946, v87, 237.

7. Gerald, P. S., and Kagan, B. M., *J. Biol. Chem*, 1951, v189, 467.

8. Reid, J., Watson, R. D., and Cochran, J. B., *Brit. Med. J.*, 1951, v2, 321.

Received April 14, 1952. P.S.E.B.M., 1952, v80.

Studies on Vi Antigen. I. Relative Vi Antigen Content of V Form Cultures. (19520)

MAURICE LANDY.

From the Immunology Division, Army Medical Service Graduate School, Washington, D.C.

It has been demonstrated that vaccines prepared from rough Vi strains of *Salmonella typhosa*(1,2), and from Vi strains of *Paracolobactrum ballerup*(3,4) and *Escherichia coli*(4), none of which contain typhoid somatic components, are effective in the immunization of mice against infection with Vi + O strains of *S. typhosa*. Although 4 bacterial species are known to possess Vi antigen, information concerning their content of this component was lacking. The object of this investigation was to compare, by mouse protective potency tests, the Vi antigen content of these bacterial species to determine which provided the richest source of this antigen.

Cultures. The V form *S. typhosa* strains 58 and Ty2 were the challenge organisms used in the mouse protection tests. Strain 58 exhibited evidence of agglutinability by typhoid O antiserum, while Ty2 was quite resistant to O agglutination. When administered intraperitoneally in mucin, both strains exhibited maximum mouse virulence ($LD_{50} < 10$ organisms). When injected in saline the LD_{50} for strain 58 was approximately 220 million, while that for Ty2 was approximately 40 million. The other V form cultures used in this study were the Kauffmann strains *E. coli* 5396/38, *P. ballerup* 7851/39 and *S. paratyphi* C (East Africa). The properties of