crystallographic properties of the resultant crystals were the same as those of the product isolated from the commercial injection, and the X-ray diffraction patterns of the material from the two sources were identical.

Anal.—Calcd. for $C_{23}H_{32}N_2O_4S$: C, 63.86%; H, 7.46%; N, 6.48%; S, 7.41%. Found: C, 64.00%; H, 7.24%; N, 6.34%; S, 7.49%.

Preparation of the Hydrochloride—Five grams of the adduct was dissolved in 20 ml. of isopropanol, and the calculated volume of 1 *N* alcoholic hydrochloric acid to form the monohydrochloride was added. After 50 ml. of acetone was added, the solution was allowed to stand in the refrigerator; 4.73 g. of the monohydrochloride was recovered. Recrystallization from isopropanol yielded crystals (m.p. 120–124°) containing one molecule of isopropanol of crystallization. Calculated for B·HCl·1-iPrOH: 11.36% isopropanol; found (loss of weight on heating at 110° under vacuum): 11.1%.

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In Vitro Binding of Salicylates to Saliva Proteins

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Abstract □ A method has been developed whereby the *in vitro* binding of salicylic acid, acetylsalicylic acid, and salicylamide to saliva proteins, may be easily determined. It was found, using gel filtration, that salicylates added to saliva are bound to the saliva proteins to the extent of from 35 to 50% after a contact time of from 0.5 to 2.0 hr. at 37°.

Keyphrases □ Salicylate binding—saliva proteins □ Saliva proteins—ninhydrin test □ Fluorometry—analysis

The salicylate drugs have been studied continuously for many years, but several important questions regarding the *in vivo* distribution and the metabolic fate of these drugs have remained unanswered. One such question deals with the degree to which the salicylates bind to the proteins in various body fluids, especially the saliva.

Many methods have been reported to demonstrate the various aspects of drug-protein interaction, but the literature is void with respect to interactions between saliva proteins and the following salicylate drugs: acetylsalicylic acid, salicylic acid, and salicylamide. Goldstein (1), in a review, stated that most of the methods could be classified into three groups: methods based upon the reduction in the free drug concentration, the alteration of the drug properties, and the alteration of the protein properties.

Klotz (2), and Davison and Smith (3), have employed dialysis to demonstrate drug-protein interactions. The latter investigators used such a procedure to show the binding of salicylic acid and related substances to purified samples of protein material.

The development of gel filtration methods between 1959 and 1964 (4–7) made possible the column separation of materials of different molecular weight. These methods were adapted to drug-protein binding studies (8–9), some of which allowed the estimation of the comparative magnitude of drug-protein binding. Potter and Guy (10) developed a spectrofluorometric method for the determination of plasma salicylate content. A dextran gel filtration procedure, rather than a solvent extraction or a protein precipitation procedure, was used for the separation of the bound from the unbound salicylate. This method also permitted the rapid estimation of the magnitude of the binding. Sturman and Smith (11) have recently expanded plasma studies to include the fractionation, by a gel filtration procedure, of protein-bound salicylate drugs from the plasma of various species of animals.

The above information shows that gel filtration procedures will adequately separate bound from unbound salicylate drugs. It has also been shown, by Lange and Bell (12), that the submicrogram concentrations of the salicylates that would be expected to be present in the saliva, can be determined by fluorometric means. This paper describes the utilization of gel filtration and fluorometry for the *in vitro* determination of the degree of salicylate-protein interaction that takes place in human saliva.

EXPERIMENTAL

Reagents and Chemicals—The reagents and chemicals used were: acetylsalicylic acid powder USP,[1] salicylic acid crystals USP,[2] sodium hydroxide USP,[2] sodium phosphate (dibasic) granular,[2] salicylamide powder,[3] synthetic polysaccharide,[4] and naphthol blue-black.[5] Ferric chloride solution, T.S., and 0.2% aqueous ninhydrin solution were used as indicators.

Apparatus—A synthetic polysaccharide (Sephadex K-25) column having an internal diameter of 25 mm. was used for the gel filtration procedure. A universal fraction collector (G.M.), equipped with a microflex timer, was used to mechanize the collection of the eluent fractions. Analyses were made using fluorometer (Turner

[1] Mallinckrodt Chemical Works.
[2] S. B. Penick and Co.
[3] New York Quinine and Chemical Works.
[4] Sephadex G-25, Coarse, Pharmacia Fine Chemicals, Inc.
[5] Allied Chemical Co.

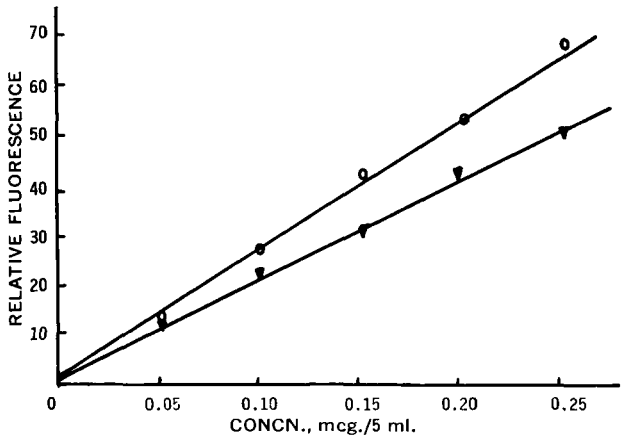

Figure 1—*Fluorescence-concentration relationship. Key:* ○, *salicylamide;* ▽, *salicylic acid.*

model 110), equipped with an all-purpose lamp, a primary excitation "sandwich" filter system consisting of two Corning No. 7-54 filters and a Wratten No. 34-A filter, and a half thickness Corning No. 5-58 secondary emission filter. The 12 × 75-mm. Pyrex cells were carefully matched and selected for low fluorescence. Appropriate Fisher transfer pipets were also used.

Methods—*Preparation of the Gel Filtration Column*—The gel was prepared by suspending 15 g. of polysaccharide (Sephadex G-25, coarse) in 45 ml. of pH 7.4 phosphate buffer. The suspension was allowed to set for 3 hr. at room temperature to insure complete swelling of the polysaccharide grains. The material was then carefully transferred to the polysaccharide (Sephadex K-25) gel filtration column and was allowed to set until there was no further reduction in the apparent volume of the gel.

Measures were taken to prevent the disturbance of the gel grains during sample application by placing a disk of filter paper, which had been cut to fit the internal diameter of the column, on the upper surface of the gel bed. The homogeneity of the column was confirmed by passing naphthol blue-black through the system. A constant head reservoir was used to insure continuous flow of the elution medium.

The In Vitro Binding of Salicylic Acid, Acetylsalicylic Acid, and Salicylamide to Saliva Proteins—Saliva samples were collected from the subjects 15 min. after their mouths had been thoroughly rinsed with 100 ml. of distilled water. One-tenth-milliliter samples of the saliva from each subject were transferred to six test tubes of 3-ml. capacity by means of an 0.1-ml. transfer pipet. Five micrograms of salicylic acid was transferred to two of the samples, an equivalent amount of acetylsalicylic acid to two samples, and an equivalent amount of salicylamide to the remaining two samples.

One sample of each drug was incubated for 0.5 hr. at 37°, and the second sample was incubated under identical conditions for 2.0 hr. After having been incubated, each sample was immediately transferred to the gel filtration column and was eluted with pH 7.4 phosphate buffer. Sixty fractions, each of 3 ml., were collected. Fractions 18 through 60 were diluted with 2 ml. of 5 *N* sodium hydroxide solution. The resulting solutions were mixed well and transferred to 12 × 75-mm. Pyrex cells that had previously been cleaned for fluorometric use. The fluorescence exhibited by the samples was then measured using the previously described conditions.

RESULTS AND DISCUSSION

It was necessary to elute known concentrations of the drugs in the preliminary work so that the number of fractions that need be collected could be determined. This portion of the work also showed that neither an increase nor a decrease in the concentration of the drug would alter the elution pattern. A simple ferric chloride detection method showed that the drugs would be eluted unbound in Fractions 18 through 60. Serum and whole saliva were also passed through the column so that the entire elution pattern could be determined. A ninhydrin test showed the proteins to be present in Fractions 8 through 14. These methods were also used to determine the void volume of the system.

Table I—Percentage of Drugs Found to Bind with Saliva Proteins

Subject	Age	% Bound — Salicylic Acid[a] 1.0[b] *hr.*	Salicylic Acid[a] 2.0 *hr.*	Acetylsalicylic Acid 1.0 *hr.*	Acetylsalicylic Acid 2.0 *hr.*	Salicylamide 1.0 *hr.*	Salicylamide 2.0 *hr.*
A	24	74.6	60.2	44.9	44.5	46.0	44.7
B	23	30.2	47.4	52.3	44.3	29.0	38.5
C	26	38.2	62.1	44.5	74.8	33.5	56.3
D	25	74.1	46.0	45.7	33.6	40.5	36.3
E	42	78.5	32.7	60.8	64.7	36.7	34.2
F	30	28.3	22.1	40.1	47.1	21.2	34.5
G	56	30.2	33.5	60.1	65.5	34.5	36.7
H	28	46.3	36.8	41.4	41.9	23.7	23.5
I	19	46.4	36.6	42.4	41.4	42.0	46.0
Mean		49.6	41.9	48.0	50.9	34.1	38.7

[a] Based on the addition of 5 mg. % of salicylic acid and an equivalent amount of acetylsalicylic acid and salicylamide to the respective samples of saliva. [b] Incubated at 37°.

Other elution media of varying pH's were also studied, but it was found that the pH 7.4 phosphate buffer effected the greatest degree of resolution when a polysaccharide (Sephadex G-25, coarse) was used as the filtering bed.

The *in vitro* determinations were carried out using the fresh saliva from nine normal, adult males, from 19 to 56 years of age. No dietary controls were enforced, but all subjects were deprived of any salicylate containing drugs or candies, *etc.*, for the 72 hr. preceding the test.

The fluorescence of each fraction was compared to standard curves that had previously been prepared. The standard curves are shown in Fig. 1. It can be seen that separate curves are necessary for salicylic acid and salicylamide as the fluorescence exhibited by equal concentrations of each is of a different magnitude. A standard curve for acetylsalicylic acid was not necessary because such analyses were made by measuring the salicylic acid content of the alkaline samples and then multiplying the result by 1.3, the molecular weight equivalence factor.

The percentages of the drugs that were found to bind to the proteins in the saliva were calculated by difference. That is, the amount of free drug eluted in Fractions 18 through 60 was subtracted from the total amount of the drug that should have been present had no binding taken place. The results are shown in Table I.

It was found by *in vitro* testing, that salicylates, when added to human saliva, are bound by the saliva proteins to the average extent of from about 35 to about 50% after a contact period of either 0.5 or 2.0 hours at 37°. The wide range in the degree of the salicylate-protein binding that was found to take place in the saliva of the nine subjects was not unexpected because of the normal variances that are found in the enzyme and protein content of human saliva. No correlation was made between the type of salicylate used, the age of the subject, the time of contact, and the degree of binding.

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Studies on the Mechanism of Action of Salicylates VI: Effect of Topical Application of Retinoic Acid on Wound-Healing Retardation Action of Salicylic Acid

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Abstract □ Oral administration of sodium salicylate or prednisone and topical application of salicylic acid or hydrocortisone in NIB retards healing and the inhibitory action of either one of these drugs can be reversed by local application of retinoic acid. Topical application of retinol, retinyl esters, or retinoic acid alone also promotes skin wound healing in the rat.

Keyphrases □ Salicylic acid effect—wound healing □ Hydrocortisone, predisone effect—wound healing □ Retinoic acid effect—drug-retarded wound healing □ Wound healing—tensile strength

In a recent report it was shown that intraperitoneal injection of retinol (vitamin A) promotes skin wound healing in rats (1). In the present study, it is shown that local application of retinol, retinyl acetate, or retinoic acid, dissolved in a nonionic base (NIB, aqueous) promotes skin wound healing. It was also shown previously that aspirin retards wound healing and this inhibitory effect can be reversed by the injection of retinol intraperitoneally (1, 2). In the present study, it was found that local application of salicylic acid or oral administration of sodium salicylate and local application of hydrocortisone or oral administration of prednisone, a dehydrogenated analog of cortisone, also retards wound healing and retinoic acid can reverse the retardation action of these anti-inflammatory agents.

EXPERIMENTAL[1]

Materials and Drugs—Retinol and retinyl esters were dissolved in NIB so that each 30 g. of NIB contained 1,000,000 USP units. The strength of retinoic acid used was 1% in NIB.

Application of NIB Preparations—NIB preparations were applied, with gentle rubbing, directly on the sutured wound immediately after wounding. The application was repeated, once a day, on the first and second days after wounding. For the control, only NIB was applied.

Administration of Drugs—Sodium salicylate, dissolved in a small amount of water, and prednisone, suspended in corn syrup, was fed to rats daily for 4 days through a short stomach tube (PE 160) connected to a blunt hypodermic needle (No. 17) attached to a 50-ml. syringe, starting 1 day before operation. The dosage levels for sodium salicylate and prednisone were 50 and 2.5 mg. per rat per day, respectively.

Wound Procedure—Sprague-Dawley male rats, weighing 230 to 240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and cutaneous muscles, at a distance about 1.5 cm. from the midline on each side. No ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread (No. 3-0) and curved needle (No. 19) were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing. On the seventh day after wounding the tensile strength of the wound was measured with a simple laboratory-made tensiometer as described previously (1).

RESULTS AND DISCUSSION

The results of the effect of retinol, retinyl acetate, and retinoic acid on skin wound healing in rats are summarized in Table I. The mean tensile strength of the control animals from Group I was 451 ± 12 g. These animals received only topical application of NIB. Results not shown here indicated that NIB does not have any effect on wound healing. The mean tensile strength of Group II animals receiving retinyl acetate was 522 ± 9 g., or 16% higher than that of the control. The mean tensile strength of Group III animals receiving local application of retinol was 515 ± 8 g. The increase in the mean tensile strength of Group IV animals receiving retinoic

Table I—Effect of Topical Application of Retinol, Retinyl Esters, and Retinoic Acid on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g.	Percent Control
I	11	NIB	451 ± 12	100
II	9	Retinyl acetate	522 ± 9	116
III	8	Retinol	515 ± 8	114
IV	8	Retinoic acid	573 ± 17	127

[1] Retinol, all *trans*, Sigma grade, Type X; retinyl acetate, all *trans*, Sigma grade, Type I; and retinoic acid, all *trans*, Sigma grade, Type XX, were crystalline synthetic compounds obtained from Sigma Chemical Co., St. Louis, Mo. Retinyl palmitate (Myvax) was obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y. Sodium salicylate, reagent grade, was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Prednisone is a product of Upjohn Co., Kalamazoo, Mich. Nonionic base (NIB, aqueous) and 1% hydrocortisone in NIB were prepared by the Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif.