rib cartilage *in vivo* (15). Aspirin (16) also decreased sulfate exchange of chondroitin sulfuric acid.

Wolf and his co-workers (18, 19) have demonstrated the role of vitamin A in the synthesis of mucopolysaccharide in rat colon segments. Recently, it was further shown (26) that a compound which appeared to be an acidic metabolite of vitamin A and vitamin acid was a component of ATP-sulfurylase (ATP: sulfate adenyltransferase, EC2.7.7.4), an enzyme involved in the first step of activation of sulfate. In the present study, it was shown that vitamin A promoted mucopolysaccharide synthesis in the granulation tissue of skin wound in rats. The effect of vitamin A and aspirin on acid mucopolysaccharide synthesis probably is another mechanism of action of these compounds on wound healing.

SUMMARY

The reversal of healing retardation action of aspirin by vitamin A and vitamin E has been demonstrated in rats. In the absence of aspirin, vitamin A promoted healing. It also increased acid mucopolysaccharide synthesis in the granulation tissue.

Inflammation and acid mucopolysaccharide synthesis are two essential features in the early stage of wound healing. Aspirin inhibited both features and vitamin A promoted both features. Two possible mechanisms of action of these two compounds are suggested.

REFERENCES

(1) Lee, K. H., *J. Pharm. Sci.*, **57**, 1238(1968).
(2) Seyle, H., in, Symposium on the Mechanism of Inflammation, Acta Inc., Montreal, 1953.
(3) Sandberg, N., *Acta Chir. Scand.*, **127**, 446(1964).
(4) Zweifach, B. W., in "Symposia on Hypotensive Polypeptides," Erdos, E. G., and Sicuteri, F., Eds., Springer-Verlag, New York, N. Y., 1966.
(5) Miller, W. S., and Smith, J. G., *Proc. Soc. Exptl. Biol. Med.*, **122**, 634(1966).
(6) Bassett, B. E., and Packer, L., *J. Cell. Biol.*, **27**, 448(1965).
(7) Dingle, J. T., Sharman, J. M., and Moore, T., *Biochem. J.*, **98**, 476(1966).
(8) De Duve, C., in "Lysosomes," Hayashi, T., Ed., Ronald, New York, N. Y., 1959.
(9) Layton, L. L., Frankel, D. R., Sher, I. H., Scapa, S., and Friedler, G., *Nature*, **181**, 1543(1958).
(10) Bollet, A. J., Goodwin, J. F., and Anderson, D. V., *Proc. Soc. Exptl. Biol. Med.*, **99**, 418(1958).
(11) White, B. N., Shetlar, M. R., and Schilling, J. A., *Ann. N. Y. Acad. Sci.*, **94**, 297(1961).
(12) Bentley, J. P., *Acta Chem. Scand.*, **19**, 15(1965).
(13) Dunphy, J. E., and Udupa, K. N., *New Engl. J. Med.*, **253**, 847(1955).
(14) Bentley, J. P., *Ann. Surg.*, **165**, 186(1967).
(15) Boström, H., Berntsen, K., and Whitehouse, M. W., *Biochem. Pharmacol.*, **13**, 413(1964).
(16) Boström, H., and Mansson, B., *J. Pharm. Pharmacol.*, **7**, 185(1955).
(17) Wolf, G., and Johnson, B. C., *Vitamins and Hormones*, **18**, 439(1960).
(18) Wolf, G., and Varandani, P. T., *Biochim. Biophys. Acta*, **43**, 501(1960).
(19) Wolf, G., Bergan, J. G., and Sundaresen, P. R., *ibid.*, **69**, 524(1963).
(20) Antonopoulos, C. A., Gardell, S., Szirmai, J. A., and De Tyssonsk, E. R., *Biochim. Biophys. Acta*, **83**, 1(1964).
(21) Scott, J. E., in "Methods of Biochemical Analysis," Clock, D., Ed., Interscience, New York, N. Y., 1960, p. 145.
(22) De Duve, C., Wattiaux, R., and Wibo, M., *Biochem. Pharmacol.*, **9**, 97 (1962).
(23) Edwards, L. C., and Dunphy, J. E., *New Engl. J. Med.*, **259**, 224(1958).
(24) Dunphy, J. E., and Jackson, D. S., *Am. J. Surg.*, **104**, 273(1962).
(25) Highberger, J. H., Gross, J., and Schmitt, F. O., *Proc. Natl. Acad. Sci.*, **37**, 286(1951).

Keyphrases

Salicylate activity—mechanism
Vitamin A effect—aspirin retarded healing
Vitamin E effect—aspirin retarded healing
Mucopolysaccharide, granulation tissue—vitamin A effect
Tensile strength—healing wound

Separation of Acetylsalicylic Acid and Salicylic Acid by Sephadex Gel Filtration

By K. H. LEE, LEON THOMPKINS*, and MICHAEL R. SPENCER

Acetylsalicylic acid and salicylic acid can be quantitatively separated by Sephadex gel filtration. A comparative study has been made by using different types of Sephadex gels.

THE AMOUNT of acetylsalicylic acid (ASA) in the biological tissues is generally determined as salicylic acid (SA) after it is hydrolyzed in the presence of the preexisted amount of SA (1). This method has large error when SA present is in great excess of ASA, a situation which often occurs in practice. A few attempts have been made to determine ASA and SA separately. Rowland and Riegelman (2) described a gas-liquid chromatography method for the determination of SA and ASA (as its silyl derivative) in plasma. Earlier, Cotty and Ederma (3) introduced a direct method for the measurement of ASA in human blood. They removed SA by reaction with ceric ammonium nitrate from salicylates mixture. These methods require two extraction procedures to remove salicylates from plasma and also require the preparation of ASA or SA derivatives. Potter and Guy (4) described a simple method for the separation of plasma protein bound SA and free SA by subjecting plasma directly to Sephadex gel filtration. This method, with modifications, can be used for the

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quantitative separation of free ASA and free SA (5). In the present paper the method and also the results of a comparative study on the efficiency of several other types of Sephadex is reported.

EXPERIMENTAL

Chemicals and Materials—The acetylsalicylic acid, ACS grade was obtained from City Chemicals Corporation and the salicylic acid used was Baker analyzed reagent. Sephadex G-25-coarse, G-25-medium, C-25, A-25, G-15, and G-10 were prepared by Pharmacia Fine Chemicals Inc.

Preparation of Column—The column was prepared in a special needle-type Pasteur pipet, 5 mm. i.d. The length of the pipet was about 3 cm. longer than the height of the gel bed. A small amount of Pyrex glass wool was placed in the bottom of the pipet and a slurry of well-soaked Sephadex was introduced into the column. The Sephadex was allowed to settle under gravity and the height of the column was adjusted. A long stainless steel wire was used to stir the column and assist in uniform packing. A standard number 19-G hypodermic needle was then placed on the tip of the Pasteur pipet. The column was washed with phosphate buffer (0.1 *M* at pH 7.4). Every eight drops of effluent is equal to 0.1 ml.

Fractionation of ASA and SA—Two-tenth milliliter of a sample containing freshly prepared ASA (2×10^{-4} *M*) and SA (8×10^{-4} *M*) in phosphate buffer was introduced to the column from a long tip pipet. The concentration of the salicylates used are within the range present in biological fluid. As soon as the sample had disappeared from the surface of the gel bed, phosphate buffer was introduced for elution. A fraction collector (GME product) with drop counter was used to collect 16-drop (0.2-ml.) portions of the effluent. The effluent in each tube was diluted to 4 ml. and assayed in the spectrophotofluorometer (Aminco-Bowman), using an activating wavelength of 305 mμ and detecting fluoresence at 405 mμ (4). All of the samples were saved in their original tubes. Acetylsalicylate does not fluoresce, but can be measured after hydrolysis. The hydrolysis was carried out by adding one drop of concentrated ammonium hydroxide to each sample and then heating in a boiling water bath for 5 min. After cooling to room temperature, the volume in each tube was readjusted to 4 ml. before assaying again in the fluorometer. For each type of Sephadex, the 100-mm. column was usually tried first and from the result obtained the decision was made to choose columns of other lengths. For each type of Sephadex the authors filled three Pasteur pipets for each column length. The types of Sephadex studied included G-25-medium (G-25-m), G-25-coarse (G-25-c), G-15, G-10, C-25 (carboxymethyl-Sephadex), and A-25 (DEAE-Sephadex).

RESULTS AND DISCUSSION

Sephadex G-25, with 100-mm. column length, as shown in Fig. 1, can separate ASA and SA into two distinct fractions, but the fractions are too close to each other. Two fractions that are farther separated as shown in Fig. 2, are of practical importance. For example, if one uses the 200-mm. column of G-25 (Fig. 2) one may just collect the first 4 ml. (the first 20 tubes) and the next 2 ml. (next 10 tubes) of the effluent in two separate tubes, which will contain exclusively ASA or SA, for analysis, either fluorometrically or colorimetrically (6).

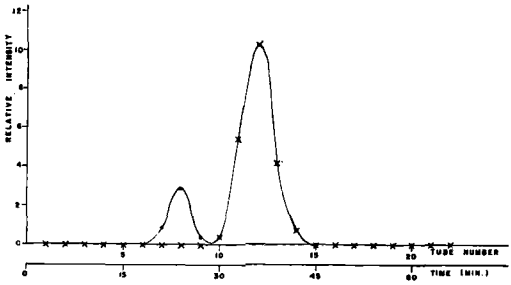

Fig. 1—Separation of ASA and SA by Sephadex G-25-m., 5 × 100 mm. Key: ×, SA before hydrolysis; O, after hydrolysis.

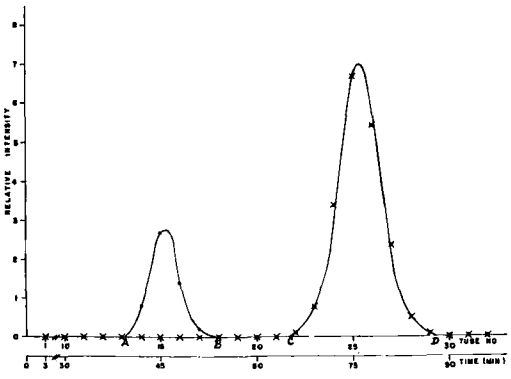

Fig. 2—Separation of ASA and SA by Sephadex G-25-m. 5 × 200 mm. Key: ×, SA before hydrolysis; O, after hydrolysis.

For the convenience of comparing resolution with all of the types of Sephadex of different column lengths studied, the results are summarized in Table I. Let A indicate the volume of effluent collected before the appearance of ASA (see Fig. 2). This volume should be much greater than the void volume (Vo) of the Sephadex bed used, if the biological fluid applied contains proteins. Plasma proteins combine with SA and they should be separated from free ASA. A small volume of effluent that comes out first, which is equivalent to the void volume, carries all of the plasma proteins, whether they are bound with drug or not. For the separation of free ASA and free SA in urine or in other nonprotein fluids, the volume of A is not important. Acetylsalicylic acid fraction is completely eluted at point B and SA begins to come out at point C and is completely eluted at point D. The difference between B and A and the difference between D and C represent the volumes of the effluent containing all of the ASA and SA, respectively. The magnitudes of (D − C) and (B − A) indicate the sizes of the fractions. The difference between C and B (C − B) should be large enough to insure the clear separation of ASA and SA fractions. Point D indicates the total volume of eluent required for the fractionation. This volume should not be too large in order

TABLE I—SEPARATION OF ASA AND SA BY DIFFERENT TYPES OF SEPHADEX GEL FILTRATION

Gel Type	Particle Size Diameter, μ	Bed Dimension, mm.	Volume of Effluent, ml., at[a] A	(Vo)	B	C	(C − B)	D	Time Required, min.
G-25-m	50–150	5 × 100	1.2	(0.79)	1.8	1.8	(0)	3.0	45
G-25-m	50–150	5 × 200	2.6	(1.57)	3.6	4.3	(0.7)	3.9	88
G-25-c	100–300	5 × 100	0.8	(0.79)	5.0	1.0	(−4)	—	—
G-15	40–120	5 × 50	0.6	(0.29)	1.4	1.4	(0)	2.6	12
G-15	40–120	5 × 100	1.2	(0.59)	2.2	3.6	(1.4)	5.8	33
G-10	40–120	5 × 50	0.6	(0.35)	1.9	2.8	(0.9)	7.2	36
G-10	40–120	5 × 100	0.8	(0.71)	2.4	5.8	(3.4)	11.6	76
C-25	40–120	5 × 100	1.2	—	2.0	2.0	(<0)	3.2	32
C-25	40–120	5 × 200	2.7	—	3.9	4.0	(0.1)	5.8	59
C-25	40–120	5 × 300	4.0	—	5.4	6.0	(0.6)	8.2	123
A-25	40–120	5 × 50	1.0	—	3.2	5.2	(2.0)	13.6	47
A-25	40–120	5 × 100	2.4	—	4.8	12.0	(7.2)	>16	—

[a] See Fig. 2 or text for denotations. Each milliliter is equal to the effluent collected in five tubes.

to avoid unnecessary dilution of the drugs and prolongation of fractionation time.

Sephadex G-10 and G-15 are less porous than G-25, and will effect a better resolution of low molecular weight substances. Indeed, Sephadex G-15 of a 100-mm. column and G-10 of only 50-mm. column separated two salicylates as well as G-25 with 200-mm. column length. Sephadex C-25 (cation exchanger) behaved just like G-25-m and Sephadex A-25 (anionic exchanger, DEAE Sephadex) separated salicylates most efficiently. A 50-mm. column of A-25 was sufficient for the separation.

It is always preferable to use a shorter column. The longer column has a slower flow rate and also it is difficult to keep the bed uniform. The flow rate of different types of gel with equal column length may not be the same. The Sephadex G-25-coarse has a higher flow rate, but it has lower resolution power. Both G-10 and A-25 only need 50-mm. column. Sephadex G-10 has a higher flow rate and A-25 gives better resolution.

The recovery of drugs was complete, since there is no irreversible retention of substance on the Sephadex column. When the amounts of ASA and SA were increased, the distribution of the drugs in the effluent was not changed, but the peaks of the fractions were increased. The time required for the filtration was very short and there was no detectable hydrolysis of ASA in the column. Sephadex gel filtration method for the separation of ASA and SA is very simple and practical.

REFERENCES

(1) Morgan, A. M., and Truitt, E. B. Jr., *J. Pharm. Sci.*, **54**, 1640(1965).
(2) Rowland, M., and Riegelman, S., *ibid.*, **56**, 717(1967).
(3) Cotty, V. F., and Ederma, H. N., *ibid.*, **55**, 837(1966).
(4) Potter, G. D., and Guy, J. L., *Proc. Soc. Exptl. Biol. Med.*, **116**, 658(1964).
(5) Lee, K. H., Unpublished data. Miles Medical Sciences Research Laboratory, Elkhart, Ind., 1964.
(6) Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol.*, **80**, 114(1944).

Keyphrases

Aspirin, salicylic acid—separation
Sephadex gels—aspirin, salicylic acid separation
Fluorometry—analysis