showed little or no effect. Higher levels of the *l* epimer showed an additive response over and above the response to the *d* epimer. If sufficient *l* epimer is fed, complete protection is achieved in the complete absence of the *d* epimer. Synergism and sparing action were not demonstrated in this experiment.

The synthetic antioxidant, DPPD, appeared to have approximately 10% of the bioactivity noted for *d*-α-tocopheryl acetate. The toxicity of DPPD in the pregnant rat has been reported by several groups(12-14).

Discussion. By the criteria of growth and delay in onset of creatinuria *l*-α-tocopheryl acetate appears to have a biopotency for the rat of up to approximately 20% of that of *d*-α-tocopheryl acetate. This is in agreement with the work of Ames *et al*(3) but somewhat lower than the value obtained by Bruggemann *et al*(4) and Weiser *et al*(5). Since neither synergism nor sparing action could be demonstrated in the present study, this would appear to represent a true biological activity for the *l* epimer.

The requirement for both *d* and *l*-α-tocopherol is similarly affected by the level of dietary selenium and methionine, an interaction which has been discussed in detail for the *d* epimer elsewhere(9).‡

Summary. The *l* epimer of α-tocopheryl acetate, appears to have approximately 20% of the biological activity of the *d* epimer in the rat by the criteria of growth and delay in onset of creatinuria as a sign of nutritional muscular dystrophy. The *l* epimer alone can replace *d*-α-tocopherol but only if sufficient is supplied. No evidence of synergism or sparing action was observed.

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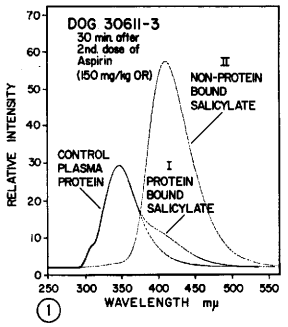
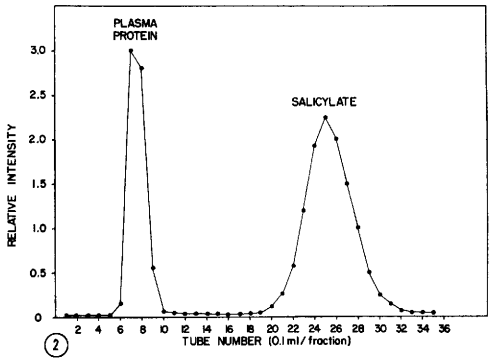
A Micro Method for Analysis of Plasma Salicylate. (29335)

GILBERT D. POTTER* AND JAMES L. GUY (Introduced by R. K. S. Lim)
Medical Sciences Research Laboratory, Miles Laboratories, Inc., Elkhart, Ind.

In recent years the use of dextran gel filtration for separation of large and small molecular weight compounds has come into widespread use(1-3). The cross linked dextran gel, (Sephadex) has also been applied to the study of protein-binding of drugs(4-6). The present report describes the application of this method in conjunction with fluorometric analysis of salicylates in plasma. Sephadex filtration eliminates the necessity

* Present address: Lawrence Radiation Laboratory, Univ. of California, Livermore.





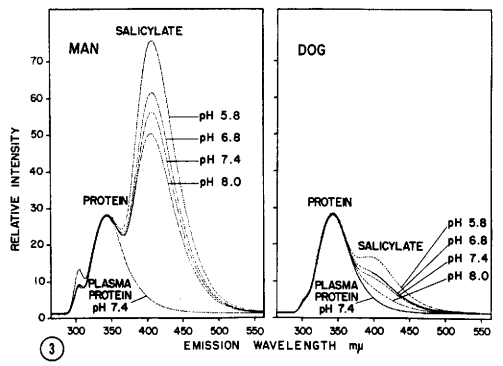


FIG. 1. Fluorescence emission spectra of Sephadex fractions I and II of dog plasma at pH 7.4 after oral aspirin.

FIG. 2. Separation of protein and salicylate in dog plasma using a 5 × 100 mm column of sephadex G25 eluted with phosphate buffer at pH 7.4 on fraction collector.

FIG. 3. Comparison of fluorescence emission spectra of fraction I (protein fraction) eluted from sephadex G25 column showing binding of salicylate in human and dog plasma at various pH values.

for solvent extraction of plasma(7) or other protein precipitation methods(8). In addition, it makes possible a rapid and simple determination of 1) protein-bound salicylate, 2) free salicylate, 3) acetylsalicylate, and 4) total salicylate in plasma.

Method. Citrated or heparinized blood samples are withdrawn from the subject, cooled, rapidly centrifuged, and the plasma removed for analysis.

Preparation of columns. The columns are prepared in special needle type Pasteur pipettes (Bellco), 5 mm inside diameter and 180 mm long. A small amount of pyrex glass wool is placed in the bottom of the tube and a slurry of Sephadex G25 medium is run into the column to 100 mm using a syringe. The Sephadex is allowed to settle and the height of the column adjusted by addition or removal of Sephadex to make a column of 100 mm. A long flattened stainless steel wire is used to stir the column and assist in uniform packing. A No. 19 syringe needle is then placed on the tip of the pipette. The columns are washed with phosphate buffers (0.1M) at the desired pH. All samples are run with 0.1M phosphate buffer at pH 7.4, except when the effects of varying hydrogen ion concentrations are studied.

Fluorometric analysis. The columns are placed in 12 ml graduated tubes. One-tenth ml of plasma is added to the column and then eluted with 1.4 ml of buffer into the first tube. This fraction(1) contains all the protein and protein-bound salicylate (Fig. 1). Fraction II, containing free salicylate, is then obtained by eluting with an additional 2 ml of buffer and collecting in a second graduated tube. The columns may be stored after filling with buffer and sealing with a No. 1 rubber vaccine vial cap; it may be used repeatedly until the flow rate becomes poor at which time stirring the column with the stainless steel wire usually improves the flow.

Fractions I and II are diluted to 4 ml in the graduated tubes and assayed in the Amin-co-Bowman spectrophotofluorometer using an activating wavelength of 305 mμ and detecting fluorescence at 405 mμ (cf. Chirigos and Udenfriend(9), activation at 310 mμ, fluorescense at 400 mμ). The method is sensitive to about 0.25 μg per ml. Acetylsalicylate does not fluoresce, but is detected only after hydrolysis. This is carried out by the addition of 0.1 ml conc. NH_4OH to the same 4 ml sample of fraction II as was used to measure free salicylate. The sample is then heated for

5 minutes in a boiling water bath, cooled to room temperature and readjusted to 4 ml before reading the fluorometer. Equilibrium dialysis studies(10) show that acetylsalicylate is not bound to protein and, therefore, would appear in fraction II.

The protein-bound salicylate (fraction I) fluoresces at the same wavelength (405 mμ) as free salicylate when activated at 305 mμ. However, native plasma protein also fluoresces when activated at this wavelength but exhibits maximal fluorescence at 345 mμ. There is a slight overlap of the protein and salicylate fluorescence spectra at 405 mμ, but this may be corrected by subtracting the protein (plasma) blank.

Results and discussion. Plasma salicylates determined by the above method were compared with values obtained, using a semimicro modification of the method of Brodie *et al*(7,11). The values for total salicylate found by both methods were comparable. At 4 and 10 mg%, the precision of the method is better than 98% and a comparison of the two methods on 74 samples of dog plasma showed no difference in the results (Sephadex 17.8 ± 0.7, Brodie 17.7 ± 0.5, $P>0.9$). Analyses on samples which had been repeatedly frozen and thawed showed no quantitative or qualitative differences.

Good separation of protein and salicylate in dog plasma was achieved using the 5 × 100 mm Sephadex column and eluting with phosphate buffer at pH 7.4. The results obtained from a fraction collector are shown in Fig. 2, in which the first peak represents plasma protein, and the second represents salicylate as measured by fluorescence using an excitation wavelength of 305 mμ and emission at 345 mμ for protein, and at 405 mμ for salicylate.

Fig. 3 illustrates the influence of hydrogen ion concentration on the extent of binding of salicylate by plasma protein of dog and of man, over a pH range of 5.8-8.0. The differences in binding of salicylate by human and dog plasma is quite striking, about 25-50% being bound at physiological pH 7.4 in human plasma, as compared with only 5% in dog plasma.

Summary. A spectrophotofluorometric method for determination of plasma salicylates is described in which a dextran-gel (Sephadex) filtration system is used instead of solvent extraction or protein precipitation for separation of non-protein-bound salicylate from plasma protein. This method permits rapid estimation of binding of drug to protein in addition to estimation of free, acetyl and total salicylate. At physiological pH very little salicylate is bound to the protein in dog plasma (<10%) whereas in the human, as much as 40-50% of the salicylate is bound at plasma levels of 20-25 mg%.

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