view of the large fecal thiamine loss encountered during oral thiamine treatment to administer the vitamin parenterally in those instances in which a rapid restoration of the blood thiamine level is desired.

It has not yet been established whether the excess thiamine observed in the stool after intake of commercial thiamine tablets is present in the bacteria or in the other fecal constituents; observations concerning this point would probably yield information of value. Thus if it can be shown that the excess thiamine in the stool under these conditions is non-filterable, this would suggest that the vitamin is incorporated in the bacteria and would presumably indicate that the intestinal bacteria had stolen the ingested thiamine. If on the other hand the thiamine can be recovered in the Seitz filtrate of the stool such finding would support the contention, recently proposed by Friedemann and his associates(3), that the intestine possesses a specific barrier against the absorption of thiamine.

Summary. The daily oral administration of 5 mg of thiamine hydrochloride in tablet form to 12 young and 21 old individuals was followed by an increase of the fecal thiamine excretion corresponding to one-half to three-fourths of the thiamine dose given. The fecal excretion of thiamine returned to the original level after the thiamine supplementation had been discontinued.

Received March 13, 1951. P.S.E.B.M., 1951, v77.

Chromatographic Separation of Estrone, Estradiol and Estriol.* (18816)

JOSEPH F. NYC, DOROTHY M. MARON, JOSEPHINE B. GARST, AND HARRY B. FRIEDGOOD. (Introduced by S. Roberts.)

From the Departments of Physiological Chemistry and of Medicine, University of California at Los Angeles.

Chief among the outstanding problems in clinical endocrinology is that concerned with the separation and quantitative estimation of the 3 natural estrogens. With the publication of Boldingh's(1) method for the use of rubber columns for the chromatographic separation of fatty acids it occurred to us that this technic might be adaptable to the foregoing problem. This communication consists of a report on the successful adaptation of rubber columns to the chromatographic separation of the steroids in question.

Physical characteristics of the column. The chromatographic tubes are 8 mm internal diameter and at least 20 cm in length. A short tip of approximately 3 mm diameter at the base of the tube is equipped with a piece of surgical rubber tubing ⅛ inch I.D. The flow of liquid through this portion of the tube is controlled by a small screw clamp. *Preparation of the rubber.* Pulverized mealorub rubber† is extracted with acetone in a continuous extractor for 24 hours. Thereafter, the rubber is air dried, moistened with methanol and ground in a mortar. It is then transferred to a 20-mesh sieve which is immersed in methanol. The particles which pass through the sieve are washed with the latter solvent and stored under it until used for chromatography.

Preparation of the column. Rubber columns are prepared in duplicate. These consist of a test column on which the estrogens are to be chromatogrammed and a blank control column through which only the solvents will be passed. The blank column which is submitted simultaneously to the same chromatographic pro-

* These studies were supported by a grant from the California State Department of Mental Hygiene, Langley Porter Clinic. We are indebted also to Ciba Pharmaceutical Products for estradiol and estrone, and to Parke, Davis and Co. for estriol.

1. Boldingh, J., *Rec. trav. chim.*, 1950, v69, 247.

† Vulcanized rubber powder obtained from Andresen Corp., Chicago.

TABLE I. Spectrophotometrically Determined Recovery of Chromatogrammed Estrogens.

Rubber column	Vol. of each solvent used, ml	% error for recovery of estrogens (400 μg each) Estriol μg	Estriol % error	Estradiol μg	Estradiol % error	Estrone μg	Estrone % error
1	20	370	— 8	393	—2	400	0
2		434	+ 9	417	+4	440	+10
3		353	—12	378	—6	392	— 2
4		356	—11	389	—3	403	+ 1
5	25	410	+ 3	408	+2	407	+ 2
6		440	+10	411	+3	384	— 4
7		420	+ 5	424	+6	404	+ 1
8		398	— 1	413	+3	387	— 3

cedures as those employed for the test column, provides the values on the basis of which corrections are made for solvents, rubber, etc. 1.6 g of rubber per column, prepared as described above, is shaken intermittently for 5 minutes with 35 ml of 10% aqueous methanol (10 parts methanol with 90 parts water, v/v). The resulting suspension is filtered through a fine mesh screen, and the retained particles are resuspended in 70 ml of 10% aqueous methanol saturated with benzene.‡ After adding 2 ml of benzene to this rubber suspension, and shaking the mixture intermittently for 5 minutes, it is poured slowly into the chromatographic tube. A glass bead resting on the constriction at the bottom of the tube allows the solvent to drain through the tube and at the same time prevents rubber particles from passing into the receiver. The rubber column is kept covered with solvent while it is being packed as well as during all of the succeeding elution procedures. As the rubber particles settle in the tube they are tamped gently with a stirring rod to insure their uniform packing. At this stage the rubber ordinarily fills the chromatographic tube to a height of about 12 to 13 cm. A glass wool plug is placed on the surface of the rubber column and then tamped with sufficient force to compress the column to a height of 11.5 ± 0.5 cm. The column is now drained until the surface of the solvent is just below the glass wool plug. The three estrogens, dissolved in 0.2 ml of methanol, are discharged into the glass wool from a micro pipette. The column is again allowed to drain until the sample solution passes from the glass wool into the upper part of the rubber column. An identical procedure is followed for the blank column, substituting 0.2 ml methanol for the sample solution. In addition to chromatogramming the 3 estrogens as a mixture, they were also treated in the same manner individually.

Selective elution of the column. It was found that the rubber columns, as defined, would handle 400 μg of each of the 3 crystalline estrogens, *i.e.*, a total of 1200 μg when these steroids were chromatogrammed as a mixture. These amounts are within the range of accurate determination spectrophotometrically(2). Twenty or 25 ml each of 20, 40 and 60% aqueous methanol (v/v) were passed successively through the column (Table I) at a rate of 0.4 ml per minute at 25°C. The total volumes of aqueous methanol eluate were collected in 12 or 15 successive 5 ml fractions from the bottom of the column as it developed. The number of fractions collected depended on the original total amount of the eluant. In the course of chromatogramming the mixture of the three crystalline estrogens, estriol is eluted by the first solvent passed through (20% aqueous methanol); estradiol by the second solvent (40% aqueous methanol); and estrone by the third solvent (60% aqueous methanol) when 20 ml of each solvent is used. The observations made as to the order of elution of the estrogens when they were chromatogrammed individually were confirmed by subjecting these steroids to the color tests developed by Bachman(3), Szego and Samuels(4), and the Zimmermann as modified by Friedgood, *et al.*(5). When 25 ml

‡ Redistilled, thiophene-free.

2. Friedgood, H. B., Garst, J. B., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1948, v174, 523.

3. Bachman, C., *J. Biol. Chem.*, 1939, v131, 463.

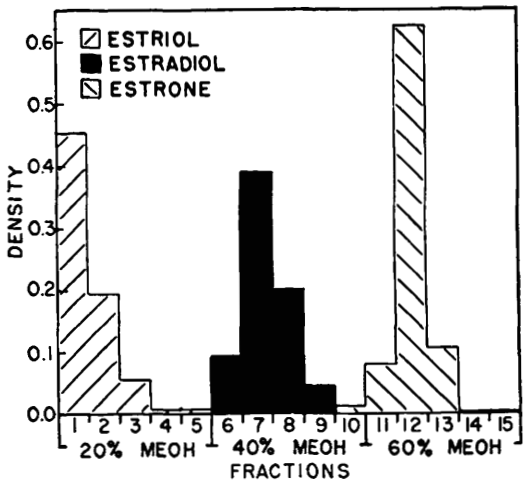

FIG. 1. Corrected densities at 280 mμ for fractions obtained from chromatographic column No. 5 (Table I). Each fraction represents 5 ml of eluant.

$$\text{Density} = \log \frac{I_o}{I_x}$$

of each eluant is passed through the column, fractions 1 through 5 contain estriol; fractions 6 through 9 contain estradiol, and fractions 10 through 15 contain estrone. In order to determine the nature of the estrogen in fraction 10 (Fig. 1), estrone and estradiol were chromatogrammed separately. It was found that fraction 10 contains only estrone. This fraction was therefore grouped with fractions 11 through 15, containing the major part of this estrogen. In view of the fact that crystalline estrogens are more soluble in 80% aqueous methanol than in 20, 40 or 60% concentrations, the columns were treated subsequently with 80% aqueous methanol in an attempt to determine whether or not a measurable quantity of estrogen remained uneluted. Within a few minutes after the addition of the 80% aqueous methanol, the rubber contracted visibly, coincidentally with the release of the bound benzene in the column. Spectrophotometric analysis of the eluate that was collected before and after contraction of the column failed to disclose a measurable quantity of any of the 3 estrogens.

4. Szego, C. M., and Samuels, L. T., *J. Biol. Chem.*, 1943, v151, 587.

5. Friedgood, H. B., and Whidden, H. L., *Endocrinology*, 1940, v27, 249.

Analysis of eluted fractions. Each of the foregoing 12 or 15 fractions was taken to dryness and dissolved in 4 ml 95% ethanol. The ultraviolet absorption of each fraction was determined at 280 mμ in a Beckman spectrophotometer. These values are shown in Fig. 1 and 2. Table I shows the per cent error for the recovery as determined by spectrophotometric analysis of the 3 estrogens eluted from rubber columns as described above. It is clear from these data that known amounts of a mixture of the three natural estrogens can be separated sharply from each other and estimated quantitatively within an experimental error of approximately ± 10%. These data have demonstrated, moreover, that one can determine the identity of an unknown estrogen by the concentration of the aqueous methanol which will remove it from a rubber column.

It should be noted that the use of the rubber column described above is not limited to the amount of estrogenic material employed in the present investigation. The accuracy with which various amounts of estrogens can be recovered from these columns and determined quantitatively will be dependent largely on the accuracy and sensitivity of the assay method employed. This chromatographic procedure can be applied to the separation of both larger and smaller quantities of estrogen by appropriately varying the amounts of rubber and solvent employed.

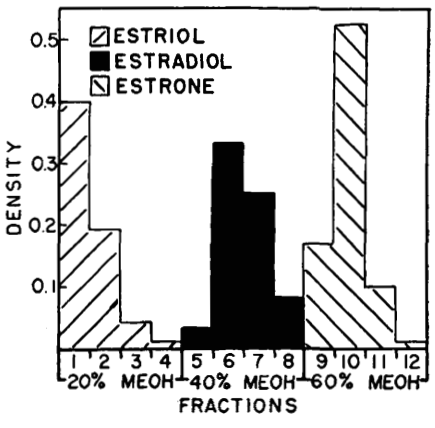

FIG. 2. Corrected densities at 280 mμ for fractions obtained from chromatographic column No. 1 (Table I). Each fraction represents 5 ml of eluant.

Summary. A column of pulverized Mealorub rubber has been adapted successfully to the quantitatively accurate chromatographic separation of a mixture of crystalline estrone, estradiol and estriol. When 20 ml each of 20, 40 and 60% aqueous methanol (v/v) are passed successively through the column, estriol is eluted by the 20%; estradiol by the 40%, and estrone by the 60% concentration of methanol.

Received May 3, 1951. P.S.E.B.M., 1951, v77.

A Non-specific Brucella Agglutinating Substance in Bovine Serum.*† (18817)

WILLIAM R. HESS‡ AND MARTIN H. ROEPKE.

From the Division of Veterinary Medicine, University of Minnesota, St. Paul.

In evaluating the results of the serum agglutination test for brucellosis in cattle, animals with titers ranging from incomplete agglutination at the 1:50 dilution to incomplete at 1:100 are designated as suspects, and herds containing one or more such animals, with all other animals negative to the test, are considered as suspicious or suspect herds. Animals with higher titers are considered infected and are designated as reactors. In a brucellosis control plan in Minnesota which is based on county areas and requires periodic testing of all the cattle in the controlled counties, a ratio of approximately two suspect herds to three reactor herds is usually disclosed. In counties where the percent of infection is very low, the number of suspect herds may exceed appreciably the number of reactor herds. Moreover, the majority of suspicious herds are so classified because of the presence of a single suspect animal. These observations coupled with the fact that approximately 85% of the animals displaying suspicious titers in suspect herds are found to be negative to subsequent retests have suggested that non-specific agglutinins may be responsible for a large portion of these reactions.

In the course of trying to demonstrate nonspecific agglutinins for Brucella a filter paper chromatographic technic was devised that made possible the detection of an agglutinating substance capable of reacting with Brucella test antigen but differing markedly from specific or high-titered agglutinins in its affinity for adsorption on cellulose. The test was based on two recently published observations; first, Franklin and Quastel(1) showed that certain proteins together in solution could be separated by filter paper chromatography when the proper developing solution and a means of localization of the fractions were employed; and second, Castaneda(2) observed that Brucella agglutinating serums were capable of fixing a stained Brucella antigen to the surface of filter paper.

Materials and methods. Filter paper. The paper used in this test was S & S 589, White Ribbon. It was cut in strips 3½ inches long and ¾ of an inch in width. *Developing solution.* The solution found to give the best results was potassium acid phthalate (0.05 M) adjusted to pH 6.2 with sodium hydroxide. *Antigen.* A hematoxylin-stained Brucella antigen prepared according to the method of Roepke(3) for use in the milk and cream ring test was used exclusively in the test. The antigen was found to give better results if the

* Paper No. 2296—Scientific Journal Series, Minn. Agric. Exp. Station, University of Minnesota.

† These studies were made possible by a grant from the Bureau of Animal Industry, U. S. Department of Agriculture.

‡ Part of thesis submitted by Mr. Hess to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology and Immunology.

1. Franklin, A. E., and Quastel, J. H., *Science*, 1949, v110, 447.

2. Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 46.

3. Roepke, M. H., *Brucellosis*, A.A.A.S., Washington, D. C., pp. 127, 1950.