complex of amphotericin B) on growth of established cell lines and chick fibroblasts in monolayer and suspension culture systems, the fungistatic concentration of 2.5 μg/ml was found to have little or no effect on the multiplication of the tissue cultures grown in serum-containing media. This concentration was tolerated by all cultures. Inactivation of Fungizone in tissue culture media was also determined.

1. McLimans, W. F., Bonissol, C., Davis, E. V., Rake, G., in *Antibiotics Annual*, 1955-1956, 690, New York, Medical Encyclopedia, Inc.

2. Bartner, E., Zinnes, H., Moe, R., *ibid.*, 1956-1957, 866.

3. Dutcher, J. D., Gold, W., Pagano, J. F., Vandeputte, J., U. S. Patent 2,906,611 (1959).

4. Gold, W., McKee, G. M., Pagano, J. F., Donovick, R., in *Antibiotics Annual* 1955-1956, 579, New York, Medical Encyclopedia, Inc.

5. Hemphill, J. J., Herman, Y. F., Young, V. M., *ibid.*, 1957-1958, 961.

6. Eagle, H., *Science*, 1955, v122, 501.

7. Ziegler, D. W., Davis, E. V., Thomas, W. J., McLimans, W. F., *Appl. Microbiol.*, 1958, v6, 305.

8. Waymouth, C., *J. Nat. Cancer Inst.*, 1959, v22, 1003.

9. Perlman, D., Giuffre, N. A., Jackson, P. W., Giardinello, F., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 290.

10. Gerke, J. R., Madigan, M. E., *Antibiot. and Chemother.*, 1961, in press.

11. Perlman, D., Giuffre, N. A., unpublished.

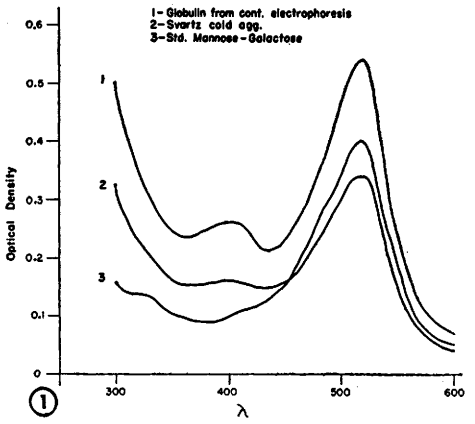
Received January 20, 1961. P.S.E.B.M., 1961, v106.

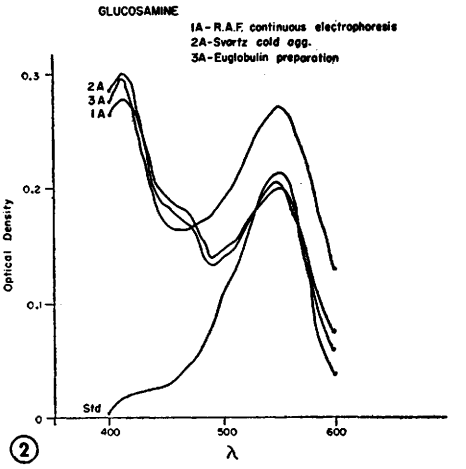
Carbohydrate Content of Euglobulins of Normal and Rheumatoid Sera. (26510)

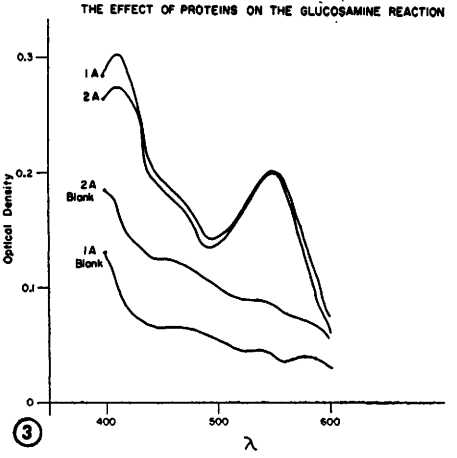
WILLARD R. STARNES (Introduced by Emmett B. Carmichael)
Metabolic Research Laboratories, Veterans Administration Hospital, Birmingham, Ala.

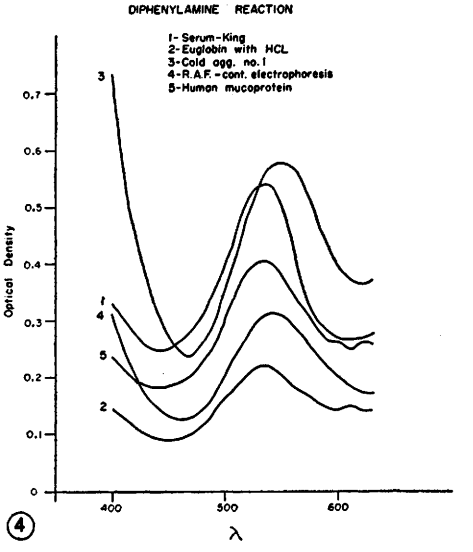
The term "rheumatoid factor" has been applied to the protein component of serum which is responsible for a group of agglutination and precipitation reactions, some of which are very important in diagnosis of rheumatoid arthritis. Up to the present, analytical studies have indicated that the rheumatoid factor is a typical 19-S gamma globulin with chemical, physical and immunological properties closely akin to 19-S antibodies(1). The present studies were undertaken to isolate euglobulins by different methods and study the carbohydrate composition. Franklin, Muller-Eberhard and Kunkel isolated and characterized, by various methods, a refined and more purified "rheumatoid factor" preparation upon which they determined the various carbohydrate fractions(1). Others have also achieved considerable purification by other technics(2,3).

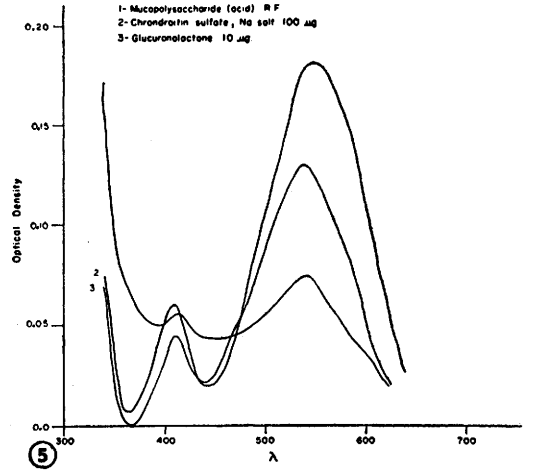
Materials and methods. Known normal pooled sera and pooled sera with a positive latex agglutination were submitted to 3 different methods for isolation and purification of the euglobulin fractions. (1) *Precipitation with 0.0027 N hydrochloric acid:* Test sera were inactivated by heating at 56°C for one-half hour. In a 100 ml cellulose nitrate tube, one volume of clear serum was diluted by the slow addition (with continuous rotation of the tube) of 9 volumes of 0.0027 N hydrochloric acid from a 50 ml burette. A pH within the range of 6.1 to 6.4 was thereby attained. The tubes were immediately placed in a refrigerated centrifuge at 4 to 8°C for 30 to 60 minutes. The euglobulin precipitate then was centrifuged in the cold at 3,000 rpm for 10 minutes. The clear supernate was discarded by decanting from the firmly packed pellet, and last traces of the supernate were removed either by draining the tube inverted over filter paper or by wiping the inner wall thoroughly with a gauze sponge. The precipitate was not washed. The concentrated euglobulin was then dissolved in an added 0.5 ml of 0.9% NaCl solution, (2) Svartz cold agglutination, (3) and isolation and purification by continuous electrophoresis in veronal buffer 0.05 M pH 8.6. The euglobulin was subjected to 3 separations on

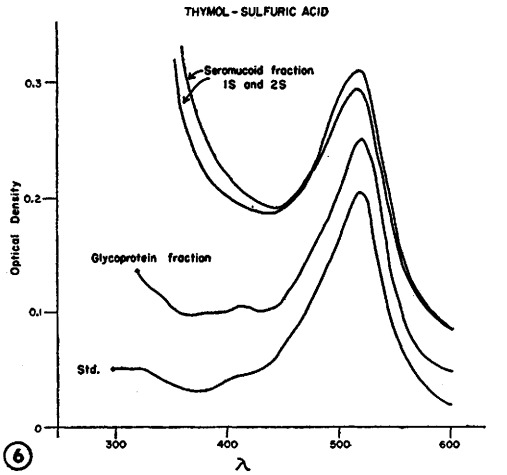

1- Globulin from cont. electrophoresis
2- Svartz cold agg.
3- Std. Mannose - Galactose
Optical Density
λ
1
2
3


GLUCOSAMINE
1A - R.A.F. continuous electrophoresis
2A - Svartz cold agg.
3A - Euglobulin preparation
Optical Density
λ
2A
3A
1A
Std


THE EFFECT OF PROTEINS ON THE GLUCOSAMINE REACTION
Optical Density
λ
1A
2A
2A Blank
1A Blank


DIPHENYLAMINE REACTION
1- Serum - King
2- Euglobin with HCL
3- Cold agg. no. 1
4- R.A.F. - cont. electrophoresis
5- Human mucoprotein
Optical Density
λ
3
1
4
5
2


1- Mucopolysaccharide (acid) R F
2- Chrondroitin sulfate, Na salt 100 µg
3- Glucuronolactone 10 µg
Optical Density
λ
2
3


THYMOL - SULFURIC ACID
Seromucoid fraction
1S and 2S
Glycoprotein fraction
Std.
Optical Density
λ

the Spinco continuous electrophoretic apparatus. Following euglobulin isolation all samples were tested with latex(4). Each euglobulin was then subjected to paper electrophoresis for protein and carbohydrate content on a Spinco Model R electrophoretic apparatus using veronal buffer 0.05 M, pH 8.6. All the above samples were gamma globulin in nature with the exception of one prepared with hydrochloric acid which contained 1.5% beta globulin.

The following tests were made for carbohydrate:

(1) Total protein bound hexose (T.P.B.H.). The thymol-sulfuric acid reaction(5). Isolation of acid mucopolysaccharide by the method of Bollet(6). (2) Hexosamine by a modification of the Elson-Morgan method(7). (3) Sialic acid by the method of Pigman, *et al.*(8). (4) Acid mucopolysaccharides by the carbazole method of Dische(9).

Results and discussion. In Fig. 1, 2 of the euglobulin preparations are compared with a mannosegalactose standard of 40 μg (20 μg of each). The 2 globulins exhibit a typical peak in the 420 mμ range which is due to presence of protein. Carbohydrate moiety shows maximum absorption at 520 mμ. This is in agreement with Shetlar and Masters(5) who found the protein curve absorbs more in the 420-440 mμ range of the curve. Absorption curves obtained when sulfuric acid reacts with protein (in absence of thymol) has some absorption at 420 mμ, most of the effect is apparently due to the reaction of sulfuric acid with proteins and does not involve the reaction with thymol.

In Fig. 2 the 3 different preparations were compared with a standard of glucosamine hydrochloride, maximum absorption being the same as glucosamine at 550 mμ. The increase in optical density from 400 to 450 mμ was due to the hydrolytic products of protein. This could be reduced greatly if hydrolyzed proteins were used as blanks. No attempt was made to purify further the glucosamine hydrolyzates because the use of the blank corrected for the increase in optical density (Fig. 3).

In Fig. 4 the euglobulin fractions were examined for their sialic acid content, utilizing the diphenylamine reaction. Specimen No. 5 was purified human glycoprotein preparation which contained 12% sialic acid. Also included was a serum to serve as a control for the reaction which was found to contain 114 mg% of sialic acid. All of the euglobulins exhibited a peak at 530 mμ as did the serum and the purified human mucoprotein fraction with the exception of No. 3 which was a cold agglutination prepared by the method of Svartz(3). This was repeatedly checked and

FIG. 1. Absorption spectrum of total protein-bound hexoses in thymol-sulfuric acid. (1) Euglobulin from continuous electrophoresis. Total protein bound hexoses, 49.8 μg. (2) Euglobulin prepared by Svartz cold agglutination method. Total protein bound hexoses, 34.3 μg. (3) Mannose-galactose standard 40 μg (20 μg each of mannose and galactose).

FIG. 2. Glucosamine absorption spectrum of euglobulin preparations. (1A) Euglobulin isolated from latex positive serum by continuous electrophoresis. Glucosamine, 53 μg. (2A) Euglobulin isolated from latex positive serum by Svartz cold agglutination method. Glucosamine, 57 μg. (3A) Euglobulin from latex negative serum. Glucosamine, 74 μg. (4) Standard (Std.). Glucosamine, 60 μg.

FIG. 3. The effect of proteins on the glucosamine reaction. 1A, 2A. Euglobulins isolated by continuous electrophoresis and Svartz cold agglutination method respectively—containing 53 μg and 57 μg. Read against Reagent Blank. 1A, 2A Blank. Hydrolytic effect of acid on proteins using distilled water as a blank.

FIG. 4. Diphenylamine reaction for sialic acid. (1) Untreated serum control, .57 mg sialic acid. (2) Euglobulin isolated with HCl, .23 mg sialic acid. (3) Svartz cold agglutination euglobulin, .56 mg sialic acid. (4) Euglobulin isolated by continuous electrophoresis, .29 mg sialic acid. (5) Mucoprotein, purified (human), .52 mg sialic acid.

FIG. 5. Carbazole reaction for uronic acids. (1) Mucopolysaccharide (acid) R.F. isolated from latex positive euglobulin, 5.1 μg. (2) Chrondroitin sulfate, sodium salt, 100 μg. (3) Glucuronolactone, 10 μg.

FIG. 6. Absorption spectrum of purified glycoprotein and seromucoid fractions utilizing the thymol-sulfuric acid reaction. (1S) Seromucoid fraction isolated with phosphotungstic acid from a latex positive euglobulin, 60.5 μg. (2S) Seromucoid fraction isolated with protamine sulfate from a latex positive euglobulin, 57.6 μg. Glycoprotein fraction isolated with perchloric acid from a latex positive euglobulin. Std. = Mannose-galactose standard, 40 μg.

TABLE I. Protein and Carbohydrate Composition of Euglobulins.

Euglobulin preparations	Total protein, g %	Total protein bound hexoses, mg %	Glucosamine, mg %	Sialic acid, mg %
Continuous electrophoresis	2.7	84.8	78.0	29.1
.0027 N HCl, pH 6.1-6.4	1.2	68.4	33.5	23.3
Cold agglutination (Svartz)	2.9	86.1	76.5	55.9
Normal euglobulin (10 donors)	1.2	33.6	18.3	16.5
Whole serum (10 donors)	7.9	182.0	—	114.0

found to show maximum absorption at 550 mμ.

When the carbazole reaction was applied to a product isolated by classical procedures for acid mucopolysaccharide-like substance (Fig. 5) it was found to have maximum absorption at 540 mμ the same as glucuronolactone and chondroitin sulfate. The cold agglutination euglobulin used for isolation of the acid mucopolysaccharide contained 144.0 mg of total protein with a total protein bound hexose of 5.6 mg. This yielded 10.2 μg of acid mucopolysaccharide calculated as chondroitin sulfate. Protamine gave no color with the carbazole reaction, confirming the results observed by Bollet(6). Badin and Schubert added chondroitin sulfate to plasma and found it in the euglobulin fraction(10).

Badin and Schubert(10) isolated euglobulin with a phosphate-citrate buffer, then precipitated the uronic acid with lysozyme. If the above is chrondoitin sulfate it is not in agreement with Badin and Schubert as they obtained higher values in rheumatoid euglobulins possibly due to the method of isolation of the acid mucopolysaccharide-like substance.

No attempt was made to identify the acidic component because of the limited amount present. Using the procedure of Bollet(6) and the carbazole method for uronic acids it appeared to be a polysaccharide containing uronic acid which exhibits the characteristics of chondroitin sulfate. In the separation of the uronic acid-containing mucopolysaccharides, the positive latex reaction was lost following reprecipitation of the euglobulin with 1.8 M perchloric acid probably due to denaturation. In checking each component from the fractionation of the acid mucopolysaccharide a loss of 14.1 mg of protein and 1.6 mg total protein bound hexose was encountered. The supernatant from the citric-phosphate buffer was dialyzed against tap water for 24 hours and 0.5 ml of 18% phosphotungstic acid was added to obtain a seromucoid fraction. The precipitate was washed with cold ethanol, then dissolved in 5.0 ml containing 1.0 ml of 1% sodium carbonate and 4.0 ml phosphate-saline buffer pH 8.2. This gave a protein high in glycoprotein material with a hexose:protein ratio at 1:5 whereas the original euglobulin was 1:25. The seromucoid fraction gave a positive thymol-sulfuric reaction similar to those observed with the original euglobulin total protein bound hexose.

In Fig. 6, "1 S" represents the seromucoid fraction treated with phosphotungstic acid and "2 S" is the same except prior treatment with protamine sulfate for precipitation of the uronic acid-containing mucopolysaccharide. The glycoprotein curve represents the precipitate following initial treatment with 0.14 M NaCl in 0.02 M NaOH and 1.8 M perchloric acid. The standard is made up with equal quantities of mannose and galactose.

Table I gives results of several analytical methods used on the various euglobulin fractions that we have isolated. The percentage of total protein bound hexoses, glucosamine and the sialic acid present in each of the euglobulin fractions is given, with mg% of each. The fractions isolated were gamma globulins by paper electrophoretic means. When these values were compared with those of normal euglobulin a marked elevation was observed.

Summary. 1. Euglobulins were prepared by 3 different methods from "latex positive" pooled serum. 2. Total protein bound hexoses, glucosamine, and sialic acid were measured on each euglobulin preparation. All were some-

what similar with the exception of the euglobulin prepared with hydrochloric acid, which had a higher per cent of hexose to protein. All showed marked increase in content when compared with euglobulins isolated from a pooled *"latex negative"* normal serum. 3. Paper electrophoretic examination reveals proteins that migrate as gamma globulins and are highly PAS positive. 4. A uronic acid-containing mucopolysaccharide was isolated from the cold agglutinating euglobulin.

1. Franklin, E. C., Holman, H. R., Muller-Eberhard, H. J., Kunkel, H. G., *J. Exp. Med.*, 1957, v105, 425.

2. Kunkel, H. G., Franklin, E. C., Muller-Eberhard, H. J., *J. Clin. Invest.* 1959, v38, 424.

3. Svartz, N., *Ann. Rheumat. Dis.*, 1957, v16, 441.

4. Singer, J. M., Plotz, C. M., *Am. J. Med.*, 1956, v21, 88.

5. Shetlar, M. R., Masters, Y. F., *Anal. Chem.*, 1957, v29, 402.

6. Bollet, A. J., Seraydarian, M. W., Simpson, W. F., *J. Clin. Invest.*, 1957, v36, 1328.

7. Winzler, R. J., *Methods of Biochemical Analysis*, 1955, v2, 279, Interscience Publishers, N. Y.

8. Pigman, W., Hawkins, W. L., Blair, M. G., Holley, H. L., *Arthritis and Rheumatism*, 1958, v1, 151.

9. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.

10. Badin, J., Schubert, M. J., *J. Clin. Invest.*, 1955, v34, 1317.

Received January 30, 1961. P.S.E.B.M., 1961, v106.

Estrogens and Progesterone in the Sea Urchin (*Strongylocentrotus franciscanus*) and Pecten (*Pecten hericius*).* (26511)

CHARLES R. BOTTICELLI, FREDERICK L. HISAW, JR.,† AND HERBERT H. WOTIZ‡
(Introduced by Frederick L. Hisaw)
Biological Laboratories, Harvard University, Cambridge, Mass., Department of Biochemistry, Boston University School of Medicine, and University of Washington, Friday Harbor Marine Station

Estradiol-17β, estrone, and progesterone have been obtained, in vertebrates other than mammals, from the ovaries of dogfish(1), while estradiol, estrone, and estriol have been found in the ovaries of laying hens(2) and lungfish(3). Extracts of gonads from many invertebrates have produced estrogenic effects in mammals(4,5), but the chemical identity of the active substances is mostly unknown. Only recently estradiol and progesterone have been identified in the ovaries of the starfish(6). This report presents the results of an effort to isolate and identify possible estrogenic steroids and progesterone from ovarian tissue of 2 other representative invertebrates: the sea urchin, *Strongylocentrotus franciscanus*, and the mollusk, *Pecten hericius*.

* Aided by grants from Nat. Science Foundation and U.S.P.H.S.

† Dept. of Zoology, Oregon State College, Corvallis.

‡ Senior Research Fellow, U.S.P.H.S.

Methods and results. The ripe ovaries (3-5 kg) of each species were collected and stored in approximately twice their volume of acetone. The acetone extract was evaporated to an oily residue, taken up by 70% ethanol and extracted 3 times with petroleum ether. Next, the ethanolic fraction was diluted with water to contain 35% of alcohol and washed with petroleum ether 3 times. These 35% ethanolic fractions were tested for estrogenic action by the Astwood method, as previously described(7,8), and all gave positive results, although it was evident that only low concentrations of hormone were present. The ethanolic fraction was then dried *in vacuo* and the residue partitioned countercurrently between 70% methanol and a 50-50 mixture of chloroform and carbon tetrachloride with 29 transfers. In brief, those tubes corresponding to authentic estradiol, estrone, and estriol were pooled and bioassayed. The results (Table I) indicated estrogenic activity only in material obtained