17. Hublé, J., Acta Endocrinol., 1957, v25, 59.
18. Moscona, M. H., Karnofsky, D. A., Endocrinology, 1960, v66, 533.
19. Blodgett, F. M., Burgin, L., Iezzoni, D., Gribetz, D., Talbot, N. D., New Engl. J. Med., 1956, v254, 636.
20. Blizzard, R. M., Wilkins, L., Arch. Int. Med., 1957, v100, 729.
21. Becks, H., Simpson, M. E., Li, C. H., Evans, H. M., Endocrinology, 1944, v34, 305.
22. Baker, B. L., Ingle, D. J., ibid., 1948, v43, 422.
23. Ducommun, P., Acta Endocrinol., 1950, v4, 343.
24. Winter, C. A., Silber, R. H., Stoerk, H. C., Endocrinology, 1950, v47, 60.
25. Bodansky, O., Money, W. L., ibid., 1954, v55, 173.
26. Cortesi, M., Rezza, E., Bruni, G., Farmaco, (Pavia), Ed. Sci., 1958, v13, 790.
27. Pratesi, V., La Cauza, C., Bartolozzi, G., Riv. Clin. Pediat., 1960, v66, 136.
28. Laron, Z., Ben-Zion, A., Kende, S., Endocrinology, 1963, v72, 470.
29. Karnofsky, D., A.M.A. Conference on Comparison of the Biological Properties of Steroids and Hormones, 1954, 218.
30. Warner, N. L., Burnet, F. M., Australian J. Exp. Biol. Med. Sci., 1961, v39, 235.
31. Faludi, G., Mills, L. C., Chayes, Z. W., Acta Endocrinol., 1964, v45, 68.
32. Faludi, G., Gotlieb, J., Meyers, J., Mills, L. C., Clin. Res., 1962, v10, 22.
33. Fielder, F. G., Hoff, E. J., Thomas, G. B., Tolksdorf, S., Perlmàn, P. L., Cronin, M. T. I., Toxicol. Appl. Pharmacol., 1959, v1, 305.
34. Tonelli, G., Haynes, J., Heyder, E., Ringler, I., Proc. Soc. Exp. Biol. and Med., 1964, v116, 501.
35. Stafford, R. O., Barnes, L. E., Bowman, B. J., Meinzinger, M. M., Endocrinology, 1955, v89, 371.
36. Finney, D. J., Statistical Method in Biological Assay, Charles Griffen & Co., Ltd., London, 1952.
37. Bliss, C. I., The Statistics of Bioassay, Academic Press, Inc., New York, 1952.
38. Ringler, I., Brownfield, R., Endocrinology, 1960, v66, 900.
39. Steelman, S. L., Morgan, E. R., Inflammation and Diseases of Connective Tissue, W. B. Saunders Co., Philadelphia, 1961, 349.
40. Van Metre, T. E., Nierman, W. A., Rosen, L. J., J. Allergy, 1960, v31, 531.
41. Decanay, E. P., Van Metre, T. E., ibid., 1963, v33, 259.
42. Dubois, E. L., Bulgrin, J. G., Jacobson, G., Inflammation and Diseases of Connective Tissue, W. B. Saunders Co., Philadelphia, 1961, 847.

Received February 10, 1965. P.S.E.B.M., 1965, v119.

Biological Properties of Guinea Pig Anti-Insulin Antibodies.* (30121)

JOSUÉ M. CORCOS† AND ZOLTAN OVARY‡
Department of Medicine, New York Medical College, and Department of Pathology, New York University School of Medicine, New York

Insulin is known to be antigenic in man (1,2) as well as in animals(3,4). It was recently shown that guinea pigs may produce two types of 7S gamma globulin antibodies against the same antigen with different biological properties(5-8). It seemed of interest to determine if similar antibodies could be produced when guinea pigs were immunized with beef insulin. Moreover, an attempt was made to establish whether the oxidized B-chain of insulin had the characteristic antigenic determinants of the entire intact insulin molecule.

Materials and methods. Antigen. The insulin used in immunization of the guinea pigs consisted of zinc crystalline beef insulin (Eli Lilly Lot No. 535664).

Iletin, a mixture of zinc crystalline beef and pig insulin, was used in the serological reactions for characterization of antibodies.

Insulin B chain was kindly provided by Dr. William Konigsberg, Yale University, New Haven, Conn. in the form of pure oxidized B chain and prepared according to Craig *et al*(9).

Animals. Hartley strain albino guinea pigs

* This investigation was carried out under the support of Grant AI 03075 of Nat. Inst. Health.
† Career Scientist, Health Research Council of City of New York, under contract #I-415.
‡ Career Scientist, Health Research Council of City of New York, under contract #I-140.

of either sex weighing 300-500 g were used for immunization as well as for passive cutaneous anaphylaxis (PCA)(10).

Immunization. Anti-bovine insulin sera were obtained from 9 guinea pigs immunized in the following manner: An emulsion of insulin in equal parts in Freund's complete adjuvant (Difco Lab Inc., Detroit), containing 0.5 mg/ml of insulin was injected in the foot pads of the animals in 0.125 ml amounts (0.250 mg of insulin per guinea pig). This was followed by an intraperitoneal injection of 5 ml of a 5% glucose solution in water, repeated 3 times at 4-hour intervals. In preliminary experiments some animals died of hypoglycemic shock in the absence of glucose injections.

Two weeks later, the animals were injected intradermally with 0.1 ml of a 1 mg/ml solution of insulin in saline in 4 sites of their freshly-shaven back. This procedure was repeated once a week for 3 weeks and the animals were exsanguinated 7 days after the last intradermal injection under nembutal anesthesia. The sera were kept frozen until used. All antisera were tested by PCA reaction, double-diffusion in agar gel, immunoelectrophoresis, hemagglutination and passive hemolysis.

Sheep cells (Probio, Inc., New York) kept in Alsever's solution were used a few days after bleeding.

Veronal buffer as described in Kabat and Mayer(11) was used throughout in complement fixation experiments.

Complement. Lyophilized guinea pig serum (Hyland Laboratories, Calif.), was reconstituted in water to the original volume and adsorbed by packed sheep cells for 10 minutes in an ice bath, centrifuged and used at a dilution of 1/10 in veronal buffer.

Double-diffusion in agar gel was carried out as described by Ouchterlony(12).

Immunoelectrophoresis. The modified method of Scheidegger(13) was used. Electrophoresis was performed in 1.2% agar gel in phosphate buffer, pH 7.6, 0.05 ionic strength.

After completion of electrophoresis, insulin in saline 0.050 to 0.100 mg/ml was placed in the troughs and the plates were left in a humid chamber for 24 to 36 hours at room temperature. The gels were washed for 24 to 36 hours in physiologic saline which was changed several times, dried, and stained for up to an hour, and the non-specific staining was removed in a decolorizing bath as described by Grabar and Burtin(14).

Zone electrophoresis on starch was carried out as described by Kunkel(15) using veronal buffer pH 8.6 ionic strength 0.1. It was performed for 24 hours at 400 V. Cuts of one-half inch were made and numbered 1 to 12 starting from the cathode. The fractions were eluted with saline, dialyzed extensively against 0.005 M pH 7.3 phosphate buffer, lyophilized and diluted in saline to an equal concentration of 0.4 mg protein per ml.

Hemagglutination of insulin-sensitized erythrocytes. The method using erythrocytes treated with bis-diazotized benzidine (BDB) as described by Pressman *et al*(16) and applied to the detection of insulin antibodies by Arquilla and Stavitsky(17) was employed. The latter method was applied as described for detection of hemagglutination of insulin-sensitized BDB-treated sheep erythrocytes by antibodies contained in whole guinea pig sera. Two series of doubling dilutions of each antiserum ranging from 1/10 to 1/1280 were prepared and to each tube of one series 0.1 ml containing 10 μg of insulin was added in order to inhibit the agglutination, and buffer of 0.1 ml was added to the other series. Hemagglutination was read by the pattern of the sensitized erythrocytes at the bottom of the tubes as well as by tapping the tubes and observing the agglutination under a magnifying glass.

Hemolytic titration of anti-insulin antibodies. A. In whole guinea pig serum. Passive immune hemolysis was studied using the same series utilized for detection of hemagglutination. The sensitized and agglutinated erythrocytes were spun down at 1500 rpm for 5 minutes and the supernatant removed. The cells were washed in veronal buffer and resuspended in 0.6 ml of the same buffer. To each tube 0.5 ml of complement diluted 1/10 was added and the tubes were incubated at 37°C for one hour with constant shaking. The cells were spun down at 1500 rpm in a cold centrifuge, the supernatant decanted and

hemolysis was read in a Beckman DU spectrophotometer at 541 mu.

B. In electrophoretic eluates. Doubling dilutions of antiserum fractions obtained by zone electrophoresis in decreasing concentration from 0.400 mg protein/ml to 0.0015 mg/ml protein were made in a volume of 0.5 ml of ice cold veronal buffer. Diluted guinea pig complement, 0.5 ml, and 0.05 ml of insulin-sensitized erythrocytes were added to all tubes which were incubated for one hour at 37°C with constant shaking. Hemolysis was read in a spectrophotometer as indicated above.

Passive cutaneous anaphylaxis (PCA) was performed as previously described(10). Four animals were used in each experiment. Different amounts of insulin varying from 1 μg to 100 μg per animal were used for challenge.

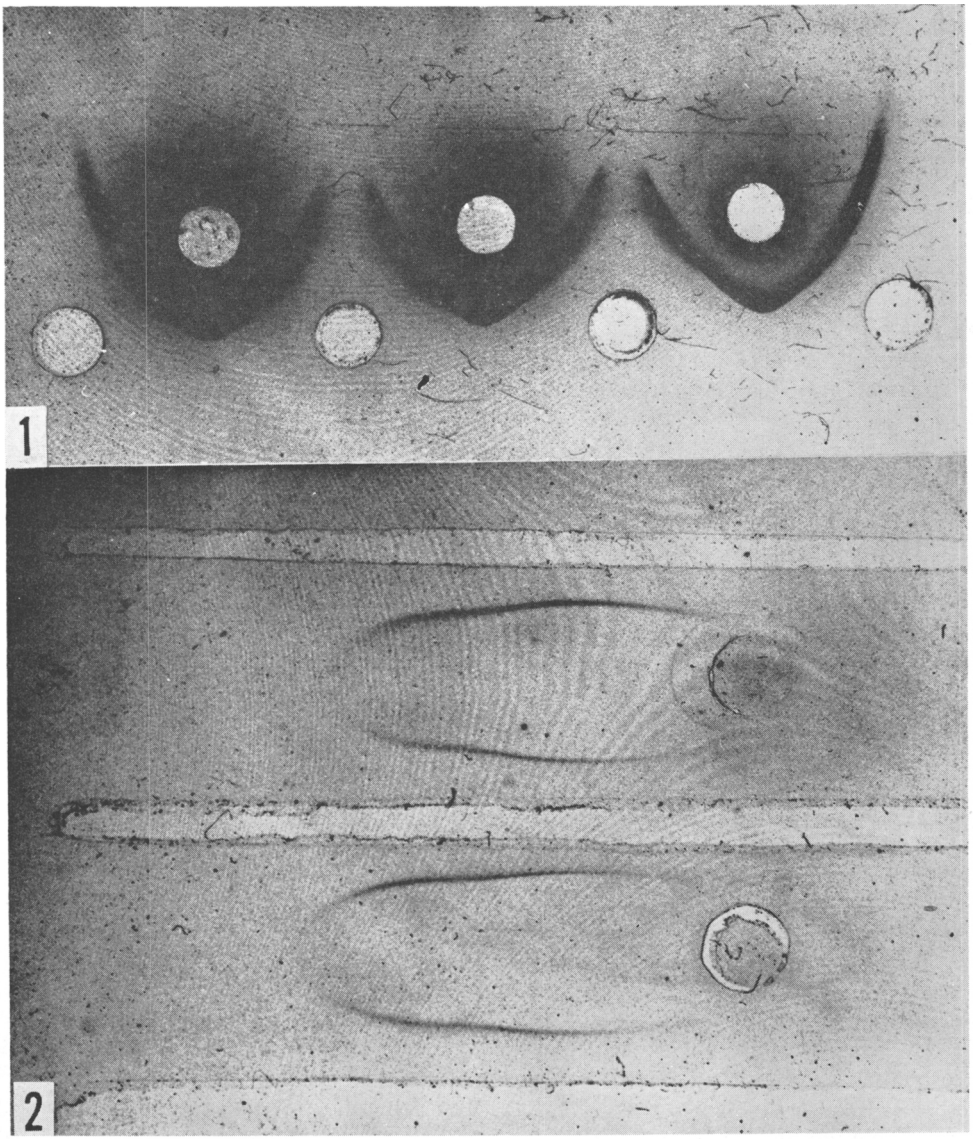

FIG. 1. Double diffusion in agar. Upper wells contain 3 different guinea pig anti-insulin sera. Lower wells contain insulin 0.1 mg/ml.

FIG. 2. Immunoelectrophoretic analysis of 2 guinea pig anti-insulin sera developed with insulin 0.1 mg/ml. A double arc of precipitation is visible in gamma globulin region.

TABLE I. Pattern of PCA in Guinea Pigs Challenged with Various Amounts of Insulin.

Dilutions of serum	Diameter of PCA reactions (mm) 1:100	1:200	1:400	1:800	1:1600	1:3200
Insulin 0.100 mg/animal	20	18	16	16	10	10
0.050 mg/animal	20	20	20	12	12	12
0.005 mg/animal	20	18	16	14	10	10
0.001 mg/animal	18	16	15	neg	neg	neg

Note: Average of 4 animals.

TABLE II. Hemagglutination and Passive Hemolysis of Insulin-Sensitized Cells Obtained with One Guinea Pig Antiserum in Presence and Absence of Excess Insulin.

Dilutions of serum	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Preincubated with 10 μg insulin									
Hemagglutination	pos	pos	neg	neg	neg	neg	neg	neg	neg
Passive lysis (*o.d.*)	0.215	0.200	0.0175	0.145	0.108	0.075	0.060	0.050	0.050
No insulin added									
Hemagglutination	pos	pos	pos	pos	pos	pos	neg	neg	neg
Passive lysis (*o.d.*)	0.260	0.260	0.255	0.240	0.190	0.160	0.175	0.108	0.070

Results. Identification of anti-insulin antibodies in whole guinea pig serum.

1. Double diffusion in agar. A single line of precipitation was obtained in all cases (Fig. 1).

2. Immunoelectrophoresis. A distinct precipitin line was obtained with all antisera. The line had a typical double-arc profile (Fig. 2) similar to that obtained with other systems(5,8). Among 9 guinea pig antisera tested, 5 gave weak reactions. No precipitin line was ever obtained when oxidized B chain was used instead of whole insulin.

3. PCA reaction was obtained with all antisera at serum dilutions varying from 1/400 to 1/3000 (Table I). Equally good results were obtained with insulin from 5 to 100 μg/animal while 1 μg/animal seemed insufficient to provoke skin reactions with high serum dilutions. No PCA reaction was obtained when the animals were challenged with chain B of insulin even when 1000 μg/animal was used.

4. Titration of anti-insulin antibodies in guinea pig sera by hemagglutination and passive immune hemolysis. Hemagglutination was obtained at serum dilutions up to 1/160 (Table II). However, when insulin was added before addition of sensitized erythrocytes, hemagglutination was completely inhibited except at the higher serum concentrations (1/5, 1/10). Hemolysis was obtained with serum dilutions up to 1/1280 (Table I). When insulin was added to the antisera prior to addition of the sensitized cells, hemolysis was partially inhibited at the higher serum concentrations, and completely inhibited at the serum dilutions between 1/320 to 1/1280. A typical pattern of hemolysis obtained with one antiserum in the presence or absence of insulin is shown in Fig. 3. No inhibition of hemagglutination or hemolysis was obtained when insulin was replaced by oxidized B chain, even at high concentrations.

Separation of antibodies of different bio-

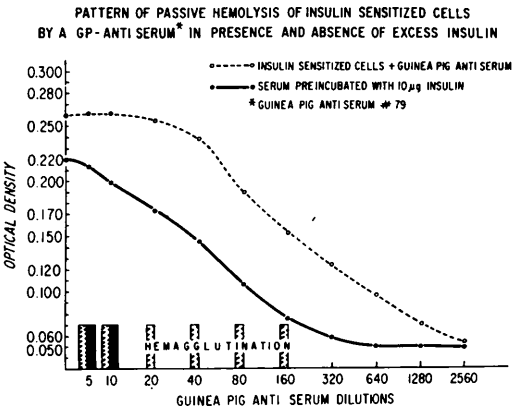

FIG. 3. Pattern of passive hemolysis of insulin-sensitized cells by a guinea pig antiserum in presence and absence of excess insulin. Hemagglutination is represented by bars at lower part of graph.

logical activity by zone electrophoresis. A pool of guinea pig antisera (GP #78 and GP #79) was separated by zone electrophoresis on starch.

1. Immunoelectrophoresis of anti-insulin antibodies separated by zone electrophoresis. Precipitin lines were obtained with fractions 4 to 12 when developed by the antigen. These lines were faint to invisible in the wet state but were identified after drying and staining of the plate (Fig. 4). All precipitin lines occurred in the zone of migration of gamma globulin.

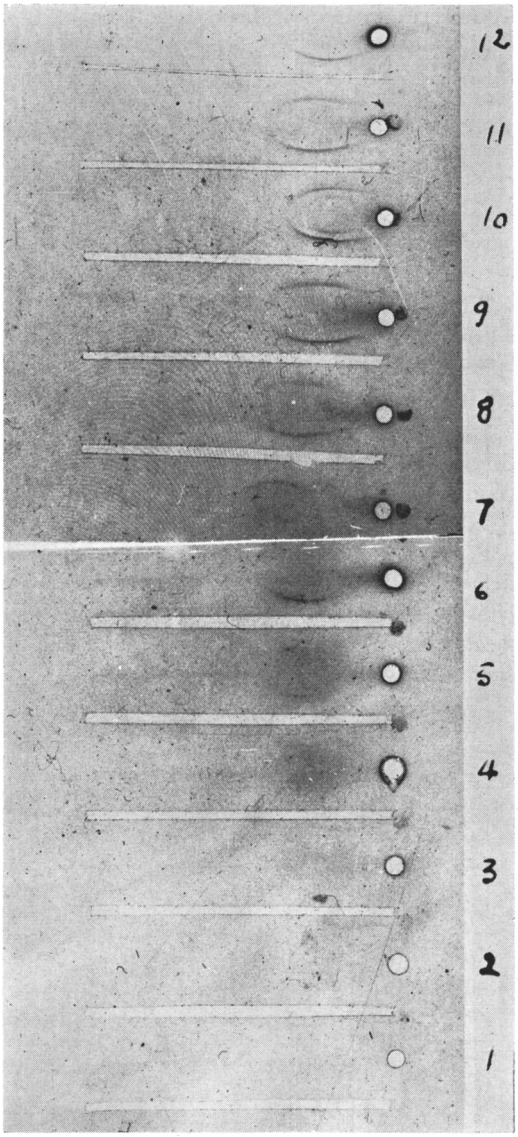

FIG. 4. Immunoelectrophoretic analysis of fractions eluted from starch block electrophoresis of a pool of guinea pig antisera (guinea pig #78 and #79) developed with insulin 0.1 mg/ml. A precipitin line can be seen at an increasing distance from the origin in fractions #4 to #12.

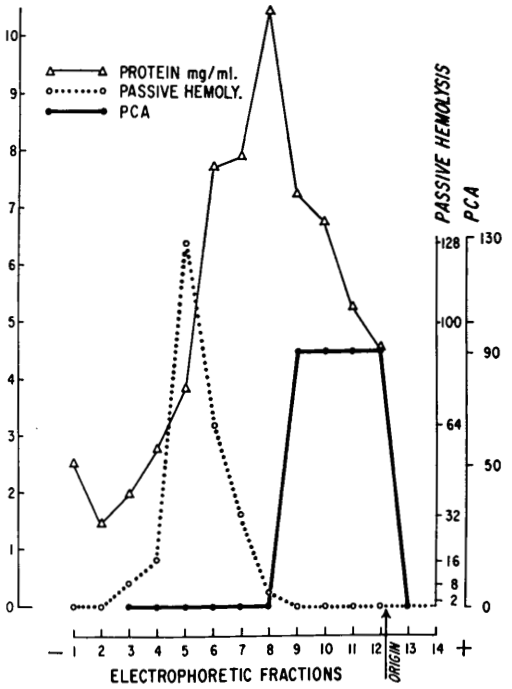

FIG. 5. Hemolytic and sensitizing properties of guinea pig anti-insulin antibodies after zone electrophoresis on starch. Numbers on right of graph represent reciprocal of dilutions of eluted fractions.

2. Titration of anti-insulin antibodies by PCA. All the animals were tested as described above and challenged with 100 μg insulin per guinea pig. Positive PCA reactions were obtained with fractions 9 to 12 with equally good results (Fig. 5). Fractions 1 to 8 were negative.

3. Titration of anti-insulin antibodies by immune hemolysis. The fractions eluted from starch after zone electrophoresis were tested for their ability to provoke immune lysis. Veronal buffer was used throughout keeping the same proportions of fractions to reagents as described for hemagglutination. All fractions were titrated for their hemolytic activity from 3 μg protein/ml to 400 μg protein/ml in 0.5 ml volume. The results are shown in Fig. 5. Hemolysis was obtained with fractions 3 to 8 with a maximum in

fraction 5 (to dilution of 1/128). No hemolysis was obtained with fractions 8 to 12.

Discussion. Precipitating antibodies have been obtained when guinea pigs were immunized with crystalline beef insulin and our results confirmed similar findings reported by others(4). A single line of precipitation was obtained at all times by double diffusion in agar against insulin. Berson and Yalow have suggested that the difficulty encountered in obtaining precipitating antibodies against insulin was due to the fact that insulin is monovalent(18,19). However, Arquilla *et al* (20) have shown that insulin has a multiplicity of antigenic determinants. On immunoelectrophoresis a double arc was obtained. This confirms the results obtained by Yagi and Pressman(21,22), who were the first to show the existence of at least 2 types of anti-insulin antibodies; however, these authors did not study the biological activity of these antibodies. The specificity of these antibodies for insulin was clearly demonstrated by the inhibition experiments. Previous incubation of the antisera with 10 μg of insulin inhibited hemagglutination in all tubes except in the first which contained an excess of antibody. Similar inhibition has been demonstrated by passive hemolysis. After electrophoretic separation on starch block slow and fast migrating populations of antibodies were demonstrated. These antibodies correspond to the gamma 1 and gamma 2 globulins already described in the guinea pig(5-7). As it was shown in other systems, anti-insulin gamma 2 antibodies are able to fix complement while gamma 1 antibodies have sensitizing properties. Passive hemolysis was obtained with the slower moving antibody population only (Fig. 5) which is similar to the results obtained with guinea pig anti-DNP-BGG antibodies(7). PCA was obtained with the faster moving anti-insulin antibodies only. This is consistent with the results obtained with the anti-DNP system (6). Oxidized B chain of insulin was ineffective in preventing hemagglutination or in provoking PCA reaction. This confirms the studies of Wilson *et al*(23), who, using hybrids of isolated A & B chains of insulin from different species, were able to demonstrate that the antigenicity of insulin resides on the A chain. Insulin is immunogenic in the guinea pig and gives rise to two types of 7S antibody populations similar to those obtained with other antigens.

Summary. 1. Precipitating antibodies to insulin have been demonstrated in the guinea pig. 2. Two types of 7S anti-insulin antibodies were separated by zone electrophoresis and were shown to have different biological activities. The slower moving gamma 2 antibodies provoked hemagglutination and hemolysis in presence of complement. The faster moving gamma 1 antibodies gave PCA reactions in the guinea pig. 3. Oxidized B chain of insulin did not share antigenic determinants with the intact insulin molecule.

We wish to express our thanks to Dr. W. Konigsberg, Yale University, for providing us with the oxidized B chain of insulin and to Eli Lilly Co. for the generous gift of crystalline beef insulin. We are grateful for the skillful technical help of Mr. Csaba de Szalay and Mr. William C. Hayes.

1. Berson, S. A., Yalow, R. S., J. Clin. Invest., 1957, v36, 642.

2. Shipp, J. C., Russell, R. O., Steinke, J., Mitchell, M. L., Hadley, W. B., Diabetes, 1961, v10, 1.

3. Moloney, P. J., Aprile, M. A., Canad. J. Biochem. Physiol., 1959, v37, 793.

4. Jones, V. E., Cunliffe, A. C., Nature, 1961, v192, 136.

5. Benacerraf, B., Ovary, Z., Block, K. J., Franklin, E. C., J. Exp. Med., 1963, v117, 937.

6. Ovary, Z., Benacerraf, B., Block, K. J., ibid., 1963, v117, 951.

7. Block, K. J., Kourilsky, F. M., Ovary, Z., Benacerraf, B., ibid., 1963, v117, 965.

8. Thorbecke, J. G., Benacerraf, B., Ovary, Z., J. Immunol., 1963, v91, 670.

9. Craig, L. C., Konigsberg, W., King, P. T., Biochem. Preparat., A. Meister, Ed., Wiley & Sons, 1961, v8, 70.

10. Ovary, Z., Passive Cutaneous Anaphylaxis, C.I.O.M.S. Imm. methods symsposium. Blackwell Scientific Publications, Oxford, 1964, v259, 284.

11. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, Charles C Thomas, Springfield, Ill., 1961.

12. Ouchterlony, O., Diffussion-in-gel methods for Immunological analysis. Progress in Allergy, Karger, Basel/N. Y., 1952, v5, p1.

13. Scheidegger, J. J., Intern. Arch. Allergy Appl. Immunol., 1955, v7, 103.

14. Grabar, P., Burtin, P., Immuno-Electrophor-

etic Analysis, Elsevier, Amsterdam, 1964.

15. Kunkel, H. G., Methods Biochem. Analysis, 1954, v1, 141.

16. Pressman, D., Campbell, D. H., Pauling, L., J. Immunol., 1942, v44, 101.

17. Arquilla, E. R., Stavitsky, A. B., J. Clin. Med., 1956, v35, 458.

18. Berson, S. A., Yalow, R. S., T. N. Y. Acad. Sci., 1962, v24, 487.

19. ———, J. Clin. Invest., 1959, v38, 1996.

20. Arquilla, E. R., Finn, J., J. Exp. Med., 1963, v118, 55.

21. Yagi, Y., Maier, P., Pressman, D., J. Immunol., 1962, v89, 442.

22. Yagi, Y., Maier, P., Pressman, D., Arbesman, C. E., Reisman, R. E., Lenzner, A. R., ibid., 1963, v90, 760.

23. Wilson, S., Dixon, G. H., Wardlow, A. C., Bioch. Bioph. Acta, 1962, v62, 483.

Received February 10, 1965. P.S.E.B.M., 1965, v119.

Comparison of ^{95}Zr and ^{95}Nb Distributions in Maternal and Fetal Rabbit Tissues.* (30122)

NORMAN S. MACDONALD, RAYMOND HAMEL, MARILYN HEPLER AND ELLIN JAMES
(Introduced by G. V. Taplin)

Laboratory of Nuclear Medicine and Radiation Biology, Department of Biophysics-Nuclear Medicine, and Department of Radiology, School of Medicine, University of California at Los Angeles

The fission products ^{95}Zr and ^{95}Nb form a radioactive "parent-daughter" pair whose metabolic behavior in animals and man is of interest because of their appearance in the environment following the detonation of nuclear devices. Peaks in the concentrations of ^{95}Zr-^{95}Nb on airborne particles were observed during early 1962 and 1963(1), and these nuclides have been identified in food(2) and humans(3,4). The early work of Hamilton and associates(5) and others(6,7) has shown that small but measurable amounts of ^{95}Zr-^{95}Nb can enter the mammalian body *via* ingestion and inhalation. ^{95}Zr and ^{95}Nb both emit gamma rays, but their energies (0.72-0.76 Mev) are so similar that it is impractical to assay the amounts of each nuclide in a mixture by γ-scintillation spectrometry. This has made it difficult to compare the metabolic behavior of the parent ^{95}Zr to that of the daughter ^{95}Nb, in the carrier-free condition. Sastry *et al*(8) approached this problem by determining the effective physical half life of γ-activity in tissues obtained from rats following intraperitoneal administration of ^{95}Zr-^{95}Nb. They utilized the fact that the physical half life of ^{95}Zr is 65 days while that of ^{95}Nb is 35, and reported that bone tissue accumulated more ^{95}Zr than ^{95}Nb. However, we have found no published data on the transplacental movement and subsequent disposition of these isotopes between a pregnant animal and its fetus. The purpose of the work now to be reported was to compare the distribution of ^{95}Zr and ^{95}Nb in an adult mammal to that in the growing fetus.

Methods. Five pregnant Dutch rabbits, estimated to be within one week of completion of full term, each received an intravenous injection of approximately 50 μc carrier free ^{95}Zr-^{95}Nb as oxalates in normal saline solution. The ^{95}Zr/^{95}Nb ratio was 1.38 expressed in terms of gamma activity of each nuclide after separation by ion exchange as described below. Twenty-four hours later each was killed with intravenous barbiturate and weighed samples of the following tissues were obtained; muscle, liver, kidney, tibial metaphysis and tibial diaphysis from the maternal body; the same tissues pooled from 3-4 fetuses of the liter; and placental tissue. All tissues were ashed at 600°C and dissolved in 15 ml of 12 N-HCl containing 0.2 ml of concentrated HNO_3. The ^{95}Zr was then separated from the ^{95}Nb in each sample by slow addition of the acid solution to an anion ex-

* These studies were supported by Contract AT(04-1)-GEN-12 between U. S. Atomic Energy Commission and University of California at Los Angeles.