the allantoic fluid of chick embryos infected with WS influenza A was found to inhibit plaque formation of EEE virus linearly in a semi-logarithmic plot between dilutions of 3:200 and 1:20. Higher concentrations of interferon, however, did not inhibit plaque formation in the predicated manner. This interferon also inhibited synthesis of cell-associated viral RNA in chick cultures infected with EEE virus. However, such inhibition was found to be consistently less than the inhibition of infectious virus replication. It is suggested that interferon does not act merely by inhibtion of viral RNA but that it interferes either directly or indirectly with the synthesis of other viral components or with the reactions underlying the assembly of these components.

Statistical analysis of some of the data was due to Dr. C. C. Li, Professor of Biometry of this School. This work was also greatly aided by the excellent technical assistance of the Misses Mary Kay Breinig, Judith Yogodnik and Becky Dordas.

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Chromatographic Separation of Glucagon and Insulin from Serum By Resin-Exchange Paper* (28091)

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During an attempt to establish a practical method for quantitative assay of glucagon in mammalian serum, a simple, easy approach to separating the hormone and coincidentally insulin from serum was evolved. This method employs descending chromatography on a specially prepared resin-exchange paper. In all commercially available resin-impregnated papers previously employed chromatographically, both hormones remain at the site of application, as does the serum. By use of a special resin-exchange paper, 30% Amberlite produced by Rohm and Haas of Philadelphia, both hormones mechanically mixed with the serum migrate independently at constant rates out of the serum into the field from the point of application.

Materials. 1. Resin-exchange paper, 30% impregnated Amberlite, IRC 50 (W.A. 1) of Rohm and Haas.† The paper was cut into strips ¾″ × 8″, each strip being pencil-marked for subsequent point of application. Before applying any test material the paper was washed for one-half hour in absolute ethanol and dried under a heat lamp. Unless this preliminary step is taken, neither glucagon nor insulin will leave the site of its application.

2. Standard chromatography chambers.

Solutions. 1. Stock solution of glucagon: to a beaker containing 10 mg of crystalline

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† The authors are grateful to Rohm and Haas Co. for their painstaking creation of a resin-exchange paper suitable to the purpose.

glucagon (Lot #258-234B-167-1)‡ was added 5 ml of physiologic saline brought to pH 8.5 by addition of one or 2 drops of 1 N NaOH. While an acid medium will also bring the hormone into solution, when such solution is subsequently combined with any serum, precipitation results. 2. Pooled serum from non-diabetic hospital patients. 3. Human gamma globulin (Lederle: Poliomyelitis Immune Globulin [Human] 165 mg/ml); animal gamma globulin. 4. Crystalline insulin (Lot J2842)‡ was placed in solution precisely as under #1. 5. Amido Black dye prepared; Amido Black 9.8 g, distilled water 180 ml, methanol 70 ml, acetic acid 10%, 10 ml. The mixture is mechanically stirred for at least one hour. 6. Solvent system for chromatography: a 12-5-6-part mixture respectively of butanol, distilled water, and glacial acetic acid.

Method. The following solutions were individually and in combination chromatographically tested, 0.01 ml of each being deposited in the usual manner at the application site(1): 1. Glucagon alone in buffered saline (final concentration 1 mg/ml). 2. Glucagon plus pooled non-specific serum (volumetrically mixed to produce a final solution of glucagon 1 mg/ml). 3. Glucagon plus non-specific gamma globulin (final concentration of glucagon 1 mg/ml). 4. Pooled non-specific serum alone. 5. Non-specific gamma globulin alone in saline, 165 mg/ml. 6. Insulin alone in buffered saline (final concentration 1 mg/ml). 7. Insulin plus pooled non-immune serum (final mixture of insulin 1 mg/ml). 8. Insulin plus non-specific gamma globulin (final concentration of insulin polypeptide 1 mg/ml; gamma globulin concentration 165 mg/2 ml. 9. Insulin plus glucagon in buffered saline (final concentration of each 1 mg/ml). 10. Insulin plus glucagon plus pooled non-specific serum (final concentration of each 1 mg/ml). 11. Insulin plus glucagon plus non-specific gamma globulin (same concentrations as in #10).

The applied solutions were permitted to dry in air at room temperature for 10 to 15 minutes. Thereafter the strips were suspended in the chromatographic system. When the solvent front reached a level 2 cm from the bottom of the strip, the papers were removed, dried in air under a heat lamp, and fixed in absolute ethanol. They were again dried and placed in the dye, where they remained for 20 minutes. Thereafter, the strips were replaced in the wash solution until the dark background completely cleared, whereupon they were again dried and examined.

Results. On the "glucagon alone" strip (Fig. 1.1), the original spot of application is clear, no protein or polypeptide remaining at this site after chromatography (as tested by Amido Black dye, as well as by elution of the original spot), all having moved with the solvent front out into the chromatographic field. The fact that all of the glucagon had left the point of application was verified by eluting the final spot and quantitatively recovering from this area all of the glucagon originally applied. The determination of recovery from the final spots was made by spectrometry using a Beckman DU 120 at 620 mμ, thereby measuring the dyed protein in the final spot.

The migration of glucagon takes place in 2 hours at room temperature (23°C). The speed of migration and distance traversed are relatively constant, for glucagon 2½″ to 3″ from the point of application.

On the strip, "Glucagon plus non-specific serum" (Fig. 1.2), all of added glucagon again travels out into the field. The R.F. value is practically identical with that in the "glucagon-alone" strip. The findings are the same when non-specific gamma globulin is substituted for the serum (Fig. 1.3). Total migration in each instance was verified as above noted.

When the above technic is applied to the polypeptide, insulin, the same findings hold (Fig. 2) except that the distance traversed by the hormone in the same time period is somewhat greater, about 3½″ from source.

On the strips "pooled serum alone" or "non-specific gamma globulin alone" (Fig. 1 and 2) there was visualized with this technic no detectable migration of protein or polypeptide. There was, however, some degree of

‡ Supplied by Eli Lilly and Co. by courtesy of Dr. William W. Bromer.

streaking (smudging) in continuity with the site of application of the serum. This always occurs with serum and is unrelated to the presence of the specific polypeptide.

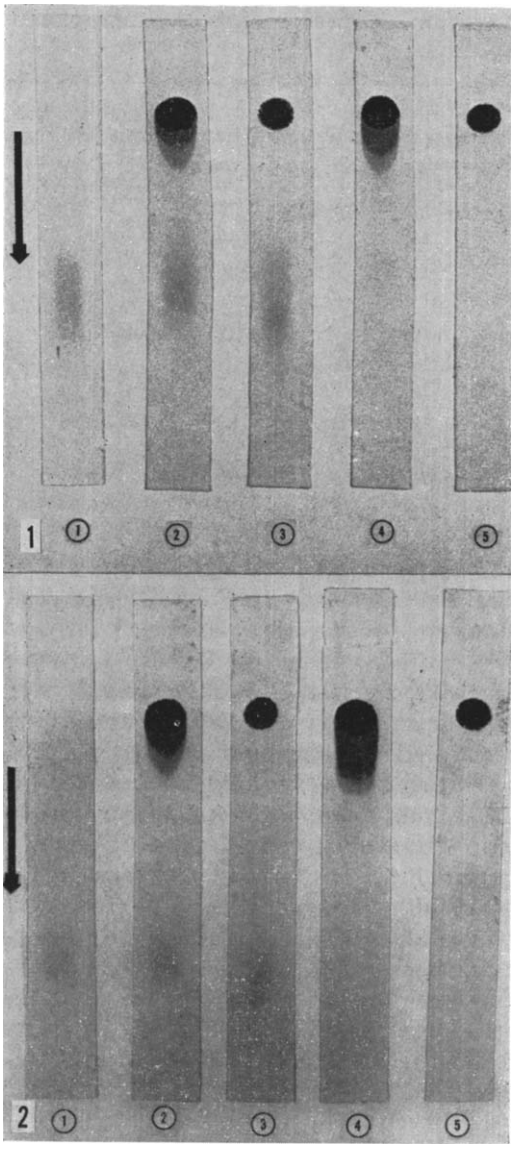

FIG. 1. Glucagon chromatographs. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.) 1. Glucagon alone in buffered saline. 2. Glucagon plus pooled non-specific serum. 3. Glucagon plus non-specific gamma globulin. 4. Pooled non-specific serum alone. 5. Non-specific gamma globulin alone.

FIG. 2. Insulin chromatographs. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.) 1. Insulin alone in buffered saline. 2. Insulin plus pooled non-specific serum. 3. Insulin plus non-specific gamma globulin. 4. & 5. Same as Fig. 1.

On the strip "Insulin plus glucagon" (Fig. 3.1), both hormones travel out into the field in 2 separate polypeptide spots, each with an R.F. value similar to that of the respective "glucagon-alone" or "insulin-alone" strip. When pooled serum or non-specific gamma globulin is added to a mixture of both hormones (Fig. 3), the same R.F. value for each polypeptide persists.

Discussion. The above findings open up a new methodologic approach for determining insulin and/or glucagon in human serum. So far as we know, it had not up to this time been possible to move either of these free hormones chromatographically (without electrophoresis) from their site of application on paper. By this dye technic, it has, however, been possible to demonstrate the movement of only relatively massive amounts of the hormones, far in excess of their physiologic presence in serum. It is likely that even the naturally existing minute quantities of the hormones also migrate out into the field, but the present system is not sufficiently sensitive to record such migration. In view of the likeli-

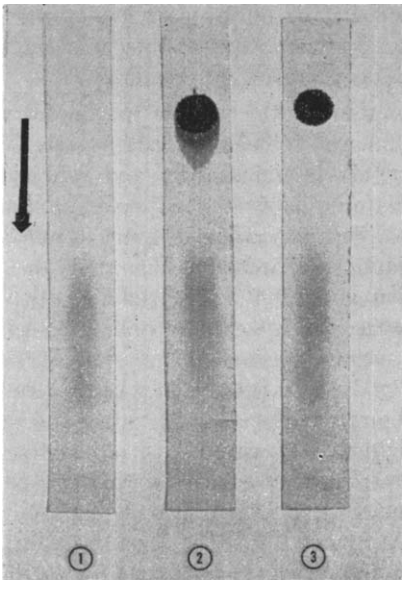

FIG. 3. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.) 1. Insulin plus glucagon in buffered saline. 2. Insulin plus glucagon plus pooled non-specific serum. 3. Insulin plus glucagon plus non-specific gamma globulin.

hood of such minute migration, we have been exploring by fluorescent-label immune technics the detection of such minute concentrations as normally occur in serum. A subsequent paper will present this report. It has, moreover, been possible by a specific immune antibody technic for us to retain both glucagon and insulin completely at their sites of application to the paper. All of the free unattached hormone otherwise migrates out into the chromatographic field.

Conclusion. By use of 30% Amberlite IRC 50 (W.A. 1) resin-exchange paper, it is possible to separate both insulin and glucagon independently from their mechanical mixtures with non-specific serum or gamma globulin.

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An Immune Chromatographic Method for Assay in Serum of Antibodies to Glucagon and Insulin.* (28092)

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A simple method is presented for qualitative and macro-quantitative assay of serum for the presence of immune antibodies to crystalline glucagon and/or insulin.

Materials and methods. Rabbits, guinea pigs and Peking ducks were immunized (using the Freund Adjuvant technic) against crystalline glucagon or insulin(1).

A solution of the antigen in concentration 1 mg/ml was injected subcutaneously below the scapula in guinea pigs and rabbits and immediately below the xiphoid process in ducks. This was repeated every 4 weeks for 3 injections. Twelve weeks after the first injection and after a 24-hour fast, the animals were bled by cardiac puncture, using a Luer syringe washed in physiologic saline. The blood was deposited in a centrifuge tube and allowed to clot at a 45° angle and stored overnight at 7°C, carefully avoiding freezing. After retraction of the clot during this period, the tubes were centrifuged for 15 minutes at 3000 rpm at 7°C. The serum was then decanted into a clean tube and recentrifuged for 15 minutes at 3000 rpm. If the serum was not immediately to be fractionated, it was rapidly frozen in an acetone-dry-ice mixture and stored at −18°C until used. The serum from each species was pooled (in view of the varying individual immune responses among the animals). Usually 4 animals were used for developing each batch of anti-serum. Fractionation of the serum was accomplished with ethyl alcohol by a method adapted by Lange from Cohn *et al.*(2). 1. One part of serum is diluted with 3 parts of distilled water. 2. pH is adjusted to the range of 7.6 to 7.8 with 5% $NaHCO_3$ or 1-N acetic acid and/or 1-N-NaOH. 3. The serum thus prepared is chilled to 0°C in an ice-bath, cautiously stirring during this period with a magnetic stirrer, carefully avoiding any bubbling. 4. While step #3 is in process, an equal volume of 50% ethanol is chilled down to −26° under magnetic stirring. Note: All separation procedures hereinafter must be performed in a cold room (−4°C). 5. The alcohol prepared in step #4 is added drop-by-drop by capillary pipette to the chilled serum (#3). 6. The mixture is stirred for 20 minutes, after all of the alcohol has been added. 7. The mixture is then centrifuged at 18,000 rpm for 10 minutes, using polypropylene centrifuge tubes for the purpose. 8. The supernatant is decanted and discarded. The

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