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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Received November 26, 1962. P.S.E.B.M., 1963, v112.

## 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

* Supported by a grant from John A. Hartford Foundation.

remaining sediment is gamma globulin. 9. Any remaining excess of the supernatant is drained off by inverting the tubes on absorbent paper for 3 to 4 minutes. 10. The sediment is resuspended in a volume of saline (0.85% at pH 7.0) equal to ⅓ the volume of the serum originally processed (adding the saline gradually). The saline for resuspension is prepared: to 1.5 liters of distilled water are added 2.75 g of sodium monobasic phosphate and 12.0 g of sodium chloride. This solution is buffered, using 1.0-1.1 ml 40% (W/V) sodium hydroxide. The pH is then checked.

The total amount of protein (gamma globulin) present is determined by the micro-biuret method(3). This gamma globulin is shell-frozen in aliquots of 0.5 ml and kept in the freezing unit until used.

*Method of testing for specific antibody.* A mixture of practically any dilution of antibody and its specific antigen will result within a 24-hour period at room temperature in a conjugation between the two substances. It is well recognized that such union between the antigen, glucagon or insulin, and its specific antibody as produced by this method does not result in a detectable flocculation or precipitation. Accordingly, in order to prove the actual presence or absence of conjugation, the following method was evolved. As pointed out in a foregoing paper(4), if insulin or glucagon is mechancally mixed with non-specific serum or gamma globulin and gravitationally chromatographed on special resin-exchange paper (30% Amberlite I.R.C. 50-WA 1 of Rohm and Haas Co.), all of the unbound or free hormone at the application site moves out into the field, no free hormone being held back. This fact is easily demonstrated by eluting both the original and final dyed protein spots, quantitating in a Beckman DU 120 at 620 mμ and recovering from the final spot all of the hormone originally deposited, no measurable hormone remaining at the point of origin. These findings held in the "hormone-alone" strips and in the final spots of the strips with the hormone added to non-specific gamma globulin or serum.

Unless the material at source contains a specific uniting antibody, all of the free hormone in the spot might be expected to move out with the solvent front. Contrariwise, the presence of a specific binding antibody at the point of application might be anticipated to combine with the antigen and prevent its migration out into the field. It has been possible to demonstrate that such retention at source actually takes place almost in linear proportion to the concentration of specific antibody to which the antigen has been exposed.

The following steps were taken to prove these premises: Crystalline insulin† and glucagon† (respectively, Lots J2842 and 258-234B-167-1) were placed in solutions of physiologic saline (pH 8.5) 1 mg/ml. Each was mixed with an equal volume of the full strength gamma globulin to be tested and in the following saline dilutions of the gamma globulin, 1:2, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160. The same volume of antigen was mixed with an equal volume of non-immune globulin as a control. The mixtures were incubated at room temperature with constant shaking for 4 hours, and then left at room temperature for an additional 20 hours. This time factor was selected after repeated demonstration that after the first 12 hours no further conjugation takes place.

After this 24-hour period, 0.01 ml of each mixture was individually applied to a strip of ion-exchange resin-impregnated paper (30% Amberlite–I.R.C. 50-WA 1) and chromatographed. As an additional control, 0.01 ml of a mixture of equal parts antigen and saline was applied to strips and chromatographed with the above. When the solvent front of the strips reached 2 cm above the free end of the strip, the strips were removed from the system and dried in air under a heat-lamp. Thereafter, they were dyed in Amido Black as originally described(4).

The same technic of eluting the final spots and of quantitating the migrated hormone in a Beckman spectrometer (as above) was employed.

The process of chromatographing was re-

† Supplied by Eli Lilly and Co. by courtesy of Dr. William W. Bromer, to whom the authors are most grateful for his interest and counsel.

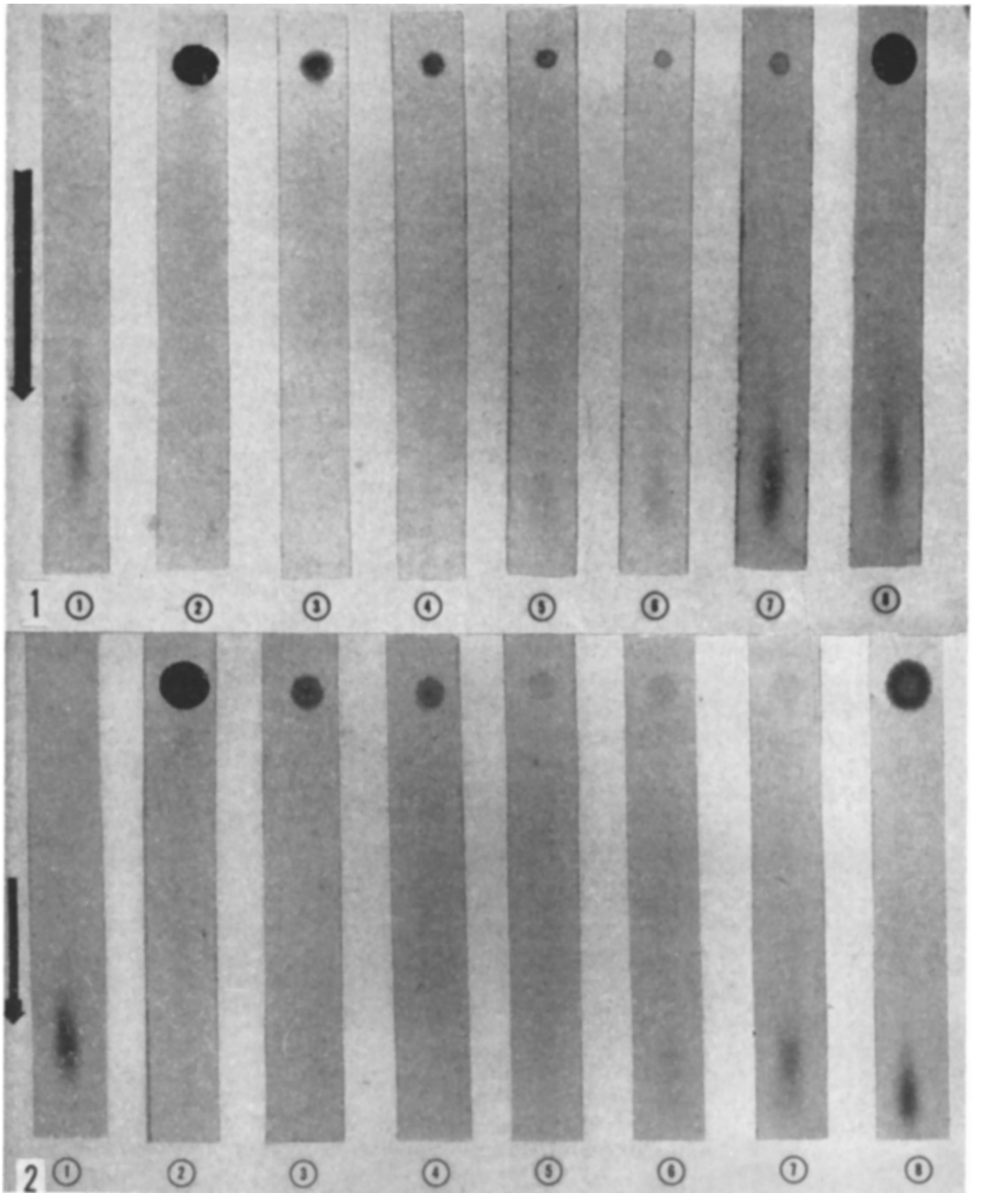


FIG. 1. Glucagon plus equal volumes of specific gamma globulin from immune serum. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.)

1. Glucagon alone in saline at application site. 2. Mixed with specific gamma globulin full strength. 3. Mixed with specific gamma globulin diluted 1 to 5. 4. Mixed with specific gamma globulin diluted 1 to 10. 5. Mixed with specific gamma globulin diluted 1 to 20. 6. Mixed with specific gamma globulin diluted 1 to 40. 7. Mixed with specific gamma globulin diluted 1 to 80. 8. Mixed with non-specific gamma globulin full strength.

FIG. 2. Insulin plus equal volumes of specific gamma globulin from immune serum. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.)

1. Insulin alone in saline at application site. 2. Mixed with specific gamma globulin full

strength. 3. Mixed with specific gamma globulin diluted 1 to 5. 4. Mixed with specific gamma globulin diluted 1 to 10. 5. Mixed with specific gamma globulin diluted 1 to 20. 6. Mixed with specific gamma globulin diluted 1 to 40. 7. Mixed with specific gamma globulin diluted 1 to 80. 8. Mixed with non-specific gamma globulin full strength.

peated, using in place of the specific gamma globulin combinations of the antigen with the unmodified serum from which the immune globulin had originally been derived.

The process was repeated, crossing both antigens and their specific antibodies.

*Results.* All of the deposited polypeptide (antigen) in the control strip ("antigen alone") migrated out into the chromatographic field (Fig. 1 and 2).

In the strips with antibody in dilution down to 1 to 5 (occasionally 1 to 10), the staining did not materially extend from the site of application. In fact, chromatographically, none of the hormone could be found in the periphery. This was verified by elution and quantitating as above. On the other hand, in higher dilutions of the antibody more antigen moved out into the field, actually in inverse proportion to the concentration of antibody in the respective application site. This is apparent from the density of the final spots on the strips but was again verified by assay of the eluted final spots (Table I).

In the strips containing antigen plus non-specific gamma globulin, all of the hormone migrated out into the field, precisely as in the "antigen alone" strip.

The same findings occurred when the unmodified original serum (from which the specific globulin had been derived) was processed. When, however, immune serum instead of the isolated gamma globulin is used, there is always an irrelevant streaking of non-specific factors in the serum, none of which has the R.F. value of the antigen(4). When the serum contains a sizable amount of immune antibody, this factor confines at source the antigen precisely as does specific gamma globulin.

In animals in which immunization failed for any reason, the extracted gamma globulin had no demonstrable holding power, all of the antigen migrating (Fig. 3).

When the antigen-antibody mixtures were crossed so that insulin was exposed to the specific antibody for glucagon and glucagon was mixed with the antibody for insulin, the results on chromatography were identical with those for non-specific antibody. There were no discoverable crossed reactions.

*Discussion.* The consistency with which

TABLE I. Reading of Elutions of Dyed Glucagon Spots in Beckman DU-120, at 620 mμ (eluting solution, ethanol, ammonium hydroxide and distilled water in the proportion of 1:0.2:0.8).

| Strips | Glucagon μg at origin | Glucagon μg in migrated spot* | Beckman readings |
|---|---|---|---|
| 1—Blank paper | 0 | 0 | |
| 2—Non-specific gamma globulin | | | .045 |
| + glucagon | 10 | 10 | .042 (.044) |
| 3—Crystalline glucagon | | | .046 |
| | 10 | 9.8 | .044 (.045) |
| 4—Non-diluted antibody | | | .002 |
| + glucagon | 10 | .34 | .003 (.002) |
| 5—1- 2 Dil. + gluc. | 10 | 2.45 | .012 |
| | | | .010 (.011) |
| 6—1- 5 Dil. + gluc. | 10 | 3.6 | .017 |
| | | | .015 (.016) |
| 7—1-10 Dil. + gluc. | 10 | 6.0 | .030 |
| | | | .025 (.027) |
| 8—1-20 Dil. + gluc. | 10 | 9.3 | .040 |
| | | | .043 (.043) |
| 9—1-40 Dil. + gluc. | 10 | 9.6 | .046 |
| | | | .040 (.043) |
| 10—1-80 Dil. + gluc. | 10 | | .042 |
| | | | .045 (.043) |

* Calculated from photometric reading—reliability or Standard Deviation ± 0.1.

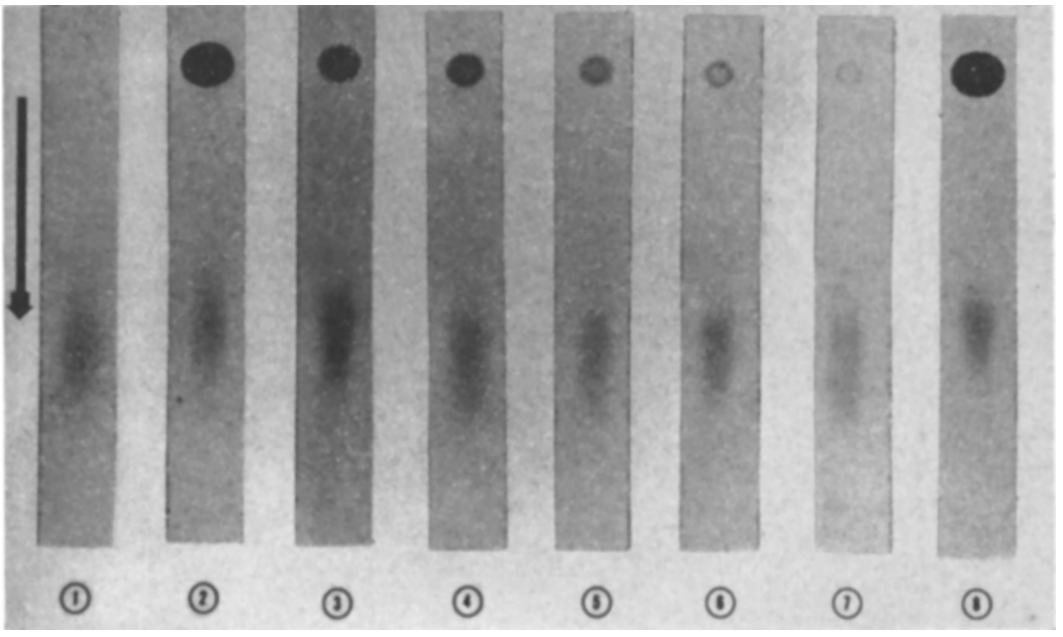

FIG. 3. Glucagon plus equal volumes of gamma globulin (non-specific) from animal in which immunization had failed. (Solvent system: butanol, distilled water and glacial acetic acid in a 12-5-6 mixture.)

1. Glucagon alone in saline at application site. 2. Mixed with (non-specific) gamma globulin full strength. 3. Mixed with (non-specific) gamma globulin diluted 1 to 5. 4. Mixed with (non-specific) gamma globulin diluted 1 to 10. 5. Mixed with (non-specific) gamma globulin diluted 1 to 20. 6. Mixed with (non-specific) gamma globulin diluted 1 to 40. 7. Mixed with (non-specific) gamma globulin diluted 1 to 80. 8. Mixed with the non-specific globulin used in Fig. 1 and 2.

the above phenomena took place in hundreds of test strips with the gamma globulins of numerous test animals (actually in 100% save where there was divergence from the above technic, or an error in the process of immunization) supports the premise that specific antibodies have been created, and that when these are adequately exposed to their specific antigens, conjugation takes place. As a result of this union at source, no free antigen for migration remains at the original spot.

The antibodies, when properly stored in frozen form, remain immunologically active for at least 6 months.

Contrary to expectation, when the antigen (glucagon or insulin) is made fluorescent with fluorescein isothiocyanate, the fluorescent antigen produces an antibody which readily combines with this new antigen. The fluorescent antigen can, however, be easily displaced from such union with its antibody by exposing the combination to unmodified crystalline antigen, competition evidently taking place for combining sites on the antibody. Parenthetically, the antibody to unmodified crystalline glucagon does not uniformly bind the fluorescent hormone. Unfortunately, the titre of antibody produced by the fluorescent antigen has in our hands been too variable for practical application of the principle to establishing a reproducible method for quantitative assay of the hormones in serum. Uniformity in antibody production with the fluorescent hormone, as well as adequate sensitivity, could conceivably by built-in standards make possible a methodology for quantitative assay of both hormones natively present in serum.

*Conclusions.* 1. By simple descending chromatography, using ion-exchange resin-impregnated paper (30% Amberlite I.R.C. 50, WA-1 of Rohm and Haas Co.), the successful production in serum of antibodies

against glucagon and/or insulin is easily demonstrable. These antibodies can be qualitatively assayed in serum by this method, which can also serve for macro-quantitative assay of the specific antibody. 2. The antibodies produced against glucagon and/or insulin are specific without cross-reaction. They are of the non-flocculating and non-precipitating type.

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Received November 26, 1962. P.S.E.B.M., 1963, v112.

## High-Powered Phase Contrast Equipment with Long Working Distance for Observation of Culture Flasks.* (28093)

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The widespread use of cell and tissue culture for cytological observations and in studies of virus-cell relationships makes direct, repeated observations of living cells necessary. Usually low power bright field microscopy is the routine method. The low degree of contrast and the limits in magnification due to the thickness of culture containers reduce the usefulness of this method. Finer structural details of individual cells can not be seen.

Phase contrast microscopy has made studies of the structure and behavior of living cells possible without the distortion and artifacts commonly encountered when fixation and staining are used. The advantages of phase contrast microscopy, however, are in many instances limited in tissue culture work unless specially constructed (expensive, fragile, and time consuming) chambers or flasks are used. The walls of such vessels have to be extremely thin and the outside thickness quite small if high magnification and proper phase effect is to be obtained, since normal high power objectives have a short working distance and ordinary phase condensers only a short focal distance. The use of fairly sturdy culture vessels and the benefits of phase contrast at a relatively high magnification was therefore highly desirable.

* Supported by a grant from Nat. Cancer Inst.

Such a system has been devised in our laboratory and has been found most useful. We have combined optical equipment from Carl Zeiss, and from Wild Heerbrugg, into a "hybrid" microscope. The objective used, in order to obtain high magnification, was a special water-immersion phase contrast 40× objective (Zeiss) with numerical aperture 0.75 and working distance 1.6 mm. It was combined with a Wild special phase condenser with long focal distance. This condenser has a maximal working distance of 20 mm and numerical aperture 0.52 and can be adapted for use on a Zeiss ordinary upright or inverted microscope. The Zeiss objective incidentally can also be used on a Wild microscope by means of an adapting collar.

The system enables us, with phase contrast, to observe and photograph with a 40× objective living cells in any flask with parallel roof and floor, when the thickness of the floor does not exceed 1.6 mm and the total thickness of the vessel 20 mm. The optical quality of Earle's T-15 and T-30 flasks(1), (Kontes Glass Co., Vineland, N. J.) has been found satisfactory. Good optical results have also been obtained with 30 ml plastic tissue culture flasks (Falcon Plastics, Los Angeles, Calif.). The latter flasks, however, we do not consider suited for work with infectious