A Method of Insulin Bio-Assay and Its Application to Human Plasma Fractions.* (21141)

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Methods have been described for the assay of minute quantities of insulin employing hypophysectomized-alloxanized rats and mice (1,2). Perhaps the most sensitive technic reported has been that of Anderson's group(2) utilizing decline of blood glucose levels following intraperitoneal injection of test material in hypophysectomized-alloxanized mice. A stable, elevated blood glucose level was induced by prior gavage with the polysaccharide, dextrin.

Certain modifications of the technic of Anderson's group are here presented. A preliminary account of the application of this modified procedure to study of human plasma fractions is also given.

Procedure. Male, 15-20 g mice of the CAF-1 strain obtained from Jackson Memorial Laboratories, Bar Harbor, Maine, are hypophysectomized by the anterior parapharyngeal approach. Anesthesia is induced by intraperitoneal injection of Nembutal (R), 75 mg per kilo. Following surgery, the mice are retained in a closed oxygen jar until their recovery from anesthesia. Diet consists of ground Purina laboratory chow and 3-5% glucose in water *ad libitum,* supplemented by lettuce, cabbage, bread crumbs and dog meal. The mice are alloxanized 7-9 days after hypophysectomy with 0.1-0.25 ml of alloxan solution in a dosage of 70 mg per kilo. This is administered into a tail vein through a No. 27 gauge hypodermic needle. Heating the mouse in a glass jar under a light bulb for 30-60 seconds dilates the tail veins sufficiently to facilitate intravenous injection. 1.25 mg of the saline suspension of the free alcohol of Cortisone are administered subcutaneously 3-4 hours before alloxanization.‡ At least 60 hours should elapse between alloxanization and use of the mice as assay subjects.

There is a marked tendency for the mice to expire in hypoglycemic shock following hypophysectomy and this problem persists after alloxanization. One ml of 5% glucose subcutaneously should be given in an effort to abort such episodes. The addition of 1.25 mg of cortisone subcutaneously may further assist the animal's survival when hypoglycemic symptoms appear. An environmental temperature of 25°-30°C, preferably 27°C, seems to be very important for the maintenance of the animals. At best, with all precautions and the most meticulous care, there is high mortality following hypophysectomy, with the attrition rate inconstant and unpredictable. Occasionally, the mortality rate may be catastrophic. In general, animals survived 1-4 weeks after operation. Mice surviving hypophysectomy beyond 2 months, in our experience, have proved to possess intact hypophyses. All animals employed for assay were examined after death for presence of pituitary tissue. Gross examination or examination with a hand magnifying lens proved to be sufficient.

The actual testing procedure commences with simultaneous intraperitoneal injection of test material and gavage with 300 mg of dextrin (Dextrin White, Mallinckrodt) in one ml of aqueous suspension. The gavage equipment consists of a one ml tuberculin syringe and a No. 20 needle, with the point removed, to which approximately 0.5 cm of polyethylene tubing is attached. The needle and tubing must be advanced into the mouse esophagus slowly and carefully. Blood samples are collected from the mouse tail vessels in 0.02 ml amounts. The tail tip is cut with shears. The tail is previously dipped into warm tap water and dried. It should be "milked" gently. Heparin powder (Hynson, Westcott, and Dun-

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TABLE I. Insulin-Like Activity of Various Substances Measured by Comparison of Blood Glucose Increments during 30 Minutes.

Material tested	No. of determinations	Mean rise in blood glucose (mg %)	Significance of difference between means by "t" test — P value — Saline	Plasma
Saline	27	146	—	—
Insulin, 0.1 mU	4	175	—	—
" 1-2 mU	4	91	>.3	—
" 4-5 mU	8	14	<.01	—
Plasma	29	106	>.2	—
Gamma-globulin	7	202	—	—
Albumin (SPPS)	13	114	>.3	—
Globulin (PGP)	45	73	<.01	>.05; <.1

Note: P not calculated if mean rise in mg % glucose for insulin or plasma fraction greater than mean rise of saline or whole plasma.

ning, Inc.) is placed on the pipette tip to prevent blood clotting. The sample of blood is diluted in 2.38 ml of distilled water. Blood glucose levels are determined by the Nelson-Somogyi method(3,4).

Results. Earlier studies indicated that it was impossible to attain a consistent plateau of blood glucose levels at any period after dextrin gavage(5). As a result, blood glucose decline at any period after 90 minutes following dextrin gavage could not readily be related to a possible insulin-like effect of the test material. However, insulin activity could be readily differentiated from spontaneous variation by employing the initial 90 minute, or ascending, portion of the blood glucose curve. The first blood glucose was usually obtained 4 ± 2 minutes after simultaneous dextrin gavage and intraperitoneal injection of test material. Succeeding blood glucose levels at 30 ± 10 and 65 ± 15 minute intervals were determined and the increments over the initial value calculated. Sufficiently diminished increments indicated insulin-like activity. However, a *precise* quantitative assay has not yet been achieved.

Experiments with fresh crystalline zinc insulin (Insulin Lilly, Iletin, U. 40), diluted in normal saline, indicated that 0.1 milli-unit showed no significant effect as compared with saline whereas 1-2 milli-units demonstrated an equivocal suppression of hyperglycemia. 4-5 milli-units showed a very significant effect, to the extent of nearly obliterating the rise in the blood glucose curve (Tables I and II).

Human plasma was prepared from 4 healthy young males who had been given 100 g glucose orally and bled 1½ hours later. The heparinized blood was spun in a refrigerated centrifuge and fresh samples were tested. Some plasma was also immediately frozen for later use. Tables I and II indicate that these plasma samples, in 0.25-1.0 ml doses, demonstrated no insulin-like activity as compared with normal saline.

Human plasma fractions tested were: a) Stable Plasma Protein Solution (SPPS), containing principally albumin and other proteins forming soluble zinc complexes; b) Protein Globulin Precipitate (PGP), a globulin-rich fraction precipitated by zinc and c) a gamma-globulin fraction extracted from PGP(6).§ The concentrations of globulin (PGP) and albumin (SPPS) fractions were 5:1 compared with the original plasma. About 4 of the gamma globulin samples represented a concentration 10-15 times that of the original plasma. Despite variation of response occurring with differing lots of mice and of plasma fractions, the data accumulated were sufficiently consistent to permit accurate statistical evaluation. Tables I and II demonstrate that the *only* statistically valid (P = <0.01) insulin-like activity, based on normal saline as the control material, was found in the globulin (PGP) fraction. The 65-minute, but not the 30-minute, increments of blood glucose reveal

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TABLE II. Insulin-Like Activity of Various Substances Measured by Comparison of Blood Glucose Increments during 65 Minutes.

Material tested	No. of determinations	Mean rise in blood glucose (mg %)	Significance of difference between means by "t" test — P value — Saline	Plasma
Saline	27	353	—	—
Insulin, 0.1 mU	4	262	>.3	—
" 1-2 mU	4	146	>.02; <.05	—
" 4-5 mU	5	29	<.001	—
Plasma	24	316	>.4	—
Gamma-globulin	7	285	>.4	>.6
Albumin (SPPS)	11	192	>.01; <.02	>.02; <.05
Globulin (PGP)	45	163	<.001	<.001

a similar clear-cut effect of globulin (PGP) as compared with whole plasma.

Effectiveness of the globulin (PGP) fraction did not appear dependent on maintenance of any particular dosage level so long as a minimum of 0.25 ml was administered. 0.3-0.6 ml was the usual dosage administered.

Discussion. With blood glucose increments measured at the 30-minute mark, there is less separation of globulin (PGP) from whole plasma and saline. This is probably a reflection of greater variation of glucose absorption and metabolism during the 30-minute period immediately following gavage. Thus, a more sensitive assay is achieved when blood glucose increments at 65 minutes are compared.

The mechanism of this insulin-like activity by globulin (PGP) may be related directly to the 5-fold concentration of the plasma fractions achieved by the fractionation procedure. However, the observation that the albumin (SPPS) and gamma-globulin samples, despite being concentrated to an equivalent or greater extent than the globulin (PGP), exhibit less insulin-like activity than globulin (PGP) suggests that it would be of value to investigate further the possible presence of more specific insulin-like factors in highly purified plasma fractions.

Summary. Modifications of the method of Anderson and associates for insulin bioassay are described. Plasma fractions, particularly globulin, demonstrate insulin-like activity by this technic.

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