Garber *et al.*(1) have also shown that a variety of methods of raising ACTH level produce rapid alteration of the JG granulation.

The results of the experiments on the effects of ACTH, hydralazine, and vasopressin in the adrenalectomized and/or hypophysectomized animals indicate that ACTH has a direct tropic effect on the renal juxtaglomerular apparatus that is independent of the presence of the adrenals. The possibility that additional adenohypophyseal principles also affect the JGA has not been ruled out.

These observations may help to explain the diversity of procedures that have been shown to produce significant morphological changes in the JGA(2,3,4). These include alteration of salt balance, of adrenal function, of blood pressure. Finally, in connection with the recent suggested localization of renin to the JGA(9), Haynes *et al.*(10) found that ACTH stimulates renin release from the kidney, and that this action of ACTH also occurs in adrenalectomized animals. This is consistent with our suggestion that the JGA may be a target structure for the direct action of ACTH.

Summary. ACTH produces hypergranulation of the renal juxtaglomerular cells even in absence of pituitary and adrenal glands, and the chronic absence of ACTH depletes juxtaglomerular granules. The renal effect of vasopressin and of hydralazine, substances which produce hypergranulation of juxtaglomerular cells, is blocked by hypophysectomy. These findings support the concept that ACTH mediates many of the responses of the juxtaglomerular cells, and that the juxtaglomerular apparatus is an extra-adrenal site for the direct action of ACTH.

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An Improved *in vitro* Method for Determination of Serum "Insulin-Like" Activity. (26189)

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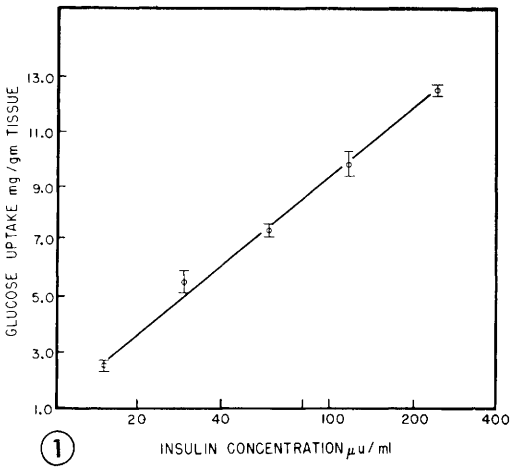
Accurate measurement of the insulin or "insulin-like" activity of biological fluids has been one of the major problems confronting endocrinological investigators. Much progress has been made since the introduction of the method utilizing uptake of glucose by the rat diaphragm. Vallance-Owen and Wright (1) have recently reviewed the available procedures. Martin *et al.*(2) have reported that the $C^{14}O_2$ production from glucose-1-C^{14} by the rat epididymal fat body was useful. Recently Beigelman(3) and Humbel(4) have utilized uptake of glucose by the epididymal fat body to measure insulin-like activity of human serum. In our studies we have modified the experimental design of the method using glucose uptake by epididymal tissue. This has resulted in a marked increase of accuracy and made the method readily amenable to statistical analysis. Although the present method was primarily designed to study the effects of various agents on the action of insulin, it should be extremely useful in determining insulin or "insulin-like" activ-

ity in biological fluids.

Methods. Male Holtzman rats (110-135 g) fed a standard laboratory diet were used. Food was withheld 2 hours prior to assay. Six animals were killed by decapitation and the epididymal fat bodies removed and placed in oxygenated Krebs-Ringer bicarbonate buffer(5) keeping the tissues from each animal in separate beakers. All handling of tissues was conducted with great care and as rapidly as possible. Eighteen 10 ml beakers were prepared, each with 2.0 ml of oxygenated Krebs-Ringer bicarbonate solution containing glucose (150 mg %), crystalline bovine serum albumin (250 mg%), and the appropriate amount of insulin or other substance to be assayed. In cases where sera were used, dilutions were made with oxygenated Krebs-Ringer Albumin Glucose (KRAG). It is highly desirable to determine concentration of glucose in the serum prior to bioassay and adjust the glucose concentration to approximately 150 mg%. It has been found feasible to reduce the incubation volume to 1.25 ml and thus conserve serum. Consequently, it is possible to obtain a 2-level assay on approximately 3 ml of serum using 1:3 and 1:12 dilutions. Eight pieces of tissue approximately 8-10 mg each were cut from the distal portion of each fat body and randomly distributed so that each of the 16 beakers had one piece of tissue from every animal. The design was similar to that used by Saffran and Schally(6) for *in vitro* assay of ACTH. Duplicate samples for each dosage level were used with 2 levels each of standard and unknowns with a 4-fold dosage increment. Two beakers contained only KRAG and served as controls for calculating uptake. With a little experience it was possible to obtain total tissue weights in each beaker within a range of ± 5-8 mg. The beakers, including the KRAG controls, were incubated in a Dubnoff metabolic shaker for 3 hours at 37-38°C using an oxygenation mixture of 95% oxygen and 5% carbon dioxide with a flow rate of 5 cubic feet per hour. At the end of incubation period the tissues were removed, blotted gently and weighed. Glucose concentrations of the incubation fluids were determined by the Somogyi-Nelson(7,8) procedure, or the ferricyanide method employing a Technicon Autoanalyzer. The latter has made it possible to double the number of assays with the same expenditure of time and personnel. Suitable control beakers should be run for each dilution of serum as well as the KRAG. Values were expressed as milligrams glucose uptake per gram of tissue.



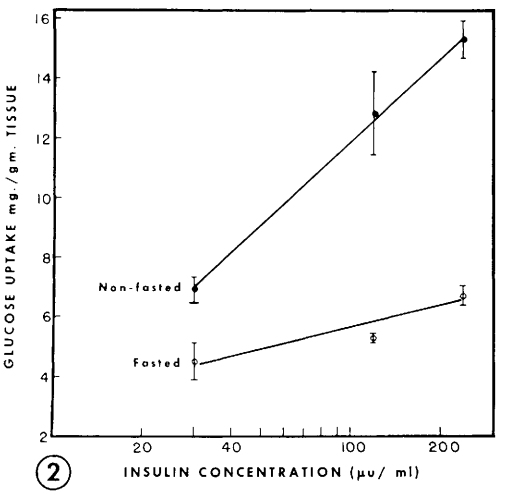


FIG. 1. Effect of insulin on uptake of glucose by the epididymal fat body. Individual points on curve represent avg response of duplicate samples together with indicated range.

FIG. 2. Effect of fasting on response of adipose tissue to insulin.

Resutls. Fig. 1 gives the dose response curve for an experiment using standard crystalline insulin (23.5 u/mg) at concentrations between 15 and 240 microunits/ml. It has been shown that higher concentrations give

TABLE I. Determination of Insulin-Like Activity in Dog Serum.

Animal No.	Serum dilutions	Insulin-like activity (μu/ml)	95% confidence limits	Index of precision (λ)*
1808	1:2, 1:8	178	105-251	.10
	1:2, 1:8	163	107-219	.08
1818	1:3, 1:12	148	118-178	.05
	1:3, 1:12	120	47-193	.14
1978	1:3, 1:12	120	50-190	.13
	1:3, 1:12	137	66-208	.12
	1:2, 1:8	149	41-257	.17

* $\lambda = \frac{s}{b}$.

essentially the same uptake as 240 microunits/ml. In fact, in many studies 120 microunits/ml was maximal. The tissue control (no added insulin) value in the above experiment was 2.26 mg/g. In other experiments we have found that sensitivity can be as low as 3.75 microunits/ml, particularly when uptake of glucose has been measured by means of uniformly labelled glucose-C^{14}. Experiments using glucose-C^{14} were found to parallel in all respects the results obtained with the above method.

To ascertain the usefulness of the assay in determining insulin-like activity in serum, 3 female beagle dogs were starved 16 hours and sufficient blood withdrawn from the jugular vein to conduct replicate assays on the sera. The sera were assayed for glucose and adjusted to approximately 150 mg %. Aliquots were then frozen for future assay. Table I summarizes the data on determination of the activity of the samples. Reproducible potencies with a high degree of precision can be obtained for a given sample. All of the assays cited were statistically valid in that there was no divergence in slope between the standard and unknown. In occasional samples a significant divergence can be noted and the assay has to be considered fundamentally invalid.

Assay limitations and calculations. On the basis of these data together with the previously reported validity of the response, it may be concluded that the method is useful for measurement of insulin or "insulin-like activity" with an excellent degree of precision.

To obtain pertinent information as easily as possible a simplified method of calculation has been devised, based upon the assumption that duplicate samples at each of 2 concentrations of standard and test preparation are employed and that the dosage increment is 4-fold. The factors used are constants valid only for these conditions. Factors for other conditions may be estimated from normal bioassay theory for symmetrical assays using the range to estimate standard deviation.

Let S_L, S_H, T_L, T_H = sum of duplicate responses of low and high doses of standard and test preparations respectively.

$$\text{Then } K = \frac{T_H + T_L - S_H - S_L}{T_H - T_L + S_H - S_L}$$

W = Sum of differences between duplicates of all doses.

$$I = \frac{\text{High Dose Std.}}{\text{High Dose Test}}$$

Relative Potency (R.P.) = I antilog 0.6 K

Index of Precision (λ)

$$\lambda = \frac{0.53\,W}{T_H - T_L + S_H - S_L}$$

$$V = \frac{W}{T_H - T_L + S_H - S_L}$$

$$F = 2.3\,V\sqrt{1 + K^2}$$

Confidence Limits (95%) = R. P. $\pm$ F (R. P.)

To test for parallelism between dose response curves of standard and unknown:

$$|P| = |T_H - T_L - S_H + S_L|$$

If $|P|$ is equal to or greater than $\frac{5W}{3}$, there is a significant lack of parallelism and the assay is fundamentally invalid. A significant lack of parallelism would indicate either that the response(s) for one of the preparations is outside the linear range of response, or that other factors such as inhibitors or potentiators are present which may modify the response of that preparation.

The usual index of precision (λ) for the assay is 0.15 or less. Occasional assays have given values of 0.05-0.07.

Discussion. To determine the validity of bioassays, it is important to be able to sub-

ject each assay to rigid statistical analysis. This is not possible with the majority of assays for "insulin-like" activity. In the case of the adipose tissue methods Beigelman(3) and Martin *et al.*(2) employ systems which compare response of one epididymal fat body to insulin and the other to the diluted or undiluted serum. This gives no information as to whether responses obtained in a given experiment are qualitatively similar. Serial dilutions of standard and unknown are necessary together with a measurement of the variability of the response. In the experimental design herein presented these factors have been taken into consideration and an assay method presented which is reproducible and accurate. A multiple dose level assay can be conducted on 3-4 ml of serum and a relative potency determined with confidence limits.

After this work was essentially completed Humbel(4) reported a method similar in design. Using animals fasted for 24 hours he found that the utilizable assay range under his conditions was 10-1000 microunits/ml in contrast to 15-240 microunits/ml reported here. From his data the assay slope was estimated to be 1.5-2.0. In contrast the authors analyzed 6 consecutive assays (non-fasted animals) conducted just prior to submission of this manuscript and found an average slope of 11.2 ± 1.2. A comparison of the responsiveness of epididymal fat bodies from 24 hour fasted and non-fasted animals was also conducted. Fig. 2 shows the marked difference in the slopes of the responses. The steeper slope increases the precision of the assay materially. The estimated lambda value $\left(\lambda = \frac{s}{b}\right)$ using Humbel's data was 0.3 or greater, whereas the present method consistently gave lambda values less than 0.15.

In addition to the length of fasting there were other differences: (a) the useful body weight range (110-135 *vs.* 120-160 g), (b) crystalline bovine serum albumin instead of gelatin in the incubation medium, (c) 150 mg % of glucose *vs.* 200 mg %, and (d) number of animals used for each assay (6 *vs.* 4). The use of 6 animals permits the potency determination of 3 samples per assay.

Summary. A highly reproducible and precise method for determination of "insulin-like" activity using glucose uptake by the epididymal fat body has been presented. It permits the assay of "insulin-like" activity in as little as 3 ml of serum with a calculation of relative potency with confidence limits as well as a test for assay validity.

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Properdin Titers in Sera from Germfree Rats.* (26190)

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Pillemer *et al.*(1) showed that normal human sera and other mammalian sera contain a protein, properdin, which in conjunction with complement and Mg^{++} constitutes a natural defense mechanism of the blood. They were of the opinion that properdin was a

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