Summary. A microculture medium for the culture of white blood cells has been utilized in which dextran-glucose has been substituted for plasma enrichment. The metaphase index obtained with this medium utilizing human and guinea pig blood as the inoculum has been almost as high as with plasma enrichment. It would appear to be particularly useful for the study of the influence of various plasma constituents on cell growth in tissue culture, as well as for the effects of a variety of substances which may be inactivated by serum or plasma inhibitors.

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Glucose Concentration and Insulin Dose-Response Curves Using Rat Fat Tissue *in vitro*.* (29411)

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Very few variables have been described as influencing the sensitivity of the response of rat epididymal fat *in vitro*. These variables have been confined either to variations between animals and between different portions of epididymal fat(1), or to the effect of fasting and refeeding rats before sacrifice (2). The sensitivity of the insulin dose-response curve is here defined as the degree of change in the metameter of response produced by a small increase in low concentrations of insulin, *i.e.*, the steeper the slope of the standard curve, the greater the sensitivity. In these terms, rat epididymal fat pad is currently recognized as the most insulin-sensitive tissue available for *in vitro* studies. Despite the increasing use of the fat pad assay, there appears to be no rational basis for the choice of glucose concentration (200-300 mg%) advocated in the literature. As there appears to be no study of a potentially major variable, glucose concentration *in vitro*, the present study was designed to test insulin sensitivity at glucose concentrations *in vitro* equivalent to hyperglycemia and hypoglycemia in the rat or in man. In addition, a modified assay superior to its original in terms of the index of precision is described, and its value assessed.

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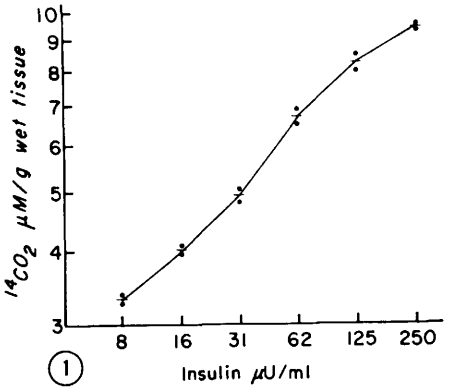
Methods. The basic insulin dose-response method of Renold *et al*(1), measuring $^{14}CO_2$ formation from ^{14}C-1-glucose was used, but the pool design proposed by Froesch *et al* (3) was adopted to minimize the error due to animal variation in any experiment. All experiments were performed in one month from December 19, 1963 to January 21, 1964, a stable period with regard to rat variation in this laboratory. Six Wistar rats, fed *ad lib* on Purina rat chow, differing less than 15 g by weight (range 140-180 g), were chosen for each dose-response curve. Each epididymal fat pad was cut into 6 pieces, so that 12 flasks received a pool of fat from all 6 rats, with an even distribution of pieces from each pair of fat pads(3). The wet weight of fat tissue per flask ranged from 80 to 140 mg in all experiments, and the weights of tissue for determination of each dose-response curve usually agreed within 25 mg. The incubation medium was Krebs-Ringer bicarbonate buffer containing 200 mg% gelatin(1) and varying concentrations of glucose. Each flask contained 2 ml of

incubation medium, with the exceptions described. Incubation(1) was shortened to one hour to reduce potential interference in the dose-response by (i) possible degradation of insulin which could affect the lower doses of insulin disproportionately, and (ii) glucose consumption, in view of the low initial glucose concentrations tested.

$^{14}CO_2$ radioactivity was determined as described by Steinke *et al*(4), except that naphthalene-dioxane-PPO†(5) was used as scintillation solution, instead of toluene with PPO† and POPOP†. Crystalline pork insulin,‡ (Lilly, lot No. PJ5589, 23.9 U/mg) was used as standard insulin.

Results. Precision of the assay. The assay design reduced inter-animal variation for each particular pool, so that the precision of the measurement of $^{14}CO_2$ production (cpm/mg wet weight) appeared to approach the limit of errors expected in weighing tissues and counting radioactivity. Fig. 1 shows a standard dose-response curve with good duplicates. The log counts per minute (shown as μM CO_2/g wet weight of fat tissue in Figures and Tables) were subjected to an analysis of variance to derive *s*, the residual standard deviation. The very low residual deviation ranged from 0.018 to 0.037, mean 0.026, in 23 standard curves, reflecting the excellent agreement between duplicates. The standard slope, *b*, was calculated in 13 assays with an initial glucose concentration of 75 mg% or more, and an insulin dose range of 16 to 125 μU§/ml, as strict linearity was reproducible only over this range. The mean *b* value was 0.417, S.D. $\pm$ 0.046. When the initial glucose concentration was 25 mg%, *b* calculated between 8-62 μU/ml was virtually identical, 0.41, S.D. $\pm$ 0.06 in 3 curves. The excellent index of precision λ (16-125 μU/ml or 8-62 μU/ml) which equals s/b in these dose-response curves, ranged from 0.041-0.091, (mean 0.060) and is attributed to the significant reduction in *s*, in comparison with *s* values obtained in this laboratory and elsewhere with other assay designs (6). Three curves with λ values of 0.112-0.128 were discarded.



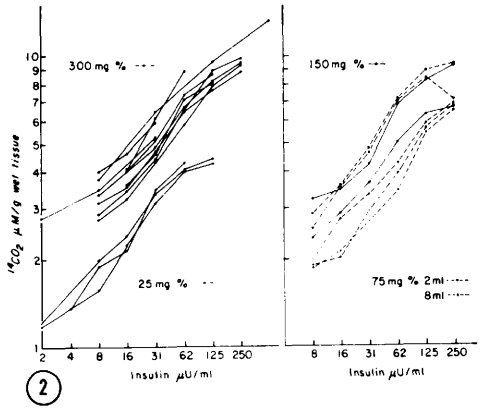


FIG. 1. Log dose-response curve. Each point shows the log counts in one flask (converted to μM CO_2/g wet weight of tissue). Horizontal line shows the mean of duplicate values. Initial glucose concentration = 300 mg%, b = 0.34, s = 0.019, λ = 0.056, μU = microunits, μM = micromoles.

FIG. 2. Log dose-response curves with different glucose concentrations. Glucose concentrations are indicated above in mg%. Each point represents mean of duplicate values.

Effect of glucose concentration. The characteristic shape of the log dose-response curves was sigmoid and remarkably similar, with whatever metameter of $^{14}CO_2$ (log or square root of cpm/unit weight of tissue) was used (Fig. 1, 2). This classical log dose-response curve does not, to our knowledge, appear so clearly in other reported fat pad-dose response curves. A higher specific activity of ^{14}C-1-glucose ensured optimal counting efficiency of $^{14}CO_2$ at low concentrations of glucose. Increasing the volume

† PPO = 2,5-Diphenyloxazole
POPOP = p-bis[2-(5-Phenyloxazolyl)]-benzene.

‡ Kindly donated by Dr. Mary A. Root, Lilly Research Laboratories.

§ μU = microunits

TABLE I. Effect of Low and High Glucose Concentrations on $^{14}CO_2$ Production. Each set represents pooled fat tissue from 6 rats in 12 flasks. Each value for CO_2 in $\mu M/g$ represents the mean value of 3 flasks. The difference between ratios A and B is not significant ($p > .10$ by the paired "t" test).

		Glucose, 25 mg %			Glucose, 300 mg %		
		Insulin (μU*/ml)			Insulin (μU/ml)		
	Set No.	0	10	Ratio (A) (10 μU/0 μU)	0	10	Ratio (B) (10 μU/0 μU)
		CO_2, $\mu M/g$			CO_2, $\mu M/g$		
1/15/64	I	1.04	1.71	1.64	2.50	3.63	1.45
	II	.99	1.54	1.56	2.35	3.20	1.36
	III	1.04	1.86	1.79	2.59	3.67	1.42
1/21/64	I	1.58	2.30	1.46	3.99	5.35	1.34
	II	1.35	2.36	1.74	3.17	4.56	1.44
	III	1.29	2.08	1.62	2.78	4.50	1.62
	Mean			1.64			1.44
	S.E.			.05			.04
	Significance			$p < .005$†			$p < .01$†

* Microunits.

† Significant effect of 10 μU/ml insulin on $^{14}CO_2$ production (probability that observed ratio could be a random sample from a population with a true ratio of 1.00).

of buffer solution from 2 to 8 ml with an initial glucose concentration of 75 mg% did not affect the dose-response curves (Fig. 2), suggesting that the flattening of the upper portion of the curve is not related to lack of available glucose because of excessive consumption of glucose at an insulin dose of 250-500 μU/ml. The absolute amount of $^{14}CO_2$ production is greater with higher initial glucose concentrations, confirming the observations of Jeanrenaud and Renold(7). This effect *simply displaces the curve upwards* (Fig. 2) *and is irrelevant in assessing sensitivity,* as defined above, in terms of insulin dose-response.

Comparison of the standard slopes using different glucose concentrations showed no statistical difference in *b* ($p > 0.1$) for the linear portion of the curves. Visual comparison of the shapes of the curves obtained with different glucose concentrations suggested that the lower initial glucose concentrations caused a small "shift to the left" (Fig. 2) appearing as (i) an early flattening of the curve at higher insulin doses and (ii) an increase in the slope with low insulin doses. A more stringent test of comparative sensitivity at the lowest insulin concentrations showed that sensitivity to 10 μU/ml was not statistically different ($p > 0.1$ by paired t test) by an initial glucose concentration of 25 mg% as opposed to 300 mg% (Table I).

Discussion. In these studies dealing with the stimulation by insulin of glucose-1-^{14}C oxidation, observations on the precision of the dose-response curves have been brief and pertinent to the aim of these studies (*i.e.*, testing sensitivity at the lowest possible insulin dose). The superior precision of a pool design, previously shown for glucose uptake (8) and net gas exchange(3), was confirmed using ^{14}C-1-glucose oxidation as the metabolic index. It is important to question whether the excellent index of precision described really increases the usefulness of this bioassay for insulin in biological fluids. The narrowing in fiducial limits by reducing *s*, has not, in our hands, alleviated the fundamental limitations of this assay method for serum insulin. This bioassay is not specific for insulin. Much of the "insulin-like" activity in sera from normal fasting humans (3) or diabetic dogs dying after pancreatectomy(9) does not behave like insulin. Therefore, there seems to be little reason for using serial dilutions of insulin as a standard curve unless serial dilutions of fasting human serum show a parallel slope. We have not been able convincingly to prove or disprove such parallelism. The advantages of the pool design for insulin may well be confined to the measurement of minute quantities of highly purified insulin.

Comparison of our results on insulin sensi-

tivity at different glucose concentrations with the data of Jeanrenaud and Renold(7) is not practicable. Their results were originally misinterpreted by us as suggesting that low glucose concentrations might increase insulin sensitivity as the ratios of CO_2 production at 100 μU/ml of insulin/basal CO_2 production were: 1.78, 1.42, 1.04, and 0.90 with glucose concentrations of 22.5, 90, 360, and 1440 mg% respectively. These authors attributed the lack of a significant insulin effect at the higher glucose concentrations to inter-animal variation. Our present results suggest that the increase in ratio with decreased glucose concentrations in their data may be explained in a similar fashion. The shape of our dose-response curves suggested a possible increase in the sensitivity for doses of less than 16 μU of insulin/ml at low glucose concentrations. However, a better test minimizing inter-animal variation showed no statistical increase in sensitivity, even though the ratio used to express sensitivity (Table II) is usually higher with 25 mg% glucose than with 300 mg%.

This striking similarity in all the dose-response curves appears to be the first evidence suggesting that insulin sensitivity of rat adipose tissue is relatively independent of glucose concentration *in vitro*. Conclusions about the physiological significance of the family of $^{14}CO_2$ dose-response curves are necessarily limited by lack of knowledge of the relationship between glucose entry into the cell, glucose oxidation, and fat synthesis at the different glucose concentrations. Although $^{14}CO_2$ production indicates entry and metabolism of ^{14}C-1-glucose within the fat cell, glucose uptake and metabolism may not be proportional to $^{14}CO_2$. Our view is that these studies cannot distinguish between rate-limiting processes governing glucose oxidation, with respect to transport, phosphorylation, or other metabolic steps without unwarranted assumptions and speculation. However, by using CO_2 as an index of insulin sensitivity, it has been shown that the quantitative increase in CO_2 associated with the increase in initial glucose concentration is irrelevant in assessing insulin sensitivity and that glucose concentrations of 25 to 300 mg% clearly do not modify insulin sensitivity.

Summary. A pooled design for adipose tissue bioassay measuring $^{14}CO_2$ production permitted an index of precision of 0.041-0.091. Comparison of dose-response curves at various glucose concentrations from 25 to 300 mg%, showed very similar classical sigmoid-shaped curves, with similar slopes. The insulin sensitivity of rat epididymal fat, in terms of the oxidation of glucose-1-^{14}C to $^{14}CO_2$, is independent of glucose concentration (25-300 mg%).

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