nomena are related. Perhaps the large initial inhibition of leucine catabolism compensates for whatever anabolic inhibition is occurring. Since less leucine is being degraded, more is available for incorporation and this effect counteracts the process of inhibition of protein synthesis. Once the catabolic inhibition reaches a plateau, the compensation no longer exists, and the inhibition of protein anabolism can assert itself. This hypothesis represents one possibility.

Another explanation is offered for the peculiar instance of PEBG-induced inhibition of protein incorporation. It is possible that there is present in the medium an unknown substance which reacts with the PEBG to form an inactive complex; inactive toward inhibition of protein synthesis, it would be active toward inhibition of CO_2 release. Once 8 μmoles of PEBG were added, all of the unknown substance would be complexed and further addition of PEBG would result in the sudden drop in incorporation seen in Fig. 2. It was originally thought that this unknown substance might be glucose, since there are 8 μmoles of glucose present in each incubation flask. The data in Table IV show the fallacy of this assumption.

Summary. Leucine-C^{14} was utilized to study the affects of 2 oral hypoglycemic agents (tolbutamide and phenethylbiguanide) on protein metabolism. Both compounds were seen to inhibit $C^{14}O_2$ production and incorporation of the amino acid into protein by rat liver homogenate. Differences in the mode of inhibition are noted and discussed. Neither compound appears to be dependent on the presence of glucose for its *in vitro* affects on protein metabolism.

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On Insulin Immunologic Activity in Livers and Kidneys of Mice Injected with Beef Insulin.* (31248)

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In mammals studied to date the I^{131} of intravenously administered Insulin-I^{131} has been found to concentrate rapidly in liver and especially in kidney, and to pass somewhat less rapidly from an original TCA insoluble state to a TCA soluble state(1,2,3,4,5). In the case of liver, the highest concentration of TCA insoluble I^{131} was shown to be in the mitochondrial and microsomal fractions(4). The decreased TCA insolubility of the I^{131} was accompanied by a loss of insulin biological (hypoglycemia-inducing) activity(2). These findings have been interpreted by some (3) to indicate that intact insulin molecules are capable of penetrating rapidly into and being concentrated by liver and kidney cells. A number of arguments have been advanced by Krahl(6), Kallee(7) and others that this interpretation has not been proved. One argument is that the TCA insoluble I^{131} found in liver and kidney after Insulin-I^{131} administration may not represent intact insulin mole-

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cules. In support of this argument it may be noted that following i.v. injection of radioiodine labeled insulin, plasma insulin values estimated by bioassay(8) or by immunoassay (9) decreased appreciably more rapidly than did plasma insulin values which were estimated by assuming that all TCA insoluble radioiodine represents undegraded insulin.

A second argument has been that I^{131}, whether or not attached to insulin, might have been present in the main extracellularly at time of sacrifice, and have been found at high concentration in mitochondrial and microsomal fractions only because of post-mortem redistribution. A third argument has been that *in vivo* metabolism might be quite different for iodinated and non-iodinated forms of insulin. This paper presents data bearing on these arguments.

In the present experiments sacrifice was made and tissue samples were taken 2 to 25 minutes after i.v. injection of beef insulin, prepared to contain 19 equimolar parts of unlabeled insulin for each part of Abbott Co. Insulin-I^{125}. Insulin concentration per tissue sample was estimated by two independent methods. In the one, tissue insulin concentration was inferred from count of I^{125} believed to be still attached, at least in the main, to insulin. In the other method, tissue insulin concentration was estimated by immunoassay, using guinea pig anti-insulin serum (AIS) and Insulin-I^{131}. It was arranged that the I^{131} counting rate should not be affected by presence or absence of I^{125}. The premises on which this paper is based are that: (a) I^{125} counts give direct information only about administered I^{125} molecules, (b) insulin immunoassay values of this paper refer mainly to unlabeled insulin molecules since most of those injected were unlabeled, and (c) insofar as the two kinds of insulin values agree, to that extent it may be inferred that I^{125} labeled and unlabeled beef insulin molecules are metabolized in similar manner.

Materials and methods. Reagents. Acid Alcohol was prepared by placing 3.75 ml concentrated HCl in a 250 ml volumetric flask and adding 75% ethanol to the mark. All other solutions were prepared, w/v, using glass distilled water. *Heparin* contained 10 mg powder per ml 0.9% NaCl. *Sucrose* (0.25 M) contained 4.95 g of the monohydrate/100 ml. *AT* contained 0.2% beef albumin and 0.1 M TRIS buffer of pH 7.2 at 25°C. *TCA* contained 10 g crystals/100 ml. *Injection Insulin* was prepared by mixing together *AT*, Abbott Co. beef Insulin-I^{125} (original specific activity >10 mc/mg) and Eli Lilly beef insulin, low in glucagon, in such a manner that each ml contained 1,000 mμg insulin derived from the Abbott Co. preparation and 20,000 mμg insulin altogether. *AIS* consisted of high titer guinea pig anti-insulin serum(10) diluted as desired with AT. Insulin-I^{131}, 10 mμg insulin per ml (on assumption that Abbott Co. data were correct) was prepared by appropriately diluting with AT Stock Abbott Co. beef Insulin-I^{131} of original activity about 10 mc/mg. This was used only in the immunoassays, wherein it competed for *AIS* with *Injection Insulin* which was present in standards or unknowns. *Cellulose Slurry* consisted of a 10% by weight suspension of MN cellulose powder 300 (Brinkman Instruments, Inc.) in 0.1 M TRIS buffer (Sigma) of pH 7.2 (25°C). *Insulinase* was prepared by homogenizing rabbit liver with ice-cold Isotonic Sucrose, 1.8 ml/g liver, and centrifuging the homogenate at $>105{,}000$ *g* and near 0°C for 15 minutes. The supernatant Cell Sap so obtained was held frozen. Just before use in those immunoassays in which Insulinase was used this Cell Sap was diluted 1:1 with 0.3 M TRIS buffer of pH 7.8 at 37°C.

Treatment of mice in the in vivo *insulin distribution experiments.* Webster strain male mice of 24-34 body weight and age 2-5 months were used. Thirty minutes before scheduled time of sacrifice each mouse was injected i.p. with Heparin, 0.01 ml/g. Each mouse was also injected by tail vein with *Injection Insulin,* 0.005 ml/g (total insulin dosage, 100 mμg/g), at 2, 5, 10 or 25 minutes before sacrifice. Three or four mice were used per sacrifice period. Before insertion of the 27-gauge needle the tail was held for a few seconds in water at 50-60°C; this increased to nearly 100% the chances of having a satisfactory injection. On sacrifice by decapitation, the blood was collected through a funnel into an ice-chilled centrifuge tube;

centrifugation of blood to obtain plasma was carried out at 4°C. Processing of excised tissues was carried out as described below.

Procedures in the whole tissue insulin concentration studies. Livers, kidneys, and plasmas from mice of a given sacrifice group were pooled separately. Each excised liver and kidney sample was frozen on dry ice immediately on excision. All tissues were held frozen to time of analyses. Each pooled liver or kidney sample was homogenized with Sucrose, 4.3 or 9.3 ml/g (assumed dilution, 5× or 10×). An aliquot of each tissue homogenate or diluted plasma sample was mixed 1:1 with TCA to give a precipitate. Each TCA precipitate was extracted twice for 30 minutes at room temperature with Acid Alcohol. The two acid alcohol supernatants per precipitate were combined and brought to desired volume. Using a biodryer (combined centrifugation and lyophilization), 0.5 or 1 ml of each acid alcohol extract was brought almost to dryness. It is in our experience crucial: (a) that complete dryness not be attained, since well dried samples failed to exhibit insulin immunological activity, and (b) that only traces of HCl and alcohol be left since either agent is capable of drastically altering the position and shape of the immunoassay curves. Each lyophilizate was shaken thoroughly with 4 or 5 ml altogether of AT and the mixture held at 4°C overnight. The supernatant obtained on centrifugation was called the AT extract. Appropriate aliquots of each such extract were used in the immunoassay and I^{125} insulin estimations.

Preparation of ultrasonic-treated liver mitochondrial-microsomal (MM) fractions. Each liver sample used was homogenized with ice-cold isotonic sucrose, and the particles between 700 *g* and 105,000 *g* obtained using a Spinco Model L ultracentrifuge. The particles were dispersed in ice-cold isotonic sucrose, a second centrifugation at 105,000 *g* for 60 minutes was carried out, the particles were dispersed again in isotonic sucrose, and each such dispersion was subjected to ultrasonication.

Radioisotope counting. Counts were obtained using 1 ml fluid or suspension per counting tube, a TracerLab Gamma/Guard spectrometer, Model GG-5331, and a setting appropriate for I^{125} or I^{131}. When I^{125} was counted, I^{131} was not present; when I^{131} was counted, whatever I^{125} was present gave no counts. Correction for quenching was not found necessary.

Insulin immunoassay procedures. The "Insulinase" method used in the present liver cell fractionation studies has been described (11). Details are given below of the method, herein called the Cellulose method, which was used for insulin immunoassay in the present whole tissue insulin concentration studies. This cellulose method is a modification to the insulin mμg range of the cellulose slurry method for separating AIS bound and free forms of both labeled and unlabeled insulin molecules described by Wright and Rivera-Calimlina (12). Their procedure is in turn an adaptation to the centrifuge tube of the classic Berson and Yalow method (13) for unlabeled insulin immunoassay, in which: (a) Insulin-I^{131} and unlabeled insulin compete for AIS, and (b) AIS bound and free forms of Insulin-I^{131} are separated and counted on paper.

Insulin standards were prepared in AT, or in an appropriate tissue extract or fraction which had been brought into AT, and which had been prepared using tissue from control mice (not injected with insulin). Solutions and immunoassay tubes were precooled in an ice bath. In each tube we placed: (a) 1 ml of AT (Blank) *or* insulin standard *or* unknown, and (b) 0.1 ml of AIS diluted with AT (7000× in Exp. 1; 6000× in Exp. 2). All tubes were held at 4°C for 24 hours. We then added to each tube 1 mμg Abbott Co. Insulin-I^{131} in 0.1 ml AT. After additional incubation at 4°C for 18 hours, 1 ml cellulose slurry was added per tube. The tubes were held at room temperature for 30 minutes and agitated vigorously at intervals. Supernatants above packed cellulose were obtained by 20 minutes' centrifugation at near 0°C and 2000 *g*. I^{131} present in 1 ml of each supernatant was then counted; for each unknown the I^{131} count obtained was used to read off amount of "unlabeled" insulin in that tube using the Standards curve of that experiment (Fig. 1). This amount times the

dilution factor (Tables I and II) then gave an insulin immunoassay value for "unlabeled" insulin concentration per g tissue.

Tissue insulin values based on I^{125} criteria. Mirsky *et al*(2) found that the action of tissues, etc. in converting the I^{131} of Insulin-I^{131} from TCA insoluble to TCA soluble condition ran parallel with their action (through "insulinase") to destroy insulin hypoglycemic action. They and many others(1,3,4,5) have used tissue action in converting I^{131} from TCA insoluble to TCA soluble condition as a

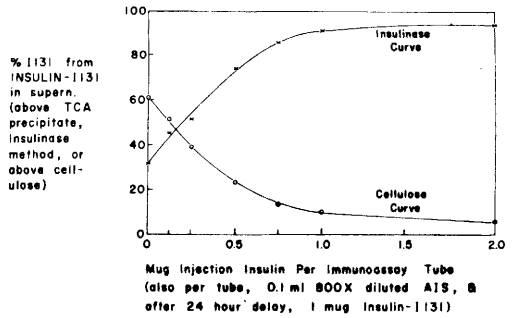

FIG. 1. Insulinase and cellulose insulin immunoassay curves prepared simultaneously.

TABLE I. Recoveries of Beef Insulin Mixed with Mouse Plasma, or with Mouse Liver or Kidney Homogenate, with These Then Subjected to TCA Precipitation, Acid Alcohol Extraction, etc.

		mμg of insulin			
		Estimated by immunoassay		Estimated from I^{125} counts‡	
Sample designation*	Dilution factor† (a)	In AT aliquot used (b)	In original mixture (a) × (b)	Using AT extract	Using TCA insoluble I^{125} extr. into acid alcohol
PC500	5000	.090	450	450	526
	2500	.205	512		
LC500	2500	.17	425	409	477
KC500	2500	.184	460	421	493

* C indicates that plasma (P), liver (L), or kidney (K) was obtained from control mice; 500 indicates that 500 mμg altogether of beef insulin was added (19 parts unlabeled, 1 part Abbott Insulin-I^{125}).

† 1/g of original tissue from which insulin estimated by immunoassay could have come.

‡ Values have been calculated on the assumption that I^{125} labeled and unlabeled insulin molecules passed through the extraction into AT in identical manner.

TABLE II. Insulin in Tissues of Mice Injected i.v. with Beef Insulin, as Estimated by Immunoassay, and by Recovery of I^{125} Administered as Insulin-I^{125}.

		Tissue insulin concentration ratios‡			
		From insulin immunoassay estimations (AT extracts)	From I^{125} counts		% of tissue I^{125} which was TCA insoluble (that inj, 90.5%)
Sample designation*	Dilution factors in immunoassays†		Using AT extracts	Using original TCA insoluble I^{125}	
2P	1000; 400	4.68; 3.80	2.91	3.88	92.2
5P	1000; 400	1.80; 1.88	1.64	2.26	83.7
10P	1000; 400	.77; .50	1.00	1.36	61.2
25P	1000; 400	.35; .30	.49	.60	32.0
2L	625; 250	5.06; Off curve	1.34	2.98	90.5
5L	625; 250	2.69; 2.06	1.19	2.04	80.1
10L	625; 250	1.03; .72	.67	1.42	72.9
25L	625; 250	.59; .48	.30	.57	59.5
2K	2500; 1000	6.25; 7.48	4.54	5.76	92.5
5K	2500; 1000	11.79; 10.32	8.79	11.81	87.9
10K	2500; 1000	12.06; 7.75	6.44	8.73	80.7
25K	2500; 1000	2.20; 1.15	1.80	2.66	63.7

* 2, 5, 10 or 25 = minutes between i.v. injection of *Injection Insulin* and sacrifice; P, L or K = plasma, liver or kidney respectively.

† 1/g of tissue from which insulin estimated by immunoassay could have come.

‡ For immunoassay estimations, concentration ratio = mμg insulin found per g tissue/100 mμg insulin inj per g mouse; for I^{125}, concentration ratio = CPM found per g tissue/CPM of TCA insoluble I^{125} inj per g mouse.

convenient measure of tissue "insulinase" activities, and TCA insoluble I^{131} as a convenient approximate measure of intact exogenous insulin. In the present experiments we have in parallel manner used TCA insoluble I^{125}, derived from administered or added beef Insulin-I^{125}, as measure of the maximum amount of unaltered exogenous insulin which could be present in each particular mouse tissue extract or fraction prepared. The equation used was:

$$\text{m}\mu\text{g insulin} = \frac{\text{TCA insoluble } I^{125} \text{ per tissue aliquot}}{\text{TCA insoluble } I^{125} \text{ per m}\mu\text{g injection insulin}}.$$

Implicit in the use of this equation is the assumption that radioiodinated and unlabeled beef insulin molecules are metabolized in the same manner. Original TCA insolubility of the Abbott Co. Insulin-I^{125} preparations used varied from 84% to 96% (Tables). The AT extracts contained no measurable TCA soluble I^{125}; hence I^{125} criteria insulin values reported for these extracts are based on total I^{125} counts.

Results. Fig. 1 shows typical immunoassay curves of the present experiments, used in estimation of the immunoassay insulin values of the Tables. The I^{131} counted was that present in supernatants. The Insulinase curve has a positive slope because the I^{131} present in the supernatants was derived only through action of insulinase on Insulin-I^{131} NOT bound by AIS. With increase in unlabeled insulin, less AIS became available to bind Insulin-I^{131}, therefore more I^{131} could be made TCA soluble by the enzyme. The cellulose curve has a negative slope because it was the I^{131} attached to AIS bound insulin which went into the supernatant above cellulose. With increase in unlabeled insulin, less AIS became available to bind Insulin-I^{131} and thus make this labeled insulin and its attached I^{131} resistant to adsorption by cellulose.

These curves were obtained using insulin standards prepared in AT, rather than through addition of insulin standards to a similar extract or fraction which had been prepared using tissues from control mice. This was possible, since use of an insulin dosage of 100 mμg per g mouse permitted us so to dilute potential tissue interfering substances prior to the immunoassays that control tissue insulin immunoassay curves differed only slightly from simultaneously prepared AT curves, and then not according to any recurring pattern. It should be noted that in all curves there was some scatter of points. Therefore, the immunoassay tissue insulin values of the Tables are only approximate, but still useful in relation to the overall purposes of this paper.

Table I presents data on recoveries of Injection insulin added to tissue homogenates and diluted plasma of control mice. These homogenate and diluted plasma samples were processed to secure AT extracts as described under *Materials and methods*. Most of the added insulin was recovered in the AT extracts. Each insulin value secured for a given AT extract through immunoassay was in fairly good agreement with the insulin value estimated for that same extract from its I^{125} count.

Table II presents data on mouse tissue insulin concentrations which were obtained in one of two experiments performed, both of which gave similar results. In these experiments mice were injected i.v. with Injection Insulin and sacrificed at times indicated. Correlation between immunoassay and I^{125} criteria insulin values of the AT extracts was not so good as had been the case when Injection Insulin had simply been added *in vitro* (Table I). In fact, for the liver extracts into AT, the immunoassay insulin values secured were so much higher than corresponding insulin values based on I^{125} counts as to suggest that mouse liver may accumulate unlabeled insulin molecules more avidly than I^{125} labeled insulin molecules. Otherwise, findings regarding insulin metabolism in the mouse were in the same direction, regardless of the assay method used: (1) insulin reached its highest concentration per tissue sooner in liver than in kidney, (2) insulin was far more highly concentrated by kidney than by liver, and (3) insulin showed decrease in concentration with passage of time in the order plasma>liver>kidney.

Table III presents data obtained for soni-

TABLE III. Accumulation of Insulin in 700 *g* to 105,000 *g* (MM) Fraction of Mouse Liver, Under Both *in vitro* and *in vivo* Conditions.

Experiment*	mμg injection insulin used		Concentration ratios† for insulin found per g liver			% TCA insoluble I^{125} in homogenate‡	
			By count of TCA insoluble I^{125}				
	Added per g liver	Inj per g mouse	Entire liver	MM fraction only	In MM fraction by immunoassay	At beginning (of cell fractionation process)	At end
In vitro, A	1500	—	.25	.011	.008	24.8	12.9
B	1500	—	.13	.003	.003	13.3	7.1
1st *in vivo*, 2′ sacrifice	—	100	1.65	.52; .51	.65; .70	83.2	—
2nd *in vivo*, 2′ sacrifice	—	100	1.39	.55	.53	67.1	49.4
2nd *in vivo*, 5′ sacrifice	—	100	1.03	.34	.20	62.2	45.6

* In the *in vitro* experiment, control mouse liver was homogenized with ice-cold isotonic sucrose, 8.3 ml/g. One volume of Injection Insulin, 1500 mμg total insulin per ml, was mixed with 9 vol of liver homogenate, to give an estimated final concentration of 1500 mμg insulin per g of original liver. In the B part of this experiment, the filled Spinco centrifuge tube was allowed to sit while all other procedures were carried out (TCA precipitation, filling of tubes to obtain total I^{125} counts, etc.). In the A part, the first centrifugation was started 2 min after filling of the Spinco centrifuge tube was completed. In the *in vivo* experiments the first centrifugation was begun as soon as possible after sacrifice.

† The mμg insulin found per g liver = Concentration ratio × 1500, the *in vitro* Exp., and × 100, the *in vivo* Experiments. Approximate estimations of mμg insulin per g of MM protein may be made by multiplying the proper concentration ratio(s) by 60,000, the *in vitro* Exp., and by 4000, the *in vivo* Experiments.

‡ In this Exp. 84% of the I^{125} of the Abbott Insulin-I^{125} used was TCA insoluble. The % TCA insoluble I^{125} at end values of Table were estimated as equal to $100\% \left(\frac{\text{Sum of TCA insoluble } I^{125}\text{, all fractions}}{\text{Sum of total } I^{125}\text{, all fractions}} \right)$.

fied mouse liver particles (Mitochondrial-Microsomal (MM) fraction). When Injection Insulin was added to control liver homogenate (*in vitro* Exp.), the insulin was mostly degraded before completion of the centrifugations. Regardless of the insulin assay procedure used, hardly any insulin was found in the washed 700 *g*-105,000 *g* fraction. However, in each of the *in vivo* experiments performed, a large fraction of the I^{125} found in the liver could be accounted for as insulin present in this MM fraction; this relation obtained whether the insulin value was based on TCA insoluble I^{125} present in this fraction or was secured through immunoassay.

Discussion. Arguments which have been advanced against the conclusion of Lee and Williams and their associates(1,3,4) that intact insulin molecules are capable of penetrating into and being accumulated by mammalian liver and kidney cells, may now be reconsidered. In our opinion the argument that the TCA insoluble radioiodine found concentrated in human and rat liver after radioiodoinsulin administration may not represent any significant amount of intact insulin has been refuted by the present finding that after radioiodoinsulin administration, mouse liver and kidney contained not only TCA insoluble radioiodine but also roughly equivalent amounts of insulin estimated by immunoassay. The argument that radioiodine might in the intact animal be concentrated at or in extracellular liver and kidney structures, and be found at high concentration in mitochondrial and microsomal structures only because of redistribution which had occurred after homogenization was for all practical purposes refuted by Lee and Wiseman(4) when they showed that the I^{131} of Insulin-I^{131} added to rat liver homogenates was rapidly transformed from TCA insoluble to TCA soluble condition and was not accumulated to any great extent by the mitochondria and microsomes, whereas such accumulation did occur when the Insulin-I^{131} was administered intravenously. In the present experiments we have confirmed their radioiodine distribution findings, and have also demonstrated that insulin estimated by immunoassay is accumulated by mouse liver 700 *g* to 105,000 *g* particles under *in vivo* but not under *in vitro* conditions.

The argument that insulin metabolism may be quite different for iodinated and non-iodinated forms of insulin would also appear to be at least partially refuted by present findings of rough parallelism between tissue exogenous insulin concentrations based on: (a) immunoassay and (b) I^{125} criteria.

Additional support for the hypothesis that intact insulin molecules penetrate into and are accumulated in significant amounts by mammalian liver cells is furnished by more recent work of Kallee(14). He perfused rat liver with Insulin-I^{131}, followed by human anti-insulin serum to wash out insulin molecules which might have been present extracellularly, and then isolated liver mitochondrial and microsomal fractions. These fractions contained I^{131} at high concentration. Much of this I^{131} was separated from the particles by incubating them with human anti-insulin serum; this I^{131} then moved electrophoretically in the same manner as the I^{131} of Insulin-I^{131} which had been reacted directly with this anti-insulin serum.

Summary. Mice were injected with a 19 to 1 mixture of unlabeled and I^{125} labeled beef insulins and sacrificed 2, 5, 10 or 25 minutes later. Insulin in especially prepared tissue extracts and fractions was estimated by: (a) immunoassay, and (b) I^{125} count. Using either assay procedure, insulin (1) reached its highest concentration in liver sooner than it did in kidney, (2) was far more highly concentrated by kidney than by liver, (3) showed decrease in tissue concentration in the order plasma>liver>kidney, and (4) was rapidly and highly concentrated in liver particles brought down between 700 *g* and 105,000 *g*. Insulin added to liver homogenate was not concentrated by these particles. These findings are in accord with the hypotheses that radioiodinated and unlabeled forms of insulin are metabolized in similar manner, and that both forms of insulin are capable of penetrating into liver and kidney cells.

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