Biochemical Pharmacology, Vol. 16, pp. 2337–2353. Pergamon Press Ltd. 1967. Printed in Great Britain

METABOLIC EFFECTS OF HYPOGLYCEMIC SULFONYLUREAS—I.

IN VITRO EFFECT OF SULFONYLUREAS ON LEUCINE INCORPORATION AND METABOLISM AND ON RESPIRATION OF RAT TISSUES*

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(*Received* 19 *July* 1967; *accepted* 28 *July* 1967)

Abstract—The hypoglycemic sulfonylureas: chlorpropamide, tolbutamide, carbutamide and glycodiazine were found to inhibit leucine incorporation into the protein of rat diaphragm, liver, kidney and adipose tissue. This inhibition was not the result of a reduced amino acid transport, an alteration in the metabolism of leucine or an impaired endogenous respiration of these tissues. A stimulation of oxygen uptake was observed in muscle and liver early during the incubation. Glucose and insulin relieve to some extent the inhibition of leucine incorporation by sulfonylureas and this could indicate that the inhibition of protein synthesis is related to an impaired energy metabolism. The oxidative decarboxylation of leucine, in the four tissues studied, is strongly inhibited by chlorpropamide and is mainly responsible for the increased cellular levels of leucine found in diaphragm after incubation with chlorpropamide and tolbutamide. The possibility of a link between sulfonylurea- and leucine hypoglycemia is discussed.

IT IS now well established that the clinically effective antidiabetic sulfonylureas induce, by an unknown mechanism, an increased release of pancreatic and possibly of extra-pancreatic bound insulin (for reviews see ref. 1–3). This effect which admittedly represents the main mechanism of action of these compounds is most prominent however after their acute administration and there seems to be no conclusive evidence of a sustained insulin synthesis and/or release during long-term sulfonylurea administration.[4–6] Furthermore, a number of hepatic and peripheral metabolic effects observed following the acute or chronic administration of sulfonylureas do not correspond to those observed after insulin.[7–10] For these reasons there remains a widespread belief that an enhanced release of insulin from the pancreas or from other sites does not constitute the sole and long-term mechanism of action of these compounds but that their therapeutic effect is, at least in part, due to a more direct action of the sulfonylureas on tissue metabolism. Several such *in vivo* and *in vitro* effects have been reported amongst which: inhibition of glucose[11–14] and amino acid nitrogen[15, 16] output by the liver, reduction of ketogenesis in diabetic patients and in liver slices from normal and diabetic rats,[17] increased incorporation of labeled bicarbonate into rat liver glycogen,[18] inhibition of lipolysis[19–20] and of the incorporation of acetate and mevalonate into cholesterol.[21]

In this paper we describe an *in vitro* effect of hypoglycemic sulfonylureas on amino acid incorporation into the protein of various rat tissues. While this work was in

* This work was supported in part by grants from the Belgian Medical Research Fund and from Chas. Pfizer & Co.

progress McDonald published his observations on the same subject.[22, 23] Our results confirm and extend his findings.

MATERIALS AND METHODS

Materials. L-Leucine-1-^{14}C (sp. act. 8·5–35 mC/mM) was purchased from the Radiochemical Center, Amersham, U.K. and used at concentrations ranging from 0·05 to 0·10 μC per ml of incubation medium. Glucagon-free crystalline pork insulin, obtained through the courtesy of E. Lilly and Co. (P-J-5589) was dissolved in 0·002 N HCl and used throughout at a concentration of 0·4 U/ml. Sulfonylureas were obtained through the courtesy of the following manufacturers: chlorpropamide from Chas. Pfizer and Co. Pfizer Ltd., U.K.; tolbutamide and carbutamide from Boehringer GmbH, Mannheim, Germany. Glycodiazin (2-Benzenesulfonamido-5(β-methoxy-ethoxy)-pyrimidin) which is not a sulfonylurea but is chemically related to this group of compounds was obtained from Schering A.G. Berlin as the Na-salt. Chlorpropamide, tolbutamide and carbutamide were dissolved in the minimum amount of N NaOH and made up to the desired concentration with either bicarbonate or phosphate buffer according to the experiments. PPO (2,5-diphenyloxazole). scintillation grade, was purchased from E. Merck A.G. Darmstadt, Germany. Hydroxide of Hyamine 10-X (1 M in methanol) was obtained from the Packard Comp. Ill., USA. All other chemicals were reagent grade.

Leucine incorporation. Male Wistar rats (125–200 g), maintained on stock laboratory diet were fed *ad libitum* before use. Animals were killed by decapitation, exsanguinated and the tissues rapidly removed and placed in cold incubation medium. Epididymal fat pads, after weighing if required, were directly transferred to the incubation vessels. Hemidiaphragms were removed by severing the central tendon and cutting around the sternal and rib boundaries. Unless otherwise indicated, one hemidiaphragm served as control for the other. Liver and kidney slices were prepared using a Stadie–Riggs microtome. Tissues were incubated in 2 ml Krebs–Ringer bicarbonate buffer containing the appropriate sulfonylureas and labeled leucine and adjusted to pH 7·3–7·4 by 30 min equilibration with 95% O_2–5% CO_2 (v/v). After introduction of the tissue, the flasks were gassed with the same mixture, tightly stoppered and incubated for two hours in a Dubnoff metabolic shaker (100 c/min).

The procedure for the isolation of labeled protein from muscle, liver and kidney was essentially that used by Manchester.[24] Following incubation, the epididymal fat pads were washed and transferred to 100 ml flasks containing 25 ml of chloroform-methanol (2:1, v/v) and shaken for three hours at 37°. The solvent was then removed and replaced by 25 ml of fresh solvent and the shaking continued for another 3 hr. The defatted pads were then removed and dried and the pads from two identical incubation flasks were combined for protein isolation according to the procedure used for the other tissues. The purified protein was suspended in 95% ethylalcohol and an aliquot was transferred to weighed stainless steel planchets and heat dried. Radioactivity was measured in a gas-flow end window Geiger counter provided with an automatic sample changer. The amount of protein was determined by reweighing the planchets after counting. All values for leucine incorporation will be given as sp. act. of the protein (cpm/mg) after correction to a uniform 10 mg level of self-absorption.

$^{14}CO_2$ *from leucine-1-*^{14}C. Tissues were incubated in rubber capped flasks fitted with a center well which contained a small removable glass cup. The incubation volume was 4 ml of bicarbonate buffer. After 2 hr of incubation, 0·6 ml of hyamine was injected through the rubber cap into the glass cup and 0·4 ml of 4 N H_2SO_4 into the main compartment to liberate carbon dioxide. The flasks were shaken for an additional 2 hr, the glass cup was removed and dropped into 14·4 ml of PPO (4 g/l.) in toluene. Radioactivity was measured in a Packard liquid scintillation counter at 4°. Results are expressed as cpm/mg wet wt. tissue.

Intracellular free leucine-^{14}C. Hemidiaphragms, after incubation as described above for leucine incorporation, were carefully blotted on wet Whatman No 50 paper and weighed. Each hemidiaphragm was homogenized in exactly 5 ml of cold 5% TCA. After centrifugation the protein precipitate was further processed to measure leucine incorporation as outlined above. A measured aliquot of the supernatant (0·2 ml) was plated on stainless steel planchets to which 0·2 ml of 95% ethyl alcohol was added to obtain, after drying, a smooth film of material giving excellent counting statistics. The number of counts obtained multiplied by the fraction of supernatant utilized for plating represents the sum of extra- and intracellular free leucine-^{14}C counts in the tissue at the end of the incubation period. To measure the concentration of leucine-^{14}C in the extracellular tissue water, which equals its concentration in the medium at the end of the incubation period, 0·5 ml of the medium of each flask was added to 4·5 ml of 5% TCA, centrifuged and 0·2 ml of the supernatant plated and counted as above. From these data, the diaphragm weight and the size of the extracellular space taken as 25 per cent of the tissue wet wt., the intracellular concentration of free leucine-^{14}C was calculated. The identity, after incubation, of the free radioactivity in the tissue with leucine-^{14}C and the size of the extracellular space under the conditions of the experiment have been established by us before.[25] Results are expressed as cpm/ml intracellular water.

Endogeneous respiration. Tissues were placed in calibrated Warburg flasks containing 2 ml of Ca-free Krebs–Ringer phosphate buffer pH 7·4 gassed with oxygen. The sulfonylureas used in these experiments were made up to the desired concentration with the same phosphate buffer and 0·25 ml was placed in the side-arm of the flasks together with 0·25 ml (0·18 μC) of leucine-^{14}C. A folded filter paper wetted with 0·2 ml of 10% KOH was placed in the center well. Side-arm contents were tipped in after a 10 min equilibration period at 37°. Oxygen uptake was followed over a 90-min period and the results are expressed as μl of oxygen uptake per 100 mg wet wt. tissue.

Statistical treatment of data. The observed values have been statistically treated as paired data. The values given in the tables represent the arithmetical means of the given number of observations; the S.E.M. difference and the probability limits are based upon Fisher's *t* test for significance.

RESULTS

Throughout the experiments a large and consistent inhibitory effect of the sulfonylureas on the incorporation of leucine into the protein of several rat tissues was noted (Tables 1–4). Tolbutamide and chlorpropamide exert the greatest effect and are almost equally potent inhibitors. At respectively 2·5 and 5×10^{-3} M concentrations their mean inhibitory effect is approximately 30 per cent and 60 per cent. For any given compound the differences in percentage inhibition observed between tissues, such as

the usually larger effect in kidney slices and the smaller effect in adipose tissue, probably relate to differences in the thickness of the particular tissue preparation (kidney slices are generally thinner than either liver slices or hemidiaphragms) and to differences in the distribution of the compounds in these tissues. The latter factor may also to some extent explain the differences in inhibition of leucine incorporation observed between sulfonylureas in any one tissue. To gain insight into the mechanism whereby sulfonylureas affect protein synthesis the effect of glucose on the sulfonylurea

TABLE 1. EFFECT OF TOLBUTAMIDE ON THE INCORPORATION OF LEUCINE-^{14}C INTO RAT TISSUE PROTEIN

Drug concentration and number of observations		Leucine incorporation (cpm/mg)		
		Control	Tolbutamide	% effect
Diaphragm	$2{\cdot}5 \times 10^{-3}$M (21)	212	161 ± 8 P < 0·001	−24
	5×10^{-3}M (18)	220	119 ± 10 P < 0·001	−46
	10^{-2}M (14)	159	29 ± 10 P < 0·001	−82
Liver	$2{\cdot}5 \times 10^{-3}$M (8)	351	245 ± 21 P < 0·01	−30
	5×10^{-3}M (8)	334	148 ± 21 P < 0·001	−56
Kidney	$2{\cdot}5 \times 10^{-3}$M (6)	394	135 ± 23 P < 0·001	−66
	5×10^{-3}M (8)	400	97 ± 25 P < 0·001	−76
Adipose Tissue	5×10^{-3}M (6)	1946	1203 ± 122 P < 0·01	−38
	10^{-2}M (6)	2453	831 ± 162 P < 0·001	−66

All values represent means ± S.E. of the number of observations given in parentheses.

TABLE 2. EFFECT OF CHLORPROPAMIDE ON THE INCORPORATION OF LEUCINE-^{14}C INTO RAT TISSUE PROTEIN

Drug concentration and numbers of observations		Leucine incorporation (cpm/mg)		
		Control	Chlorpropamide	% effect
Diaphragm	$2{\cdot}5 \times 10^{-3}$M (8)	239	168 ± 16 P < 0·01	−30
	5×10^{-3}M (8)	382	130 ± 36 P < 0·001	−66
Liver	$2{\cdot}5 \times 10^{-3}$M (7)	335	283 ± 16 P < 0·02	−16
	5×10^{-3}M (8)	224	96 ± 8 P < 0·001	−69
Kidney	$2{\cdot}5 \times 10^{-3}$M (8)	498	289 ± 38 P < 0·001	−42
	5×10^{-3}M (8)	424	112 ± 24 P < 0·001	−74
Adipose Tissue	$2{\cdot}5 \times 10^{-3}$M (6)	2825	2229 ± 127 P < 0·01	−21
	5×10^{-3}M (6)	2731	1637 ± 188 P < 0·01	−41

All values represent means ± S.E. of the number of observations given in parentheses.

inhibited incorporation of labeled leucine was examined. In order to compare paired tissues, one hemidiaphragm from each rat was incubated in the presence of the drug and served as control while the drug under study and glucose was added to the other hemidiaphragm. In each series of these experiments, hemidiaphragms from one rat were incubated one without additions and the other with the appropriate drug to ascertain that the drug effect was present in the control groups.

TABLE 3. EFFECT OF CARBUTAMIDE ON THE INCORPORATION OF LEUCINE-^{14}C INTO RAT TISSUE PROTEIN

Drug concentration and number of observations		Leucine incorporation (cpm/mg)		
		Control	Carbutamide	% effect
Diaphragm	$2{\cdot}5 \times 10^{-3}$M (12)	205	183 ± 15 $0{\cdot}1 < P < 0{\cdot}2$	−11
	5×10^{-3}M (10)	227	169 ± 15·5 $P < 0{\cdot}01$	−26
Liver	$2{\cdot}5 \times 10^{-3}$M (11)	286	300 ± 12 $P > 0{\cdot}2$	+ 5
	5×10^{-3}M (11)	373	289 ± 26 $P < 0{\cdot}01$	−23
Kidney	$2{\cdot}5 \times 10^{-3}$M (11)	338	266 ± 21 $P < 0{\cdot}01$	−21·5
	5×10^{-3}M (8)	307	192 ± 15 $P < 0{\cdot}001$	−37·5
Adipose Tissue	$2{\cdot}5 \times 10^{-3}$M (10)	2026	1942 ± 103 $P > 0{\cdot}3$	− 4
	5×10^{-3}M (9)	2718	2511 ± 114 $0{\cdot}1 < P < 0{\cdot}2$	− 8
	10^{-2}M (8)	2692	1880 ± 204 $P < 0{\cdot}01$	−30

All values represent means ± S.E. of the number of observations given in parentheses.

TABLE 4. EFFECT OF GLYCODIAZINE ON THE INCORPORATION OF LEUCINE-^{14}C INTO RAT TISSUE PROTEIN

Drug concentration and number of observations		Leucine incorporation (cpm/mg)		
		Control	Glycodiazine	% effect
Diaphragm	5×10^{-3}M (8)	156	134 ± 13 $0{\cdot}1 < P < 0{\cdot}2$	−14
	10^{-2}M (8)	219	182 ± 12 $P < 0{\cdot}02$	−17
Liver	5×10^{-3}M (7)	194	197 ± 8 $P > 0{\cdot}7$	+ 1·5
	10^{-2}M (7)	229	173 ± 10 $P < 0{\cdot}01$	−25
Kidney	5×10^{-3}M (8)	291	273 ± 20 $P > 0{\cdot}3$	− 6
	10^{-2}M (8)	313	208 ± 11 $P < 0{\cdot}001$	−34
Adipose Tissue	5×10^{-3}M (8)	2427	2418 ± 189 $P > 0{\cdot}9$	− 0·4
	10^{-2}M (6)	2262	1940 ± 121 $0{\cdot}05 < P < 0{\cdot}1$	−14

All values represent means ± S.E. of the number of observations given in parentheses.

Glucose stimulated leucine incorporation from 10–29% (Table 5) in the presence of sulfonylureas.

The well known stimulation of amino-acid incorporation into rat diaphragm protein by insulin was examined in the presence of chlorpropamide and tolbutamide. The data in Table 6 show that at all drug concentrations studied, insulin retains its stimulatory effect ranging from 12 to 70 per cent. Except at the highest drug concentration with tolbutamide the effect is statistically significant.

TABLE 5. EFFECT OF GLUCOSE, IN THE PRESENCE OF CHLORPROPAMIDE AND TOLBUTAMIDE, ON THE INCORPORATION OF LEUCINE-^{14}C INTO RAT DIAPHRAGM PROTEIN

Drug concentration and number of observations	Leucine incorporation (cpm/mg)	
	Sulfonylurea (control)	Sulfonylurea + Glucose (3×10^{-2} M)
Chlorpropamide		
5×10^{-3} M (9)	42	49 ± 4 $0{\cdot}1 < P < 0{\cdot}2$
Tolbutamide		
$2{\cdot}5 \times 10^{-3}$ M (7)	115	126 ± 4 $P < 0{\cdot}01$
5×10^{-3} M (16)	34	45 ± 3 $P < 0{\cdot}02$

All values represent means ± S.E. of the number of observations given in parentheses.

TABLE 6. EFFECT OF INSULIN ON CHLORPROPAMIDE AND TOLBUTAMIDE INHIBITED LEUCINE-^{14}C INCORPORATION INTO RAT DIAPHRAGM PROTEIN

Drug concentration and number of observations	Leucine incorporation (cpm/mg)			
	Control	Insulin 0·04 U/ml	Sulfonylurea	Sulfonylurea + insulin
Chlorpropamide				
$2{\cdot}5 \times 10^{-3}$ M (9)	68	107	47	80 ± 6 $P < 0{\cdot}001$
5×10^{-3} M (6)	85	130	30	45 ± 6 $0{\cdot}05 < P < 0{\cdot}1$
Tolbutamide				
$2{\cdot}5 \times 10^{-3}$ M (8)	80	119	54	75 ± 6 $P < 0{\cdot}02$
5×10^{-3} M (10)	86	153	40	45 ± 2 $P < 0{\cdot}05$
10^{-2} M (5)	—	—	29	33 ± 2 $0{\cdot}1 < P < 0{\cdot}2$

In the experiments with tolbutamide at 10^{-2} M paired hemidiaphragms were used. In all other experiments diaphragms from two rats were cut in quarter pieces and randomized; each flask contained two quarter diaphragms. All values in the insulin and sulfonylurea columns are statistically different from their corresponding control with $P < 0{\cdot}01$. Statistical data in the last column are calculated versus the data of the previous column. Figures in parentheses indicate the number of observations.

The data in Table 7 show the combined effect of insulin and glucose on the incorporation of labeled leucine into diaphragm protein in the presence of sulfonylureas. A statistically significant stimulation ranging from 26 to 43 per cent is again observed. This stimulation does not seem to be greater than the one noted with insulin alone.

TABLE 7. EFFECT OF INSULIN, IN THE PRESENCE OF GLUCOSE, ON CHLORPROPAMIDE AND TOLBUTAMIDE INHIBITED LEUCINE-^{14}C INCORPORATION INTO RAT DIAPHRAGM PROTEIN

Drug concentration and number of observations	Control	Insulin 0·04 U/ml	Sulfonylurea	Sulfonylurea + insulin
Tolbutamide				
$2{\cdot}5 \times 10^{-3}$ M (5)	192	264	133	173 ± 10 $P < 0{\cdot}02$
5×10^{-3} M (8)	194	305	89	127 ± 10 $P < 0{\cdot}01$
Chlorpropamide				
5×10^{-3} M (12)	—	—	42	53 ± 5 $P < 0{\cdot}05$

In the experiments with tolbutamide tissues were randomized as described in Table 6. Paired hemidiaphragms were used in the chlorpropamide experiments. All flasks contain 3×10^{-2} M glucose.

All values in the insulin and sulfonylurea columns are statistically different from the corresponding control with $P < 0{\cdot}01$. Statistical data in the last column are calculated versus the data of the previous column. Figures in parentheses indicate the number of observations.

As the inhibition of leucine incorporation into tissue protein could be the result of an effect of the sulfonylureas on the transport of labeled leucine into the tissues the intracellular concentration of the label, at the end of a 2-hr incubation period, was measured in diaphragm. From the results, shown in Table 8, it can be seen that after incubation of diaphragm in the presence of either chlorpropamide or tolbutamide there is a substantial increase in free intracellular labeled leucine, dependent upon the concentration of the drug used. No such increase was noted after incubation in the presence of carbutamide. In parallel experiments with insulin a significant decrease in the cellular concentration of labeled leucine occurred while the incorporation of leucine into protein was stimulated. This insulin effect was shown by us before.[25] These results indicate that the inhibitory effect of sulfonylureas on protein synthesis is apparently not due to their interference with the transport of leucine.

Leucine decarboxylation, another parameter of leucine metabolism which could be relevant to the inhibition of its incorporation was examined in several tissues in the presence of sulfonylureas. A uniform and dose dependent inhibition by chlorpropamide of $^{14}CO_2$ formation from leucine-1-^{14}C was observed (Table 9) and will be discussed later.

As the possibility was envisaged that the sulfonylureas, at the concentrations utilized, might have a toxic effect upon tissue respiration, the latter was measured in diaphragm, liver and kidney. The results are shown in Figs. 1–3. While in diaphragm and liver chlorpropamide and tolbutamide stimulate the endogenous respiration sometimes significantly during the first hour of incubation, no such effect is noted in kidney slices where a uniform inhibition of endogenous respiration is observed.

DISCUSSION

Among the first reports of an effect of hypoglycemic sulfonylureas on protein metabolism was the observation by Recant and Fisher[26] of an increased incorporation, *in vitro*, of labeled glycine into liver protein of tolbutamide pre-treated rats. This effect was interpreted as the result of an increased over-all utilization of glucose by the

TABLE 8. EFFECT OF CARBUTAMIDE, CHLORPROPAMIDE AND TOLBUTAMIDE ON THE INCORPORATION INTO PROTEIN AND INTRACELLULAR CONCENTRATION OF FREE LEUCINE-^{14}C IN RAT DIAPHRAGM

Drug concentration and number of observations	Intracellular concentration of free leucine-^{14}C (cpm/ml intracellular water)		Incorporation of leucine-^{14}C (cpm/mg)	
	Control	Drug	Control	Drug
Carbutamide				
5×10^{-3} M (8)	15·932	15·172 ± 852 P > 0·3	110	77 ± 13 P < 0·05
Chlorpropamide				
5×10^{-3} M (5)	10·333	20·942 ± 1288 P < 0·01	188	89 ± 19 P < 0·01
10^{-2} M (6)	15·514	35·043 ± 3493 P < 0·01	139	20 ± 5 P < 0·001
Tolbutamide				
5×10^{-3} M (11)	20·037	28·407 ± 1718 P < 0·001	310	127 ± 14 P < 0·001
10^{-2} M (7)	17·426	28·651 ± 877 P < 0·001	162	24 ± 14 P < 0·001
Insulin 0·04 U/ml (5)	18·379	13·878 ± 997 P < 0·02	213	298 ± 10 P < 0·01

All values are means ± S.E. of the number of observations given in parentheses. For experimental details see Materials and Methods.

liver leading, in turn, to an enhanced protein synthesis. Manchester and Young[27] however found that tolbutamide at 10^{-2} M strongly, and carbutamide to a lesser extent, inhibited alanine incorporation into rat diaphragm protein in the presence of glucose or pyruvate. This result was not expected since Bornstein[28] had demonstrated an inhibition of liver alanine aminotransferase by carbutamide and tolbutamide. This should have led to a diminished dilution of ^{14}C-alanine by non labeled alanine, formed from glucose or pyruvate by transamination, and consequently to a higher specific activity of the diaphragm protein. Inhibition of amino acid incorporation into diaphragm protein was later confirmed by Jarett and Butterfield[29] who observed an 80 per cent inhibition of leucine incorporation by 10^{-2} M tolbutamide. No explanation for this effect was offered.

While this work was in progress, DeChatelet and McDonald[22] reported the inhibition of leucine incorporation into the protein of rat liver homogenate. The effect was practically linear with drug concentration. Recently the same authors[23] reported a similar effect of tolbutamide and chlorpropamide on *in vitro* protein synthesis in microsomes from liver of normal and alloxan-diabetic rats.

Our results confirm these findings. In addition they show that the inhibition of amino acid incorporation is a common property shared by the 4 drugs studied and extends to rat kidney and adipose tissue besides liver and diaphragm. Large differences

TABLE 9. EFFECT OF CHLORPROPAMIDE ON $^{14}CO_2$ PRODUCTION FROM LEUCINE-1-^{14}C IN RAT TISSUES

Chlorpropamide concentration	$^{14}CO_2$ from leucine-1-^{14}C (cpm/mg wet wt. tissue)							
	Diafragma		Liver		Kidney		Adipose tissue	
	Co.	Chlorprop.	Co.	Chlorprop.	Co.	Chlorprop.	Co.	Chlorprop.
$2{\cdot}5 \times 10^{-3}$ M	174 (6)	121 ± 11 $P < 0{\cdot}01$	63 (8)	52 ± 3 $P < 0{\cdot}02$	790 (6)	633 ± 30 $P < 0{\cdot}01$	257 (6)	86 ± 33 $P < 0{\cdot}01$
5×10^{-3} M	196 (5)	81 ± 17 $P < 0{\cdot}01$	52 (7)	37 ± 4 $P < 0{\cdot}05$	982 (6)	549 ± 78 $P < 0{\cdot}01$	307 (5)	62 ± 23 $P < 0{\cdot}001$
10^{-2} M	189 (6)	37 ± 13 $P < 0{\cdot}001$	65 (7)	32 ± 4 $P < 0{\cdot}001$	983 (7)	346 ± 51 $P < 0{\cdot}001$	244 (7)	16 ± 38 $P < 0{\cdot}001$

All values are means ± S.E. of the number of observations given in parentheses.

are noted however in the potency of these drugs to inhibit leucine incorporation and these differences are not paralleled by similar differences in the hypoglycemic activity of these compounds. Glycodiazin, which is clearly several times less potent than either tolbutamide or chlorpropamide as inhibitor of leucine incorporation, has a clinical efficacy similar to that of tolbutamide[30] and its effective serum concentration in man

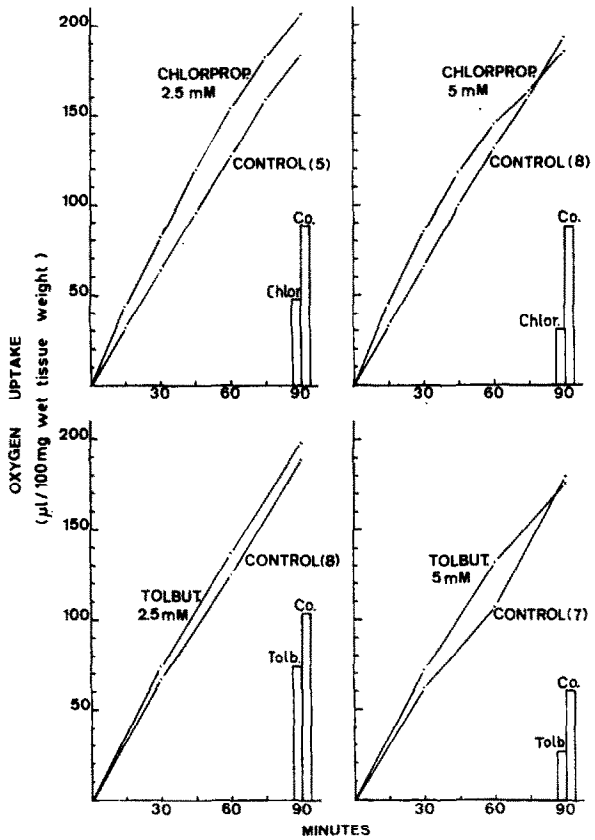

FIG. 1. Effect of chlorpropamide and tolbutamide on the endogenous respiration of isolated rat diaphragm. All values for chlorpropamide $2{\cdot}5 \times 10^{-3}$ M up to 90 min and for chlorpropamide 5×10^{-3} M up to 60 min are statistically higher than the corresponding control values with $P < 0{\cdot}05$. None of the values for tolbutamide are statistically different from the control. Bars at 90 min represent leucine incorporation of the same tissue at the end of the incubation period. Height of the bars represents cpm/mg and refers to the same scale as oxygen uptake. Figures between parentheses represent number of observations.

averages 10 mg %.[31] The corresponding range of the therapeutically effective serum concentrations of the sulfonylureas has been reported: between 3–14 mg % for chlorpropamide,[32] 5·3–9·6 mg % for tolbutamide[32] and from 6 to 8 mg % for carbutamide.[1] Furthermore it should be noted that the lowest drug concentration, at which in our experiments leucine incorporation is still affected in most tissues ($2{\cdot}5 \times 10^{-3}$ M), exceeds approximately 8–10 times the average serum concentration in good responders on chlorpropamide therapy.[32] As concentration of these drugs by tissues has not been reported and their distribution volumes, *in vivo*, are similar, it would

seem that at least in acute experiments, there is no direct causal relationship between their hypoglycemic and protein synthesis inhibitory effect. On the other hand, because of the many intangibles inherent to the interpretation of physiologic activity *in vivo* on the basis of short-term *in vitro* observations, it cannot be concluded without further investigation that the inhibition of protein synthesis and the hypoglycemic effect are

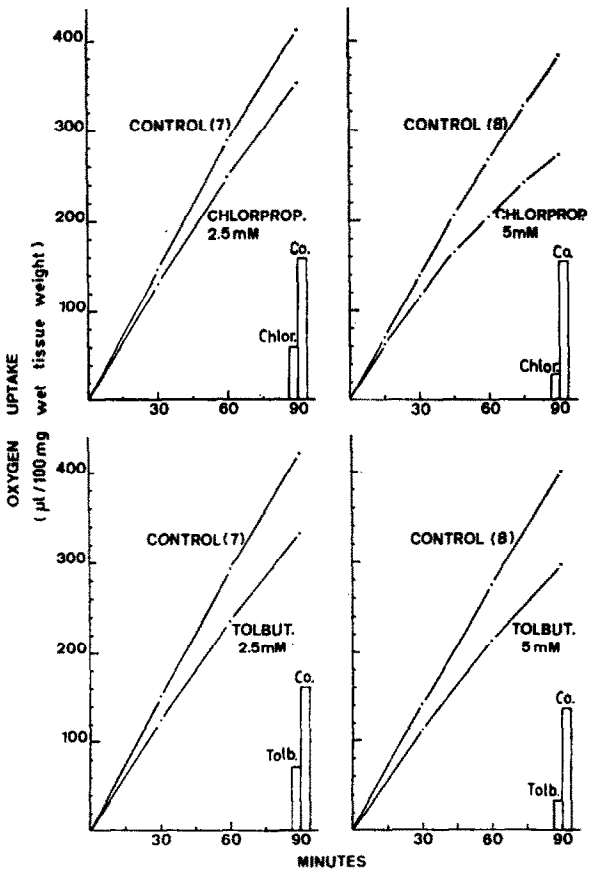

FIG. 2. Effect of chlorpropamide and tolbutamide on the endogenous respiration of rat kidney slices. All values for chlorpropamide and tolbutamide both at $2{\cdot}5 \times 10^{-3}$ M and 5×10^{-3} M concentration, are statistically different from the corresponding control values with $P < 0{\cdot}01$. See also legend Fig. 1.

entirely unrelated properties of these drugs. They, as well as other unexplained *in vivo* and *in vitro* effects including the unknown mechanism whereby the sulfonylureas release pancreatic insulin, may be related and point to a more fundamental effect of these drugs upon cell metabolism.

Our data indicate that the addition of glucose stimulates leucine incorporation in the presence of sulfonylureas but the magnitude of this effect is not such that it entirely relieves the inhibitory action of the sulfonylureas. A similar effect was not noted by DeChatelet *et al.*[22] although it occurs consistently in our experiments. Furthermore, our data in Tables 6 and 7 show the usual stimulatory effect of insulin

on amino acid incorporation and the persistence of this effect when diaphragm is incubated in the presence of sulfonylureas with or without added glucose as substrate. The mechanism whereby insulin stimulates protein synthesis is unknown but in view of the already increased cellular levels of labeled leucine in diaphragm incubated with tolbutamide or chlorpropamide (Table 8) our results would preclude an enhanced

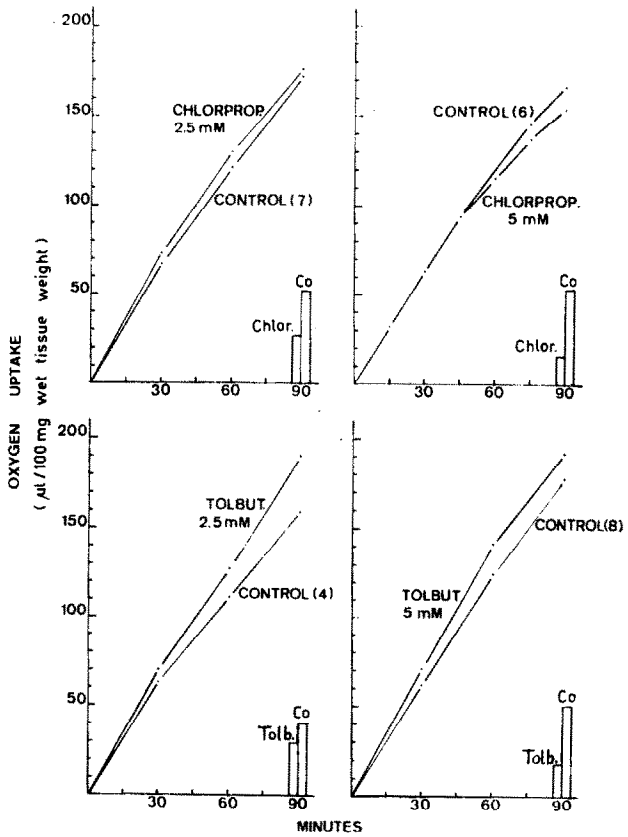

FIG. 3. Effect of chlorpropamide and tolbutamide on the endogenous respiration of rat liver slices. Only the values for tolbutamide at $2{\cdot}5 \times 10^{-3}$ M and 5×10^{-3} M at 30 and 60 min are statistically different from the corresponding control values with $P < 0{\cdot}05$. See also legend Fig. 1.

uptake of leucine as the basis for the insulin effect on protein synthesis. This is in agreement with earlier observations by us[25] and others.[33, 34] The fact that both glucose and insulin relieve to some extent the inhibition of protein synthesis by sulfonylureas and that the stimulatory effect of insulin on amino acid incorporation in diaphragm was shown to be dependent upon carbohydrate utilization,[35] suggest that an improved energy metabolism may underly this insulin effect. The idea that the common link between most of the known effects of insulin appears to be a requirement for energy has been supported by several authors.[36–38] It would be tempting to speculate that the sulfonylureas at the concentrations utilized, affect the energy metabolism of the cell and therefore inhibit protein synthesis and other energy requiring processes.[21] In order to elucidate the mechanism whereby protein synthesis

is inhibited by sulfonylureas, the effect of these drugs on leucine uptake in diaphragm and on decarboxylation in several tissues was examined. As our data reveal a large excess of intracellular free leucine after incubation with tolbutamide or chlorpropamide the inhibition of amino acid incorporation is clearly not the result of a decreased transport of leucine. To the contrary, the decreased utilization of the amino acid in protein synthesis could, at first sight, be responsible for its increased cellular concentration. The latter is however far greater than could be accounted for by the decreased leucine incorporation and this indicates that the sulfonylureas must affect leucine metabolism besides and independent of the inhibition of leucine incorporation.

In this context it is worthwhile to mention that the inhibition of amino acid incorporation by 2-deoxyglucose also increases the cellular concentration of the amino acid,[25] while in the presence of insulin, which stimulates incorporation, the cellular amino acid content decreases. The rate of protein synthesis therefore undoubtedly affects cellular amino acid concentration in these experiments but in the case of insulin and sulfonylureas the net effect is a composite one. Indeed sulfonylureas strongly affect CO_2 formation from leucine. An inhibition by 10^{-2} M tolbutamide was observed by Jarett and Butterfield in diaphragm.[29] Recently DeChatelet and McDonald reported a linear decrease of $^{14}CO_2$ production from leucine-1-^{14}C in rat liver homogenate incubated with increasing concentrations of tolbutamide.[22] The effect they observed was substantially smaller however than the one we found in liver slices by chlorpropamide. Our results show that the inhibition of leucine decarboxylation by chlorpropamide occurs in the four tissues examined, is statistically significant at the lowest dose level of $2{\cdot}5 \times 10^{-3}$ M utilized and is most pronounced in diaphragm and adipose tissue where it reaches considerable proportions. Further observations in our laboratory (to be published) indicate that this effect extends to other hypoglycemic sulfonylureas and other, but not all, amino acids currently under study. It is obvious that this inhibition of leucine decarboxylation by sulfonylureas is not responsible for the observed inhibition of leucine incorporation, although it raises the more pertinent question of a possible analogous inhibition of the decarboxylation of other keto acids such as pyruvic- and ketoglutaric acid. Furthermore, since the cellular concentration of leucine after incubation with sulfonylureas significantly exceeds the control values, the decreased production of labeled CO_2 is not the result of a decreased substrate level but of some other metabolic effect of the sulfonylureas.

The sites at which sulfonylureas could affect CO_2 production from carboxyl labeled leucine are either the transamination step to α-ketoisocaproic acid or the subsequent decarboxylation of this keto acid. The reported *in vitro* inhibition of alanine aminotransferase from liver by tolbutamide and carbutamide affords, by extension of this effect to other transaminases, a possible explanation.[28, 39]

In a very recent study, which came to our attention when this work was nearly completed, Pentillä showed that tolbutamide and chlorpropamide strongly inhibit an aminotransferase which in muscle and liver, catalyzes the reaction between the branched chain amino acids and α-ketoglutarate.[40] The inhibition was non-competitive, was observed in both directions and was significant at 5×10^{-4} M concentration of the drugs. In tissue experiments these drugs, at 10^{-3} M or higher, inhibited the mitochondrial oxidative decarboxylation of α-ketoisocaproic acid. In sonicated mitochondria and with ferricyanide or dichlorophenol-indophenol as artificial electron

acceptors the inhibition was not observed indicating that the branched chain keto acid decarboxylase itself is not affected by chlorpropamide but that the inhibition of carboxylation is due to an impaired mitochondrial function.

Since in diaphragm leucine transamination and the subsequent decarboxylation of ketoisocaproate account for 75 per cent of the leucine which disappears,[40] the increased cellular levels of leucine we observed must be due more to the inhibition of leucine catabolism than to a decreased incorporation into protein. This is in agreement with our data of Table 8 as far as tolbutamide and chlorpropamide are concerned but does not explain why we did not observe an increased cellular level of labeled leucine after incubation with carbutamide. It is possible that this compound, at the concentration used, is a less potent inhibitor of leucine catabolism as it is also of leucine incorporation.

As to the possible significance of the inhibition of leucine catabolism by sulfonylureas it might be recalled that this amino acid has been shown to produce hypoglycemia in sensitive humans, patients with islet cell tumours of the pancreas and to a lesser extent in normal subjects. This hypoglycemic effect is potentiated by sulfonylureas and insulin.[41, 42] Cochrane[43] suggested that blood leucine controls to some extent insulin release by the pancreas and it is now widely accepted that insulin release at least contributes to the mechanism of leucine hypoglycemia. Although Jarett and Butterfield[29] were unable to detect in autoradiographic studies an increased uptake of labeled leucine by pancreatic islets after the administration of tolbutamide or insulin to rats, our results indicate that elevated cellular levels of this amino acid occur *in vitro*. If sulfonylurea concentrations in the islets cells would somewhat exceed those in blood, a significant effect on leucine levels in this tissue could be expected and could contribute to the release of insulin. In addition, if leucine has metabolic effects on the liver which are relevant to its hypoglycemic activity,[44, 45] the inhibition of its catabolism would again be expected to augment these effects. In this respect it should perhaps be mentioned that hypoglycin, a hypoglycemic amino acid from *Blighia sapida*, and its keto analogue inhibit the oxidation of leucine[46] in liver slices. Their effect is however not on the early steps of leucine degradation but occurs after its conversion to ketoisocaproic acid and decarboxylation to *iso*valeryl-CoA. It is not known if hypoglycin increases the tissue levels of leucine. In view of all this the inhibition of leucine metabolism by hypoglycemic sulfonylureas could have physiological significance but if so, it is not clear why carbutamide failed to increase tissue levels of leucine in diaphragm. Further studies on this are in progress.

Our results on leucine uptake and catabolism did not explain its impaired incorporation and we therefore examined the effect of chlorpropamide and tolbutamide on the endogenous respiration of rat tissues. The very strong inhibition of protein synthesis suggested indeed that these drugs, at the concentrations utilized, might act as general cell poisons. Earlier observations by Clarke *et al.*[47] had shown that carbutamide inhibits oxygen uptake by rat liver slices in the absence of substrate, but not in the presence of pyruvate. These results however were not uniform and their significance was not stated. A significant stimulation of oxygen uptake by rat diaphragms, after 60 min incubation in the presence of glucose and 2×10^{-3} M tolbutamide was noted by Pletscher and Gey.[48] Similar observations were reported by Mohnike *et al.*[49] Clarke,[50] on the other hand, found that chlorpropamide caused a reduced

endogenous respiration in kidney slices and a reduced oxygen uptake by liver homogenate fortified with succinate.

Our results indicate that the endogenous respiration of different rat tissues is not affected in a uniform manner. In kidney slices a significant inhibition of oxygen uptake is noted at all time intervals reaching 25–30 per cent after 90 min incubation. This is in agreement with the results of Clarke.[50]

In diaphragm, the endogenous respiration during the first hours is increased and a similar effect is noted in most experiments with liver slices. At later time intervals an inhibition of oxygen uptake becomes apparent. The overall results indicate however that the concommitant impairment of leucine incorporation is not due to a toxic effect of the sulfonylureas on tissue respiration. In fact the observed increase in oxygen uptake in muscle and liver has led us to study the effects of these drugs on mitochondrial respiration and energy metabolism, the results of which will be detailed in a forthcoming publication.

In view of the strong inhibition of *in vitro* protein synthesis the question may arise if these drugs affect protein synthesis *in vivo*. More than a decade after their introduction and a very widespread use, there is no doubt that the sulfonylureas are largely free of toxic effects. This is probably related to the fact that at the usual dosage their concentration in blood is around 5×10^{-4} M which is below the level known to affect *in vitro* protein synthesis although at blood levels exceeding 10^{-3} M during two weeks no subjective symptoms have been observed in man.[51] Several authors[52, 53] reported either no untoward effects or an improvement of the condition of the liver after sulfonylurea therapy in diabetic patients with a previous or actual history of a minor degree of liver pathology. On the basis of an increase of hepatic glycogen in animals given sulfonylureas, these compounds have even been used for therapeutic purposes in selected cases of chronic liver disease and hepatitis.[54, 55] Furthermore a trophic effect of sulfonylureas on the pancreatic islets, suggesting and increased protein synthetic activity has been reported.[56, 57]

Following very high doses of chlorpropamide in mice, rats, cats and dogs the most obvious symptoms are general weakness associated with ataxia and incoordination.[58] Muscular weakness has also been observed in patients receiving an overdose of chlorpropamide and these effects are probably related to a direct action of the drug rather than to its hypoglycemic effect. Growth retardation was noted in young animals given 10–20 times the normal dose of sulfonylureas over weeks or months.[50, 58] In experiments with transplanted tumours moderate but significant growth inhibition of the latter was noted after chlorpropamide dosage levels between 250–1000 mg/kg/day.[59] Growth of HeLa cells in tissue culture was inhibited by $1{\cdot}5 \times 10^{-3}$ M chlorpropamide and the drug proved to be lethal after 5 days of incubation at 5×10^{-3} M chlorpropamide.[59] It is nevertheless surprising that in chronic toxicity studies in animals rather few toxic effects have been noted in spite of doses far exceeding the therapeutic one and blood levels of drugs probably at least as high as the concentrations used in our experiments.[58, 60] This apparent discrepancy between *in vivo* and *in vitro* effects at nearly identical drug levels in blood or incubation fluid, may be due to other metabolic conditions prevailing *in vivo* which protect the tissues from lethal effects. Our observations that both glucose and insulin significantly reduce the inhibition of protein synthesis is an indication in this direction and other hormones and substrates are probably active as well. Furthermore, due to impaired permeability

barriers, concentrations of sulfonylureas may be significantly higher in tissue slices and cut diaphragm than they ever are *in vivo*. It is indeed known that the distribution volume of the sulfonylureas *in vivo* does not exceed the extracellular space and concentration of the drugs by tissues must therefore be low.[32]

Acknowledgements—The author wishes to thank Miss H. Verhelst and Miss H. Souren for their excellent technical assistance; Dr. L. Debeer for his contribution in the preparation of this manuscript, Chas. Pfizer & Co., Boehringer GmbH and Schering A.G. for the generous supplies of chlorpropamide, tolbutamide, carbutamide and glycodiazin; E. Lilly and Co. for their gift of insulin.

REFERENCES

1. J. D. H. SLATER, *Progr. med. Chem.* **1**, 187 (1961).
2. L. J. P. DUNCAN and B. F. CLARKE, *Ann. Rev. Pharmac.* **5**, 151 (1965).
3. W. CREUTZFELDT and H. SÖLING, *Orale Diabetes Therapie*. Springer, Berlin (1961).
4. H. S. SELTZER, *J. clin. Invest.* **41**, 289 (1962).
5. M. A. ROOT, *Diabetes* **6**, 12 (1957).
6. R. S. YALOW, H. BLACK, M. VILLAZON and S. A. BERSON, *Diabetes* **9**, 356 (1960).
7. J. ASHMORE, G. F. CAHILL and A. S. EARLE, *Ann. N.Y. Acad. Sci.* **71**, 131 (1957).
8. J. W. CRAIG, W. R. DRUCKER, M. MILLER, H. WOODWARD JR. and V. MOLZAHN, *Ann. N.Y. Acad. Sci.* **74**, 537 (1959).
9. D. C. KVAM, *Proc. Soc. exp. Biol. Med.* **115**, 904 (1964).
10. H. L. DOBSON, H. GUILAK and J. A. GREENE, *Ann. N.Y. Acad. Sci.* **74**, 575 (1959).
11. W. MALAISSE, R. BELLENS and J. R. M. FRANCKSON, *Archs int. Pharmacodyn. Thér.* **149**, 290 (1964).
12. P. SCHAMBYE and F. TARDING, *Ann. N.Y. Acad. Sci.* **74**, 557 (1959).
13. E. S. EGELI and H. ALP, *Klin. Wschr.* **39**, 437 (1961).
14. A. KÁLDOR and G. POGÁTSA, *Acta med. hung.* **18**, 69 (1962).
15. Y. GOTO and F. D. W. LUKENS, *Diabetes* **10**, 52 (1961).
16. G. POGÁTSA and A. KÁLDOR, *Diabetes* **14**, 209 (1965).
17. B. R. BOSHELL, G. R. ZAHND and A. E. RENOLD, *Metabolism* **9**, 21 (1960).
18. H. D. SUMM, W. CREUTZFELDT and K. WALLENFELS, *Klin. Wschr.* **38**, 85 (1960).
19. D. B. STONE and J. D. BROWN, *Diabetes* **15**, 314 (1966).
20. D. B. STONE, J. D. BROWN and C. P. COX, *Am. J. Physiol.* **210**, 26 (1966).
21. H. J. MCDONALD and J. E. DALIDOWICZ, *Biochemistry N.Y.* **1**, 1187 (1962).
22. L. R. DECHATELET and H. J. MCDONALD, *Proc. Soc. exp. Biol. Med.* **122**, 765 (1966).
23. H. J. MCDONALD and L. R. DECHATELET, *Life Sci.* **6**, 183 (1967).
24. K. L. MANCHESTER and F. G. YOUNG, *Biochem. J.* **70**, 353 (1958).
25. P. J. DE SCHEPPER, Ph.D. Thesis, University of Maryland, School of Medicine, Baltimore, Md.. (1964).
26. L. RECANT and G. FISCHER, *Ann. N.Y. Acad. Sci.* **71**, 62 (1957).
27. K. L. MANCHESTER and F. G. YOUNG, *Biochem. J.* **70**, 297 (1958).
28. J. BORNSTEIN, *Nature, Lond.* **179**, 534 (1957).
29. R. J. JARETT and W. J. BUTTERFIELD, *Br. Med. J.* **1**, 865 (1964).
30. H. GUTSCHE and G. BOENICKE, *ArzneimittelForsch.* **14**, 409 (1964).
31. E. GERHARDS, H. GIBIAN and K. H. KOLB, *Arzneimittel. Forsch.* 14, 394 (1964).
32. J. SHELDON, J. ANDERSON and L. STONER, *Diabetes* **14**, 362 (1965).
33. I. G. WOOL and M. E. KRAHL, *Biochim. biophys. Acta* **82**, 606 (1964).
34. W. S. STIREWALT and I. G. WOOL, *Science* **154**, 284 (1966).
35. P. J. DE SCHEPPER, M. TOYODA and S. P. BESSMAN, *J. biol. Chem.* **240**, 1670 (1965).
36. S. P. BESSMAN, *Am. J. Med.* **40**, 740 (1966).
38. R. B. CONN, *Nature, Lond.* **211**, 195 (1966).
39. L. TROPEANO, A. NUNNARI, S. DISTEFANO and F. BELFRIORE, *Minerva Med., Roma* **50**, 2502 (1959).
40. I. M. PENTTILÄ, *Ann. Med. exp. biol. Fenn.* **44**, *suppl.* 11 (1966).
41. S. S. FAJANS, R. F. KNOPF, J. C. FLOYD JR., L. POWER and J. W. CONN, *J. clin. Invest.* **42**, 216 (1963).

42. A. R. COLWELL JR., *Diabetes* **15**, 560 (1966).
43. W. A. COCHRANE, *J. Dis. Child.* **99**, 476 (1960).
44. S. PENSUWAN, F. ZAXHAREWICZ, R. YERRY and T. F. FRAWLEY, *Metabolism* **12**, 773 (1963).
45. G. REAVEN and R. E. GREENBERG, *Metabolism* **14**, 625 (1965).
46. B. I. POSNER and M. S. RABEN, *Biochim. biophys. Acta* **136**, 179 (1967).
47. D. W. CLARKE, M. DAVIDSON, E. SCHÖNBAUM and H. SENMAN, *Can. med. Ass. J.* **74**, 966 (1956).
48. A. PLETSCHER and K. F. GEY, *Experientia* **13**, 447 (1957).
49. G. MOHNIKE, K. W. KNITSCH, H. BOSER, G. WERNER and S. WERNER, *Dt. med. Wschr.* **82**, 1580 (1957).
50. D. W. CLARKE, *Ann. N.Y. Acad. Sci.* **74**, 478 (1959).
51. M. CARLOZZI, D. G. IEZZONI and L. SILVER, *Ann. N.Y. Acad. Sci.* **74**, 788 (1959).
52. W. CREUTZFELDT, U. DEUTICKE and H. D. SÖLING, *Klin. Wschr.* **39**, 790 (1961).
53. B. A. BEBER and M. BEBER, *Am. J. med. Sci.* **238**, 433 (1959).
54. B. KNICK, *Klin. Wschr.* **40**, 266 (1962).
55. I. SINGH, K. B. SEHRA and S. P. BHARGAVA, *Lancet* **i**, 1114 (1961).
56. A. LOUBATIERES, C. FRUTEAU DE LACLOS, M. H. HOUAREAU and R. ALRIC, *C.r. Séanc Soc. Biol.* **157**, 1652 (1963).
57. W. SANDRITTER, K. FEDERLIN and E. F. PFEIFFER, in *The Structure and the Metabolism of the pancreatic Islets*, (Eds. S. E. BROLIN, B. HELLMAN and H. KNUTSEN), p. 67. MacMillan, New York (1964).
58. J. A. SCHNEIDER, E. D. SALGADO, D. JAEGER and C. DELAHUNT, *Ann. N.Y. Acad. Sci.* **74**, 427, (1959).
59. J. L. DAVENPORT, unpublished observations; J. L. SMITH Memorial Institute, Maywood, New Jersey, U.S.A.
60. A. BÄNDER, A. HÄUSSLER and J. SCHOLZ, *Dt. med. Wschr.* **82**, 1557 (1957).