Clearance and Metabolic Action of Insulin and Nonsuppressible Insulin-like Activity (NSILA) in the Isolated Perfused Rat Heart

Solomon S. Solomon, M.D., David R. Challoner, M.D., John W. Ensinck, M.D., and Robert H. Williams, M.D., Seattle

SUMMARY

Several aspects of glucose-U-C-14 metabolism were studied in the isolated perfused rat heart to compare the activities of nonsuppressible insulin-like activity (NSILA) and crystalline insulin. Like insulin, porcine NSILA prepared on a Dowex-50 column caused significant glucose disappearance and glycogen deposition. While 50 per cent of the insulin was cleared from the perfusate (immunoassay and bioassay) over sixty minutes, NSILA concentration, measured by the isolated fat cell bioassay, remained constant.

These results establish myocardium as a tissue on which NSILA has an action similar to insulin. Furthermore, these studies show an action of NSILA via a "normal" vascular tree and support the possibility that it may have a physiologic role. The lack of clearance of NSILA suggests that it may exert its insulin-like action over long intervals. DIABETES *16*:545-50, August, 1967.

The insulin-like activity (ILA) of human serum has been extensively investigated with widely differing methods of preparation and assay.[1-3] To describe the component of ILA which is not identified as insulin by immunological and physicochemical criteria, the terms "bound" insulin,[2] "atypical" insulin,[1] and nonsuppressible insulin-like activity (NSILA),[3] have been used.

Kipnis and Stein[4] first suggested that these forms of ILA might be identical. Recent investigations from this laboratory support this concept.[5-7] Because there is as yet no conclusive evidence relating ILA to insulin structurally, we have elected to use the term NSILA as suggested by Froesch and colleagues.[8,9] This material has been partially purified and some of its biological properties studied.[3,10,11] Recent reports have varied, however, with respect to changes in serum concentration of this material,[1,8,12] and investigators have speculated on its physiologic significance.[3,13]

As part of a series of investigations into the possible biologic consequences of NSILA in serum, the present studies were undertaken to compare the clearance and biologic activity of NSILA with insulin in the isolated perfused rat heart. The results indicate that NSILA, like insulin, exerts a potent metabolic effect on myocardium. But, in contrast to insulin, there is little destruction or removal of this material from the perfusate.

METHODS

A. *Preparation of NSILA.* Pork serum was used in these studies because of the lack of availability of sufficient rat or human serum. Porcine serum was found to contain 60 to 100 μU. nonsuppressible insulin-like activity per milliliter when measured at 2.5 per cent on the isolated fat cell assay of Gliemann.[14] NSILA was partially purified from pooled porcine serum by means of a modification of the method of Antoniades.[7,15] The serum was applied to a column of Dowex 50 × 8 (100-200 mesh) (V/V) equilibrated in 0.15 M NaCl. The majority of the serum proteins were eluted in 0.15 M NaCl (3 Vol) and the residual protein on the resin was eluted with 0.02 N NH_4OH (2 Vol) into 0.2 N H_2SO_4 (.02 Vol), maintaining the *p*H at 7.4 by addition of 0.02 N H_2SO_4. The protein eluted with NH_4OH was dialyzed against running tap water (24° C.) and deionized water over twenty-four hours. The lyophilized protein contained 250 to 300 μU. insulin-like activity (ILA) per mg. of dry extract, as measured independently in the isolated fat cell assay[14]

From the Department of Medicine, University of Washington, Seattle, Washington. Address requests for reprints to Dr. John W. Ensinck, Department of Medicine, University Hospital, Seattle, Washington 98105. Present address of Dr. Challoner: Dept. of Medicine, University of Indiana Medical Center, Indianapolis, Indiana.

and the isolated hemidiaphragm bioassay.[16] In both systems, none of the ILA was neutralized by insulin specific antiserum nor was insulin detected by immunoassay.

B. *Isolated rat heart perfusion system.* Hearts were removed from 250 to 300 gm. ad libitum fed male Wistar rats and perfused after the technic of Morgan.[17] Twenty milliliters of a modified Krebs-Ringer bicarbonate buffer[18] were recirculated in each experiment at a constant temperature of 37.5° C. Prior to introduction to the recirculating system, each heart was preperfused for five minutes with buffer. Equilibration with 95 per cent O_2 - 5 per cent CO_2 was maintained by bubbling. To prevent foaming, one spray of Dow Corning Antifoam A into the reservoir chamber was used in each experiment. Venous pO_2 in this preparation was never below 100 to 150 mm. mercury.

Additions to the buffer are described in Results. The albumin used was bovine albumin fraction V (Lot B23809),* which contained no assayable NSILA on the isolated fat cell assay. Crystalline pork insulin† and uniformly labeled C-14 glucose‡ were used.

At the completion of the metabolic studies, the hearts were immediately removed, opened, blotted twice, weighed and introduced into hot 30 per cent KOH. Samples of the perfusate were frozen for later assay.

C. *Assays for IRI, ILA, and NSILA.* (1) Immunoreactive insulin was determined on aliquots of the perfusate by the double antibody assay of Morgan and Lazarow.[19] (2) ILA and NSILA were determined by the isolated fat cell bioassay of Gliemann[14] as adapted for measurement of NSILA by Hepp et al.[20]

D. *Glucose, glycogen and C-14 determinations.* (1) Perfusate glucose levels were measured by the ferrocyanide method with a Technicon AutoAnalyzer. (2) Myocardial glycogen was determined, after double precipitation with ethanol, by the anthrone technic.[21] An aliquot of the glycogen pellet was dissolved and introduced into Bray's solution[18] for liquid scintillation counting. (3) Disappearance of glucose C-14 label from the perfusate was followed by incubating 50 μl. of the perfusate with .2 ml. of hydroxide of Hyamine 10-X§ at 37° C. for thirty minutes and then adding 10 ml. of Bray's solution and counting as above.

The clearance data are presented as the percentage of initial NSILA or IRI present. All data are presented as mean ± S.E.M. where appropriate. The per cent of glucose uptake incorporated into glycogen was calculated from the true glucose disappearance and the milligram C-14 glucose equivalent converted to glycogen (as determined from the initial specific activity of glucose in the perfusate and the C-14 in glycogen).

RESULTS

The adsorption of insulin to glassware and a protective effect of albumin have been demonstrated.[23] Since the perfusates in this system are exposed to both glass and plastic surfaces, the clearance characteristics of the system without the heart were first studied using both NSILA and insulin with and without albumin (figure 1). The presence of 200 mg. per 100 ml. bovine fraction V albumin clearly prevented significant loss of both NSILA and ILA in the control system over sixty minutes of recirculating perfusion. In the absence of albumin, however, NSILA disappeared from the perfusate. All studies with the heart, therefore, were carried out in the presence of albumin.

With the heart present in the perfusion system, approximately 50 per cent of the insulin, measured both as ILA and IRI, was cleared during a sixty-minute perfusion period (figure 2). In contrast, NSILA remained at 100 per cent of the initial assayed concen-

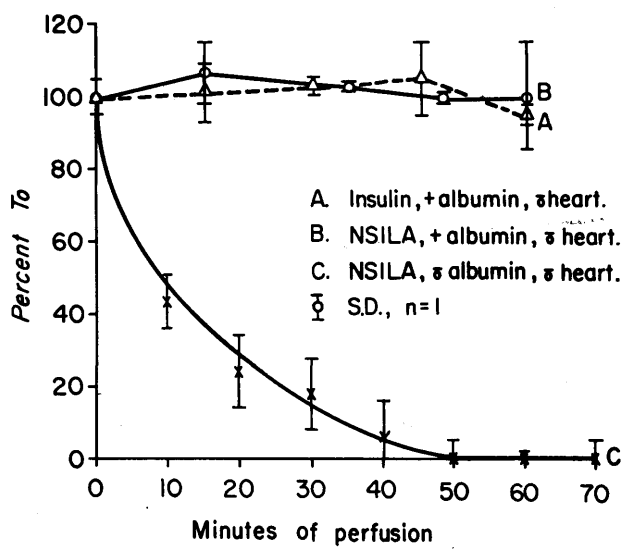

FIG. 1. Protective effect of albumin (200 mg. per 100 ml.) on disappearance of NSILA and insulin (200 μU./ml.) from the perfusion system in the absence of a heart. Values are expressed as the per cent of the initial concentration (T-O) ± S.D. of replication in the fat cell assay system.

*Armour Pharmaceutical Company, Kankakee, Illinois.
†Sigma Chemical Company, St. Louis, Missouri.
‡New England Nuclear, Cambridge, Massachusetts.
§Packard Instruments. La Grange, Illinois.

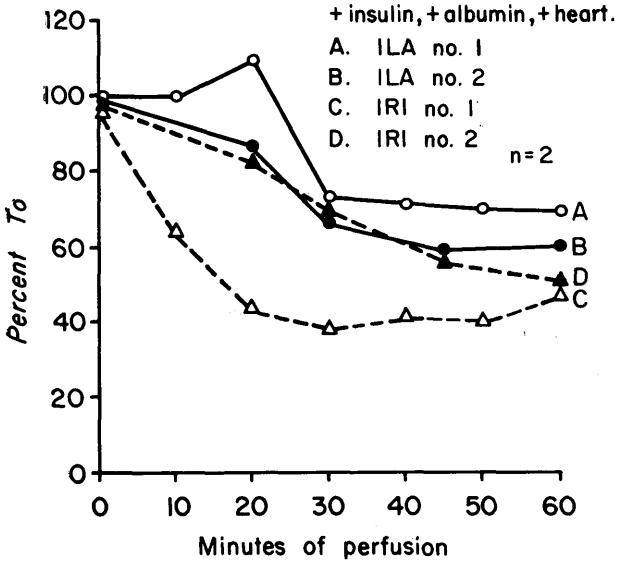

FIG. 2. Disappearance of insulin (200 μU./ml.) during two heart perfusions followed by both bioassay (A, B) and immunoassay (C, D).

tration (figure 3). Furthermore, in contrast to liver,[7] NSILA was not released from the heart during perfusion (figure 4).

In order to compare the action of insulin and porcine NSILA on myocardium, several aspects of glucose and

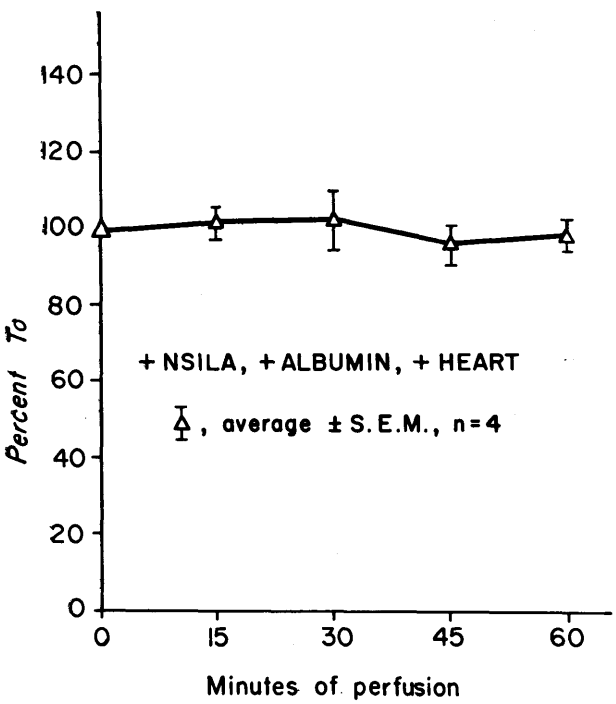

FIG. 3. Disappearance of NSILA (200 μU./ml.) during four heart perfusions. Values are expressed as the mean per cent of initial concentration (T-O) ± S.E.M.

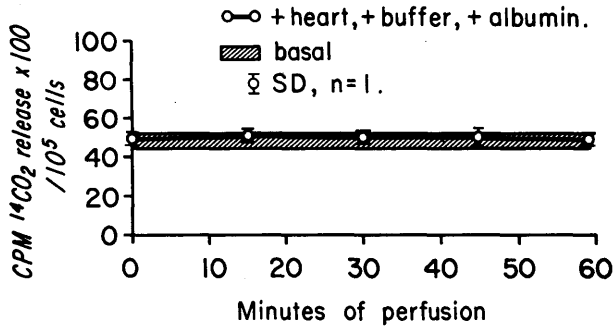

FIG. 4. Assay for insulin-like activity appearing in the perfusate from a heart. The perfusate was assayed at a 1:15 dilution on isolated fat cells and the ILA is expressed as C-14-O_2 release, counts/minute/100,000 cells ± S.D. of assay replication for ready comparison with baseline activity of the assay.

glycogen metabolism were measured. Six hearts in each group were perfused for thirty minutes with Krebs Ringer bicarbonate containing 140 mg. per 100 ml. glucose U-C-14 and 200 mg. per 100 ml. albumin (control buffer); control buffer plus insulin (1,000 μU. per ml.); or control buffer plus porcine NSILA (1,000 μU. per ml.). Muscle glycogen C-14 in glycogen and the disappearance of glucose and C-14 were determined. From these data, glucose uptake and incorporation into glycogen were calculated (see Methods). The glycogen content of a group of hearts processed at the end of the five-minute preperfusion period was determined as the initial or T-O glycogen (figure 5, table 1).

Glucose uptake and C-14 disappearance were stimulated by 1,000 μU. of NSILA and insulin (figure 6, table 1). The increase over control appeared to be of equal magnitude with both substances. The greater disappearance of glucose than C-14 (figure 6) is accounted for by the return to the perfusate of labeled glucose metabolites such as pyruvate and lactate.[18] The rate of uptake fell off during the second fifteen-minute period in all groups, possibly secondary to some decrease in heart rate and contractility.[18]

The glycogen level dropped during perfusion in the absence of insulin, but both NSILA and insulin maintained glycogen levels similar to those at T-O during thirty minutes of perfusion (figure 5). Incorporation of C-14 into glycogen was clearly stimulated by both NSILA and insulin (figure 5). Insulin was found to triple, and NSILA double, the control proportion of the glucose uptake appearing in glycogen (table 1).

DISCUSSION

The present studies indicate that myocardium may be

TABLE 1

	Period (minutes)	ō	Additions to buffer: 1,000 μU./ml. insulin	p*	1,000 μU./ml. NSILA	p*
Glucose uptake gm./gm. wet weight	15	3.77 ± 0.27	5.07 ± 0.32	0.02	5.00 ± 0.24	0.01
	30	5.79 ± 0.53	7.65 ± 0.28	0.02	7.56 ± 0.68	0.1
Per cent glucose uptake to glycogen	30	4.27 ± 0.69	12.42 ± 1.37†	<0.001	8.02 ± 0.60†	0.005

*p value as compared to no buffer addition (ō); n = 6 for each condition.
†p = 0.02, comparison of the two values marked (†), insulin and NSILA.

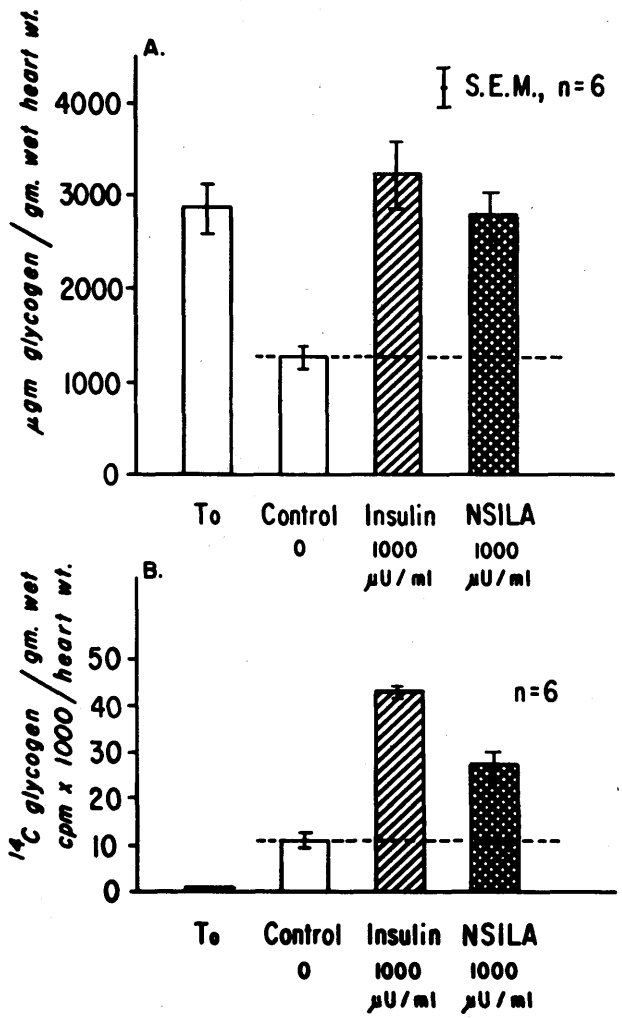

FIG. 5. Effects of insulin and NSILA (1,000 μU./ml.) on glycogen and glycogen C-14 during a thirty-minute heart perfusion.

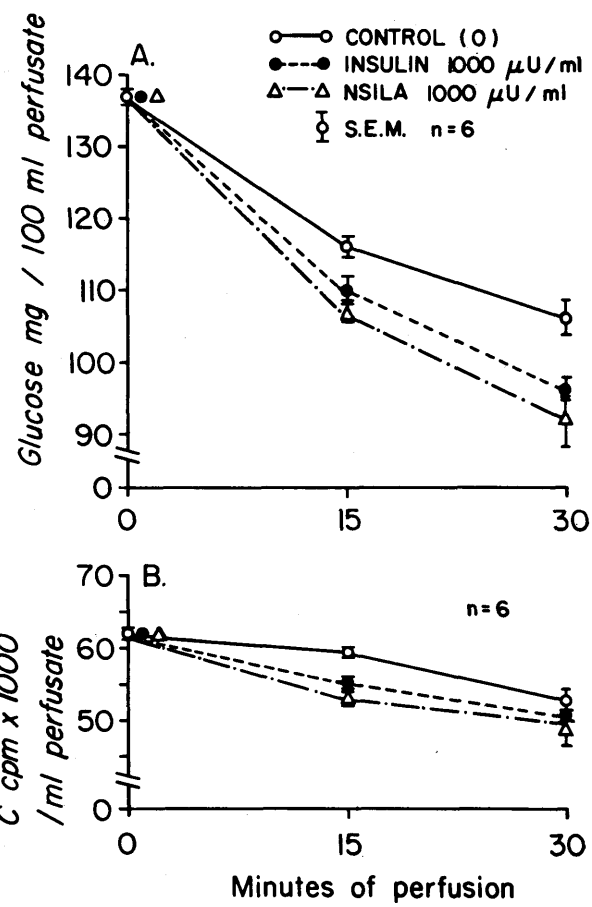

FIG. 6. Comparative effects of insulin and NSILA (1,000 μU./ml.) on the disappearance of glucose and glucose-U-C-14 from the perfusate during a thirty-minute heart perfusion.

included with adipose tissue and hemidiaphragm as tissues on which NSILA has in vitro metabolic effects qualitatively similar to those of insulin. The quantities of NSILA used in these studies were equivalent to 1,000 μU. per ml. of insulin as measured on the isolated fat cell. These concentrations of NSILA and insulin also produced comparable effects on the glucose uptake, glycogen levels and C-14 glycogen incorporation by the myocardium.

In three tissues insulin and NSILA now demonstrate qualitatively similar actions on such parameters as glucose uptake, glycogen deposition, C-14-CO_2 production from glucose and on antilipolysis.[3,20,24] These similarities suggest a common primary mode of action of these two substances, though they add nothing to the debate about what this mode of action might be.[25]

However, the present experiments also suggest the possibility of quantitative differences between these substances in the control of substrate flow through alternate pathways. While glucose uptakes were similar, the per cent incorporation into glycogen was significantly greater with insulin than with NSILA (table 1). It should be noted, however, that the per cent incorporations in the present studies can be considered only as estimates without direct measurement of the specific activity of the glucose-1-phosphate precursor pool. Similar quantitative differences have been reported in other tissues.[24,26,27]

Of special interest was the observation that, although NSILA exerted a potent metabolic effect on the myocardium, it was apparently not removed from the perfusion medium. This is in contrast to a clearance of insulin, approximating 50 per cent under the same conditions. Many hormones, including insulin, are probably partially "deactivated" or cleared at or near their sites of action.[28] This does not seem to be the case in this particular in vitro situation, although it is certainly possible that NSILA was cleared at a rate undetectable by the limits of the bioassay. It is at least definitely slower than insulin clearance. If this finding can be extrapolated to in vivo situations, it may suggest that NSILA exerts insulin-like action over long intervals.

Further evidence bearing on discussions regarding the physiologic significance of NSILA can be deduced from these studies. NSILA, with a molecular weight of greater than 40,000[5,29] is similar in size to albumin and much larger than insulin (MW = 6,000). Based upon the fact that large, normally intravascular proteins are nonphysiologically and directly exposed to cell membranes in standard bioassay procedures, Rasio, Soeldner and Cahill question a physiologic role for ILA.[13] Froesch and associates are also concerned with the problem of the passage of NSILA through normal capillaries.[3] They do suggest, however, that NSILA crosses the capillary membrane under conditions of increased permeability, i.e., inflammation. They have also demonstrated biologic effects of this material in vivo after intravenous injection.[3] The isolated perfused rat heart is a preparation in which the uncut tissue is exposed to perfusate by means of its intact coronary vascular bed and simulates conditions occurring in vivo. That this is not completely comparable to the in vivo situation is inferred by some accumulation of edema in the heart during perfusion,[18] demonstrating the possibility of increased permeability to large molecules. However, this is not a necessary postulate for NSILA effects as both Froesch[3] and ourselves (unpublished results) have demonstrated an in vivo effect after injection. With these reservations, our data demonstrate a metabolic cellular action of this high molecular weight substance supplied via a "normal" vascular tree.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Dieter Hepp for his criticism and Miss Ellen Laschansky and Mrs. Gail Movius for their technical assistance.

This work was supported by U.S. Public Health Service Grants AM-02456, T1-5020-12 and AM-10215-01, an Institutional Research Grant from the American Cancer Society to the University of Washington, Boeing Good Neighbors Fund, and Fund 171 from the University of Washington, and a grant from the United Health Foundation, and U.S. Public Health Service Fellowships 5 F2 AM-30, 112-02 and 1 F3 AM-31, 618-01.

REFERENCES

1 Samaan, N. A., Fraser, R., and Dempster, W. J.: The typical and atypical forms of serum insulin. Diabetes *12*:339, 1963.

2 Antoniades, H. N., Huber, A. N., Boshell, B. R., Saraves, C. A., and Gershoff, S. N.: Studies on the state of insulin in blood: properties of circulating "free" and "bound" insulin. Endocrinology *76*:709, 1965.

3 Froesch, E. R., Müller, W. A., Bürgi, H., Waldvogel, M., and Labhart, A.: Nonsuppressible insulin-like activity of human serum. II. Biological properties of plasma extracts with nonsuppressible insulin-like activity. Biochim. Biophys. Acta *121*:360, 1966.

4 Kipnis, D. M., and Stein, M. F.: Ciba Foundation Colloquia on Endocrinology, Vol. 15. Boston, Little, Brown and Co., 1964, p. 156.

5 Poffenbarger, P. L., Ensinck, J. W., Hogan, R. A., and Williams, R. H.: Studies characterizing the nonsuppressible insulin-like activity (NSILA) from normal human plasma (abstract). Diabetes *15*:542, 1966.

6 Poffenbarger, P. L., Ensinck, J. W., Hepp, D. K., and Williams, R. H.: The nature of human serum insulin-like activity. J. Clin. Invest. In press.

7 Solomon, S., Poffenbarger, P., Hepp, D., Fenster, F., Ensinck, J., and Williams, R. H.: Quantitation and partial characterization of nonsuppressible insulin-like activity in serum and tissue extracts of the rat. Endocrinology *81*: August, 1967.

8 Froesch, E. R., Bürgi, H., Ramseier, E. B., Bally, P., and Labhart, A.: Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity. J. Clin. Invest. *42*:1816, 1963.

9 Ensinck, J. W., Poffenbarger, P. L., Hogan, R. A., and Williams, R. H.: Studies of insulin antagonism. I. An artifactual antagonist to insulin and plasma nonsuppressible

insulin-like activity in preparation of "albumin." Diabetes *16*:289, 1967.

[10] Antoniades, H. N., Huber, A. M., and Gershoff, S. N.: "Bound" insulin: in vivo and in vitro biologic activity. Diabetologia *1*:195, 1965.

[11] Antoniades, H. N., and Gershoff, S. N.: Inhibitory effects of "bound" insulin on insulin uptake by isolated tissues. Diabetes *15*:655, 1966.

[12] Antoniades, H. N., Bougas, J. A., Camerini-Davalos, R. F., and Pyle, H. M.: Insulin regulatory mechanisms and diabetes mellitus. Diabetes *13*:230, 1964.

[13] Rasio, E. A., Soeldner, J. S., and Cahill, G. F., Jr.: Insulin and insulin-like activity in serum and extravascular fluid. Diabetologia *1*:125, 1965.

[14] Gliemann, J.: Insulin-like activity of dilute human serum by an isolated adipose cell method. Diabetes *14*:643, 1965.

[15] Antoniades, H. N.: Studies on the state of insulin in blood. The state and transport of insulin in blood. Endocrinology *68*:7, 1961.

[16] Vallance-Owen, J., and Hurlock, B.: Estimation of plasma-insulin by the rat diaphragm method. Lancet *1*:68, 1954.

[17] Morgan, H. E., Henderson, M. J., Regan, D. M., and Parks, C. R.: Regulation of glucose uptake in muscle. J. Biol. Chem. *236*:253, 1961.

[18] Opie, L. H., Ship, J. C., Evans, J. R., and LeBoeuf, B.: Metabolism of glucose-U-C-14 in perfused rat heart. Amer. J. Physiol. *203*:839, 1962.

[19] Morgan, C. R., and Lazarow, A.: Immunoassay of insulin. Two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats. Diabetes *12*:115, 1963.

[20] Hepp, D. K., Poffenbarger, P. L., Ensinck, J. W., and Williams, R. H.: Effects of nonsuppressible insulin-like activity and insulin on glucose oxidation and lipolysis in the isolated adipose cell. Metabolism *16*:393, 1967.

[21] Seifter, S., Dayton, S., Novi, B., and Muntwyler, E.: The estimation of glycogen with the anthrone reagent. Arch. Biochem. *25*:191, 1950.

[22] Bray, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. *1*:279, 1960.

[23] Sonksen, P. H., Ellis, J., Lowy, C., Rutherford, A., and Nabarro, J. D. N.: A quantitative evaluation of the relative efficiency of gelatine and albumin in preventing insulin adsorption to glass. (Abstract). Diabetologia *1*:150, 1965.

[24] Leonards, J. R., Landau, B. R., and Bartsch, G.: Assay of insulin and insulin-like activity with rat epididymal fat pad. J. Lab. Clin. Med. *60*:552, 1962.

[25] Schwartz, I. L., and Hechter, D.: Insulin structure and function. Amer. J. Med. *40*:765, 1966.

[26] Antoniades, H. N.: Rat serum "bound" insulin, in vivo biologic effects in rats. Diabetes *15*:889, 1966.

[27] Gener-Segui, J., and Wolfe, A. J.: Studies of "bound" insulin by epididymal fat pad assay. Diabetes *15*:894, 1966.

[28] Elgee, N. J., Williams, R. H., and Lee, N. D.: Distribution and degradation studies with insulin I-131. J. Clin. Invest. *33*:1252, 1954.

[29] Bürgi, H., Müller, W. A., Humbel, R. E., Labhart, A., and Froesch, E. R.: Nonsuppressible insulin-like activity of human serum. I. Physicochemical properties, extraction and partial purification. Biochim. Biophys. Acta *121*:349, 1966.