Effects of Nethalide on Insulin Activity and Binding by Rat Muscle and Adipose Tissue

P. D. BEWSHER, C. C. HILLMAN, AND J. ASHMORE

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana

(Received January 4, 1966)

SUMMARY

Preincubation of rat adipose tissue and hemidiaphragm with nethalide, an adrenergic blocking agent, inhibits the effect of insulin on glucose uptake and $^{14}CO_2$ production from ^{14}C-labeled glucose. With the use of ^{131}I-labeled insulin, it is possible to demonstrate that blocking of the metabolic effects of insulin is not accompanied by any reduction in ^{131}I bound by the tissue. Addition of guinea pig anti-insulin serum does not affect the proportion of bound radioactive material when rat adipose tissue is incubated with radioinsulin, but markedly reduces insulin binding to rat diaphragm muscle. Incubation of the fat pad with radioinsulin for 90 min reduces the proportion of immunologically active insulin in the medium by about 10%. Experiments described indicate a lack of correlation between the metabolic effects of insulin and the binding of this hormone to tissues.

INTRODUCTION

Previous studies from this and other laboratories have indicated that lipolytic effects of hormones such as the catecholamines, adrenocorticotropin, thyroid-stimulating hormone, and glucagon on isolated adipose tissue can be effectively inhibited by adrenergic blocking agents and insulin (1–6). If insulin and the adrenergic blocking drugs have a common point of interaction, the possibility existed that the metabolic effects of insulin on adipose tissue might be inhibited by adrenergic blockers.

In the present study nethalide was selected as the sympatholytic agent of choice. This drug has been found to be an effective adrenergic β-receptor blocker free from intrinsic sympathomimetic activity. These properties have been confirmed in cardiovascular studies in animals and man (7, 8), and the rise in plasma free fatty acids following injection of epinephrine in human subjects is effectively blocked by nethalide (9). In the present study, isolated rat diaphragm muscle and epididymal adipose tissue were tested for insulin responsiveness in the presence and absence of nethalide.

The demonstration that nethalide blocked the metabolic action of insulin prompted further experiments to investigate the phenomenon of insulin binding to tissues. The theory that a hormone, in order to exert its effects, forms a chemical attachment to tissue has received considerable support. However, recent studies with labeled insulin indicate that binding of insulin may be unrelated to its metabolic action.

METHODS

Male albino rats, which had been maintained on laboratory chow and tap water, were killed by stunning and exsanguination. The epididymal fat pads were removed and bisected, one-half acting as control for the other. The diaphragm was removed and bisected, and the membranous part was

discarded. For the experiments using nethalide, the tissues were incubated at 37° in 4 ml of Ringer-bicarbonate buffer containing 1% bovine serum albumin, glucose-1-^{14}C (1 mg/ml) and nethalide (10^{-3} M). After 15 min, insulin (100 μU/ml) was added to the flasks, and the incubation was continued for a further 75 min. Medium glucose concentrations were measured by the glucose oxidase method (10) and $^{14}CO_2$ produced from labeled glucose was plated as $BaCO_3$ (11) and counted in a gas flow counter. Incorporation of ^{14}C into muscle glycogen was determined by the method described by Rafaelsen *et al.* (12).

In the experiments on the binding of insulin to tissues, fat pads and hemidiaphragms were incubated for 90 min, unless otherwise stated, in 5 ml and 2 ml, respectively, of Ringer bicarbonate medium containing 1% bovine serum albumin. ^{131}I-Labeled insulin was used (specific activity 9–11 mC/mg)in varying concentrations, and the concentration of nethalide was again 10^{-3} M. Anti-insulin serum (AIS) was prepared as described by Robinson and Wright (13). After incubation, the amount of bound ^{131}I was determined by washing the tissues three times by gentle agitation for 1 min in 25 ml of insulin-free medium, similar to the method used by Stadie *et al.* (14). After blotting, the tissues were placed in 5 ml of Bray's solution and the bound radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. The amounts of radioactive material bound by the tissues have been expressed as percentages of the total medium radioactivity bound per gram of wet tissue.

Any change in the concentration of insulin in the medium was estimated by adding excess AIS to aliquots of the medium, using the same dilution of normal serum as the control. Free insulin and antibody-insulin complex were separated by the addition of an equal volume of a 10% w/v suspension of finely powdered cellulose (MN-300, Macherey, Nagel and Co., Duren, Germany). Free insulin adsorbs to the cellulose, and after centrifugation the supernatant contains antibody-insulin complex plus a small amount of degraded insulin which may be present.

RESULTS

The effects of insulin and nethalide on glucose uptake by isolated adipose tissue have been summarized in Fig. 1. Insulin

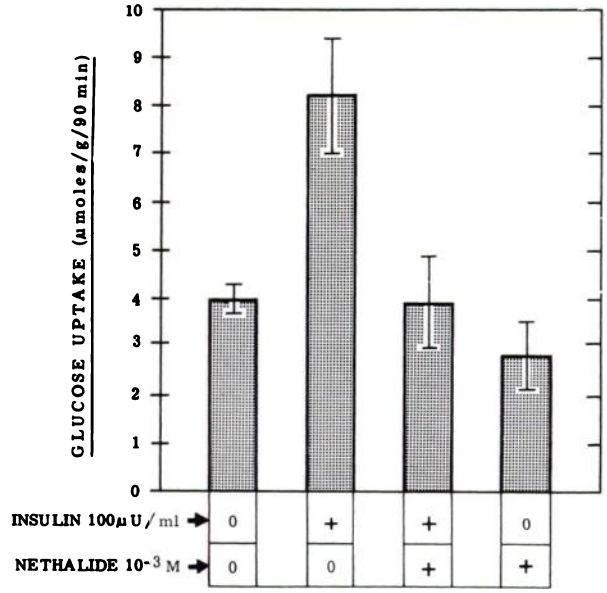

FIG. 1. *The effect of insulin and nethalide on glucose uptake by rat epididymal fat pad* Values are means ±SEM of 8 observations.

increased glucose uptake twofold ($P < 0.01$), but this increase was abolished if nethalide was present in the medium ($P < 0.01$), and nethalide alone had no significant effect on the basal uptake of glucose. A similar effect on $^{14}CO_2$ production from labeled glucose is shown in Fig. 2. The increased CO_2 formation induced by insulin ($P < 0.01$) was partially blocked by the nethalide ($P < 0.01$). Since the blocking effect of nethalide may have been due to decreased binding of insulin with the tissue, the incubations were repeated with ^{131}I-labeled insulin (Fig. 3). The reduced metabolic effect of insulin in the presence of nethalide is again demonstrated, but the amount of ^{131}I bound by the fat pad was not altered by the nethalide. When the tissues were incubated with nethalide and different concentrations of labeled insulin (1000 μU/ml and 100 μU/ml) a similar small percentage of the total radioactivity was bound in each case, and nethalide did not reduce the binding of radioactivity; in fact, the binding appeared to be slightly increased in the presence of nethalide, and this was statistically significant when the concentration of insulin was 1000 μU/ml (Fig. 4).

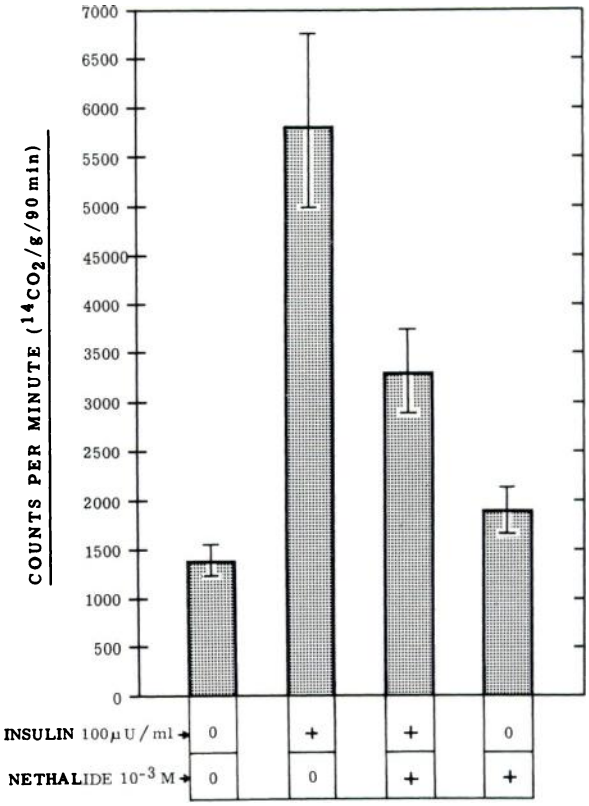

FIG. 2. *The effect of insulin and nethalide on $^{14}CO_2$ production from glucose-1-^{14}C by rat epididymal fat pad*

Values are means ±SEM of 8 observations.

The blocking effect of nethalide was further demonstrated when the hemidiaphragm was substituted for adipose tissue and the insulin effect was measured by the incorporation of ^{14}C-labeled glucose into tissue glycogen. The results shown in Table 1 indicate that inhibition of the insulin effect was not accompanied by any reduction in insulin binding, as measured by the amount of bound radioactivity after washing. In fact, as had been observed with the

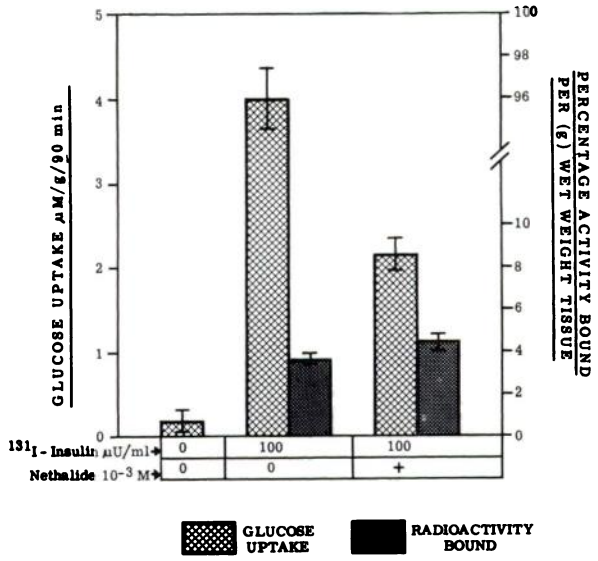

FIG. 3. *Effect of nethalide on ^{131}I-insulin stimulated glucose uptake and binding of radioactivity by rat adipose tissue*

Values are means ±SEM of 8 observations.

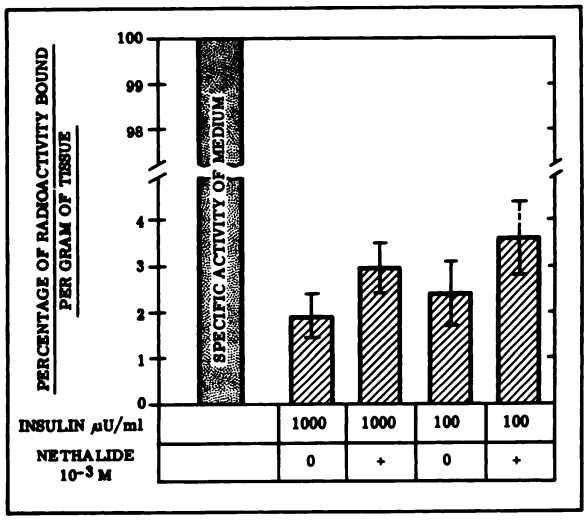

FIG. 4. *Effect of nethalide on binding of radioactivity by rat epididymal fat pad at 2 different concentrations of ^{131}I-insulin (100 and 1000 μU/ml)*

Values are means ±SEM of 8 observations.

fat pad, the amount of radioactivity bound to the tissue was increased in the presence of nethalide.

The apparent discrepancy between the binding of insulin and its metabolic activity led to further experiments on ^{131}I-insulin binding to the rat epididymal fat pad and the rat hemidiaphragm. The biological activity of insulin both *in vivo* and *in vitro* can be effectively inhibited by addition of serum from guinea pigs previously immunized against insulin.

Anti-insulin serum (AIS) was added to the incubation flasks in amounts far in

TABLE 1
Effect of nethalide and insulin on incorporation of ^{14}C-glucose into rat diaphragm glycogen and binding of radioactivity from ^{131}I-insulin

Parameter	Control	Insulin	Insulin + nethalide	
Incorporation of ^{14}C-glucose into diaphragm (cpm per gram tissue)	7100 ± 1300[a]	11,500 ± 350	7400 ± 700	$P < 0.001$
Bound radioactivity (^{131}I-insulin) (percentage of total medium activity bound per gram wet tissue)	—	12.3 ± 0.52	20.0 ± 1.22	$P < 0.001$

[a] Values are means ± SEM of 8 observations. Insulin concentration, 100 μU/ml.

excess of that required to neutralize the ^{131}I-insulin (100 μU/ml) present. After preincubation for 15 minutes, the tissues were added and the incubation was continued for a further 75 minutes. The amounts of bound ^{131}I are shown in Table 2. The phragms in a medium containing a 5% suspension of cellulose, to which insulin adheres, significantly reduced the amount of radioactive material bound to both tissues (Table 3). Using the fat pad, attempts were made to remove bound

TABLE 2
Effect of preincubation of ^{131}I-insulin with AIS on binding of radioactivity

Tissue	Excess AIS	Normal serum	
Epididymal fat pad	2.94 ± 0.28[a]	3.25 ± 0.3	NS
Hemidiaphragm	1.69 ± 0.21	12.9 ± 0.13	$P < 0.001$

[a] Expressed as percentage of total medium ^{131}I bound per gram of wet tissue. Values are means ± SEM of 8 observations. ^{131}I-Insulin concentration, 100 μU/ml. NS = not significant.

formation of an insulin–antibody complex was confirmed in those flasks containing AIS by addition of a cellulose suspension to aliquots of the medium, centrifugation, and counting the radioactivity in the supernatant fraction as described in the section on methods. The presence of excess AIS did not interfere with the binding of radioactivity by the fat pad, but markedly reduced the amount bound by the hemidiaphragm.

Incubation of fat pads and hemidiaradioactivity by washing the tissue with medium containing cellulose or AIS. The results of these washes are shown in Table 4. Neither the cellulose nor the AIS were able to remove radioactivity from the tissue in excess of the control. The difference between the amount of bound radioactivity in experiments with cellulose and AIS washing may be related to the radioactive age of the labeled insulin. To determine whether the ^{131}I which binds to the tissue was insulin, rather than some

TABLE 3
Effect of cellulose and ^{131}I-insulin on binding of radioactivity

Tissue	Cellulose	Control	
Epididymal fat pad	0.78 ± 0.03	2.92 ± 0.13	$P < 0.001$
Hemidiaphragm	1.61 ± 0.07	16.5 ± 0.8	$P < 0.001$

[a] Expressed as percentage of total medium ^{131}I bound per gram of wet tissue. Values are means ± SEM of 8 observations. ^{131}I-Insulin concentration, 100 μU/ml.

TABLE 4
Effect of washing with cellulose and AIS on radioactivity bound to fat pad

Wash	Per cent radioactivity/gram tissue after wash	Control wash	
Cellulose	1.90 ± 0.12	1.91 ± 0.09	NS
AIS	3.18 ± 0.19	2.73 ± 0.2	NS

[a] Expressed as percentage of total medium ^{131}I bound per gram of wet tissue. Values are means ± SEM of 8 observations. ^{131}I-Insulin concentration, 100 μU/ml. NS = not significant.

degradation product such as ^{131}I-labeled polypeptides, ^{131}I-labeled tyrosine, or even molecular $^{131}I_2$, incubation was carried out with ^{131}I-insulin which had been treated with "insulinase" of liver homogenates. In these experiments all the ^{131}I-insulin was degraded, as determined by trichloroacetic acid precipitation and binding to AIS and cellulose. Heat-inactivated insulinase served as a control. The percentages of radioactivity bound by fat pad and hemidiaphragm are given in Table 5.

TCA. When radioinsulin was added to the tissue immediately before homogenization, only 15 ± 3.5% of the radioactivity was TCA soluble. Labeled insulin, in the absence of homogenate, is wholly TCA precipitable. It seems, therefore, that a large proportion of the radioactive material bound to the tissue after 90 min incubation represents some degradation product of insulin.

Any change in the concentration of insulin in the medium following incubation with adipose tissue was estimated as described in the section on methods. In the absence of tissue, 7% of the radioactivity in the medium after 90 min incubation was degraded insulin (Fig. 5), but in the presence of adipose tissue, the degraded insulin had increased to 17%, with a complementary fall in immunoreactive insulin. It appears that during incubation with adipose tissue some degradation of insulin does occur.

Fat pads were incubated with ^{131}I-insulin (100 μU/ml), and to some of the flasks, nonradioactive insulin (400 μU/ml) was also added at the same time as the

TABLE 5
Effect of insulinase-treated ^{131}I-insulin on binding of radioactivity

Tissue	Insulinase	Inactivated insulinase	
Epididymal fat pad	2.18 ± 0.1	3.24 ± 0.27	$P < 0.005$
Hemidiaphragm	17.3 ± 0.6	11.3 ± 0.6	$P < 0.001$

[a] Expressed as percentage of total medium ^{131}I bound per gram of wet tissue. Values are means ± SEM of 8 observations. ^{131}I-Insulin concentration, 100 μU/ml.

Again, a difference between the behavior of the fat pad and the hemidiaphragm was observed, since the amount of radioactive material bound to the fat pad was slightly but significantly reduced after insulinase treatment of the radioinsulin, whereas the radioactivity on the hemidiaphragm was increased. The radioactivity bound to hemidiaphragm was further investigated by homogenizing the tissues after 90 min exposure to ^{131}I-insulin (400 μU/ml). The homogenates were treated with trichloroacetic acid (TCA), and 62 ± 18% of the tissue radioactivity was not precipitated by labeled insulin. Glucose uptake increased in response to the radioactive insulin, and a greater response was obtained by those tissues to which the unlabeled insulin had been added (Fig. 6). However, the amount of bound ^{131}I was unaltered, indicating lack of competition for available receptor sites. The same effect was obtained if the amount of unlabeled insulin is increased to 1×10^6 μU/ml.

The effect of *N*-ethylmaleimide (NEM), a sulfhydryl blocking agent, on insulin binding was investigated by preincubating hemidiaphragms with two concentrations of

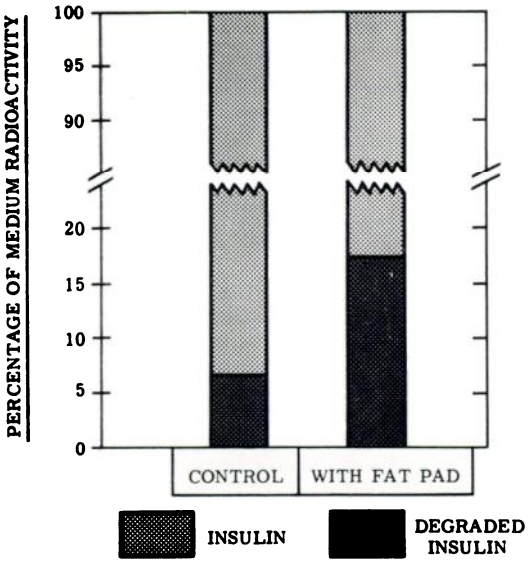

FIG. 5. *Analysis of medium radioactivity after 90 min incubation with and without rat fat pad*

this compound for 15 min prior to adding ^{131}I-labeled insulin (200 μU/ml). In the absence of NEM the ^{131}I bound was 11.5%, and with the concentration of NEM at 10^{-4} M, this figure was not significantly altered at 11.3%. When the concentration of NEM was raised to 10^{-3} M the radioactivity of the tissues increased to 22.6% (Table 6).

TABLE 6
Effect of N-ethylmaleimide (NEM) on insulin binding by rat hemidiaphragm

Insulin	11.5 ± 0.55[a]
Insulin + NEM (10^{-4} M)	11.3 ± 0.82
Insulin + NEM (10^{-3} M)	22.6 ± 0.97[b]

[a] Expressed as percentage of total medium ^{131}I bound per gram of wet tissue. Values are means ± SEM of 8 observations. ^{131}I-insulin concentration 200 μU/ml.

[b] $P < 0.001$.

DISCUSSION

The results of the experiments on insulin binding, using radioactive insulin, indicate that there is little, if any, correlation between the binding of this hormone to a tissue and its metabolic activity.

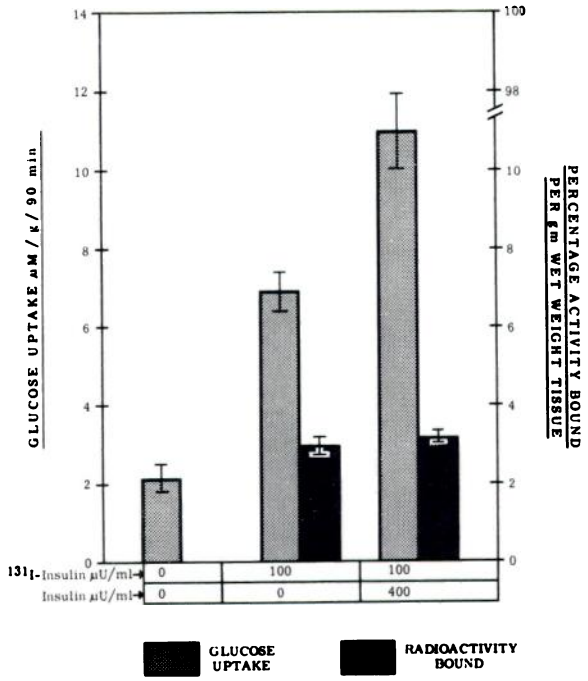

FIG. 6. *Effect of addition of unlabeled insulin to ^{131}I-labeled insulin on glucose uptake and binding of radioactivity by rat epididymal fat pad*

Values are means ±SEM of 8 observations.

The blocking effect of nethalide on insulin stimulation of glucose utilization by rat adipose tissue and hemidiaphragm is not accompanied by any reduction of insulin binding, as estimated by the amount of radioactivity bound to the tissues after washing.

Stadie and his co-workers, initially using unlabeled insulin, and later, insulin labeled with ^{131}I and ^{35}S, showed that after exposing tissues, usually rat diaphragm, to varying concentrations of insulin for 1 min, washing, and reincubating in insulin-free medium, the insulin effect, measured as glycogen synthesis, was related to the concentration of insulin during the initial brief exposure (14–19). The experimental procedure described in this paper differs from that of Stadie *et al.* chiefly in the duration of exposure of the tissues to insulin, which was for periods up to 90 min. The concentrations of insulin used in the present experiments ranged from 100 to 1000 μU/ml, and correspond to the upper limits of what is presently considered to be physiological concentrations. Previous workers have used insulin concentrations ranging from 10^3 to 10^6 μU/ml, and indeed, Stadie *et al.* were unable to find evidence for insulin "binding," determined as biological activity of the bound insulin, when concentrations less than 10^4 μU/ml were employed.

Though excess AIS will neutralize insulin and prevent its metabolic effects *in vitro*, AIS did not reduce the binding of radioactivity to the fat pad (Table 2), a result suggesting that the amount of ^{131}I-insulin bound is unrelated to its metabolic effect; or that the bound radioactivity is not, in fact, insulin. The results with hemidiaphragm indicate that AIS may prevent the binding of insulin. However, if the bound radioactive material is not insulin, but degraded insulin, the discrepancy with the diaphragm may be the result of protection of insulin, by the formation of insulin–antibody complexes, from insulin-degrading systems which are more active in muscle than adipose tissue. However, the addition of cellulose, which strongly adsorbs insulin, to the incubation medium markedly reduced the radioactivity bound by both tissues. Like the experiment using AIS, this result may be interpreted in two ways. First, if the bound material is insulin, the cellulose has prevented it from binding with the tissues; or secondly, the bound radioactivity may be degraded insulin and the low values in the presence of cellulose may reflect protection of the hormone from breakdown by the tissue.

If the bound radioactivity is entirely degraded insulin, we would expect a marked increase if the hormone was pretreated with insulinase. An increase did occur when the hemidiaphragm was investigated, but by much less than would have occurred if the amount of binding was related to the proportion of radioactive material which was degraded insulin. But there was a small, but significant, decrease in binding by the fat pad after insulinase treatment of the medium. This is difficult to understand, since if the reduction is due to the nonavailability of insulin following its destruction by insulinase, then the remaining radioactivity in the tissue must be degraded insulin—which should have increased, since much more is present after insulinase treatment of the insulin.

The results shown in Table 6, indicate that most of the ^{131}I bound to the hemidiaphragm after 90 min incubation is not precipitable by trichloroacetic acid. In fact, from these figures, the maximum bound radioactivity which is true insulin is approximately 38%, the remainder being degraded insulin. The period of incubation for this experiment was long, and the results are not necessarily the same as might occur after a brief exposure to insulin. Because of the insulin-degrading activity of muscle, it is quite possible that the proportion of radioactive material which is insulin may decrease with increasing duration of incubation.

The apparent lack of correlation between insulin binding and its metabolic effect, indicated by these experiments, supports conclusions reached by other workers. Newerly and Berson (20) remarked on the lack of specificity of binding of insulin by rat diaphragm and showed that the

amount bound was always small, but proportional to the total radioactivity to which the tissue was exposed. Similar results have been obtained when the metabolically active tissue was substituted by inactive tissue or even glass.

Although much evidence exists to indicate a lack of correlation between insulin binding and biological activity, there are also data that suggest that there may be a relation between insulin binding and action. Ball and Jungas (21), using rat adipose tissue and insulin concentrations in the region of 0.1 U/ml, were able to demonstrate continued metabolic activity after exposing the tissue to insulin, washing extensively and reincubating in insulin-free medium. This latter medium, at the end of incubation, was able to exert an insulin effect on fresh tissue, indicating that insulin, bound to the tissue during the initial exposure, and resistant to removal by washing, had been released into the medium during the second incubation.

Malaisse and Franckson (22) have confirmed that the radioactivity bound to muscle after incubation with ^{131}I-insulin, is directly related to the concentration of insulin to at least 10^6 μU/ml, but an increased metabolic effect was not obtained with concentrations greater than 10^3 μU/ml. These workers have shown that increasing concentrations of AIS in the medium proportionally decreased radioactivity bound to the isolated rat diaphragm muscle. In this case bound radioactivity was shown to be proportional to the surface area of the tissue.

Wardlaw and Weidinger (23) confirmed the results of Stadie *et al.*, using concentrations of insulin as low as 0.5 mU/ml, but did not obtain an effect on glycogen synthesis below a concentration of 1 mU/ml. In addition, they demonstrated that, for any given increase in glycogen synthesis, a 5-min exposure to insulin required ten times the concentration of insulin that would be needed if insulin was present throughout the incubation.

The hypothesis that the action of vasopressin on the kidney and the toad bladder is mediated by a thiol-disulfide reaction between a disulfide bond in the hormone and a sulfhydryl group on the receptor, has been extended to the action of insulin. The insulin-like action of synthetic oxytocin on rat adipose tissue would indicate a similar receptor site for both hormones (24). The disulfide linkage concerned connects two cystine molecules on the A chain. Fong *et al.* (25), using thiol compounds to displace radioactivity from tissues, have produced evidence to support this hypothesis. Further supporting evidence is provided by the experiments of Cadenas and coworkers (26), who were able to block the effect of insulin on glucose utilization by the perfused rat heart, by pretreatment with *N*-ethylmaleimide. The same treatment reduced the binding of radioactivity from ^{131}I-labeled insulin in the perfusion fluid, to one half the control level. On the other hand, Mirsky and Perisutti (27) were able to inhibit the metabolic action of insulin on rat adipose tissue with NEM but were unable to affect the binding of radioactive insulin with rat hemidiaphragm and rat adipose tissue. We have confirmed their result on the rat diaphragm when the concentration of NEM was 10^{-4} M. When the concentration was raised to 10^{-3} M, the binding of ^{131}I with the tissues was significantly increased; since we have shown that NEM causes breakdown of insulin, the radioactive material bound may be degraded insulin.

The experiments outlined in this report confirm the lack of correlation between the action of insulin and its binding with tissues, as measured by bound radioactivity from ^{131}I-labeled insulin after rather lengthy incubations *in vitro*. The results and the conclusions drawn from them do not deny the possibility of a combination between hormone and effector site in order that insulin may produce its metabolic effect, but assuming that insulin labeled with ^{131}I behaves in the same way as unlabeled insulin, our data do not support this hypothesis. It is possible that only the briefest exposure to insulin is sufficient to trigger a chain of reactions that will continue for some as yet undetermined time, the magnitude of this reaction being related to the

concentration of insulin to which the tissue was exposed. Any insulin bound to the tissue after washing may well be non-specifically adherent and completely unrelated to the metabolic state induced by the initial exposure.

REFERENCES

1. R. S. Gordon, Jr., and A. Cherkes, *Proc. Soc. Exptl. Biol. Med.* **97**, 150 (1958).
2. M. C. Schotz and I. H. Page, *J. Lipid Res.* **1**, 466 (1960).
3. R. J. Havel and A. Goldfien, *J. Lipid Res.* **1**, 102 (1959).
4. E. Wertheimer, M. Hamosh and E. Shafrir, *Am. J. Clin. Nutr.* **8**, 705 (1960).
5. W. C. Love, L. Carr and J. Ashmore, *J. Pharmacol. Exptl. Therap.* **140**, 287 (1963).
6. C. H. Hollenberg, M. S. Raben and E. B. Astwood, *Endocrinology* **68**, 589 (1961).
7. J. W. Black and J. S. Stephenson, *Lancet* **ii**, 311 (1962).
8. A. C. Dornhorst and B. F. Robinson, *Lancet* **ii**, 314 (1962).
9. T. R. E. Pilkington, R. D. Lowe, B. F. Robinson and E. Titterington, *Lancet* **ii**, 316 (1962).
10. A. S. Huggett and D. A. Nixon, *Lancet* **ii**, 368 (1957).
11. M. L. Karnovsky, J. M. Foster, L. I. Gidez, D. D. Hagerman, C. V. Robinson, A. K. Solomon and C. A. Villee, *Anal. Chem.* **27**, 852 (1955).
12. O. J. Rafaelsen, V. Lauris and A. E. Renold, *Diabetes* **14**, 19 (1965).
13. B. H. B. Robinson and P. H. Wright, *J. Physiol. (London)* **155**, 302 (1961).
14. W. C. Stadie, N. Haugaard, J. B. Marsh and A. G. Hills, *Am. J. Med. Sci.* **218**, 265 (1949).
15. W. C. Stadie, N. Haugaard, A. G. Hills and J. B. Marsh, *Am. J. Med. Sci.* **218**, 275 (1949).
16. W. C. Stadie, N. Haugaard and J. B. Marsh, *J. Biol. Chem.* **189**, 53 (1951).
17. N. Haugaard and J. B. Marsh, *J. Biol. Chem.* **194**, 33 (1952).
18. W. C. Stadie, N. Haugaard and M. Vaughan, *J. Biol. Chem.* **199**, 729 (1952).
19. W. C. Stadie, N. Haugaard and M. Vaughan, *J. Biol. Chem.* **200**, 745 (1953).
20. K. Newerly and S. A. Berson, *Proc. Soc. Exptl. Biol. Med.* **94**, 751 (1957).
21. E. G. Ball and R. L. Jungas, *Rec. Progr. Hormone Res.* **20**, 183 (1963).
22. W. Malaisse and J. R. M. Franckson, *Arch. Intern. Pharmocodyn.* **155**, 484 (1965).
23. A. C. Wardlaw and H. J. Weidinger, *Can. J. Physiol. Pharmacol.* **43**, 943 (1965).
24. I. A. Mirsky and G. Perisutti, *Endocrinology* **71**, 158 (1962).
25. C. T. O. Fong, L. Silver, E. A. Popenoe and A. F. Debons, *Biochim. Biophys. Acta* **56**, 190 (1962).
26. E. Cadenas, H. Kaji, C. R. Park and H. Rasmussen, *J. Biol. Chem.* **236**, Pc 63 (1961).
27. I. A. Mirsky and G. Perisutti, *Biochim. Biophys. Acta* **62**, 490 (1962).