# Ligands for Insulin Receptor Isolation†

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ABSTRACT: Biotinylated insulins are bivalent molecules having the ability to bind to insulin receptors on the one hand and to "avidins" on the other. In order to be useful as ligands for insulin receptor isolation, biotinylated insulins must be developed that have the capacity to bind simultaneously to both avidin and insulin receptor. The present investigation addresses this problem. A series of biotinylated and dethiobiotinylated insulins has been prepared in which the distance between the biotin carboxyl group and the insulin varies from 7 to 20 atoms. These compounds form complexes with succinoylavidin. The dissociation rates ($K_{-1}$) of these complexes have been determined from the [$^{14}$C]biotin exchange assay. The dissociation kinetics of most of these complexes are biphasic, and the kinetic constants reported are those corresponding to the slow rate. Ligands containing dethiobiotin dissociate more rapidly than the corresponding biotin derivatives. The interposition of a spacer arm substantially decreases the rate of dissociation. The [$^{14}$C]biotin exchange assay could not be used with streptavidin complexes of the above ligand since biotin dissociates more rapidly from streptavidin than from succinoylavidin. However, the relative dissociation rates of a series of ligands could be determined and were as follows: 6-(dethiobiotinylamido)-hexanoic acid > dethiobiotinyl-A1-insulin > biotinylinsulin > biotinyl-A1-insulin > biotinyl-A2-insulin. Dethiobiotin and its amide failed to form complexes with streptavidin. The affinity of the ligands for insulin receptors was determined by measuring their ability to stimulate $^{14}CO_2$ formation from [1-$^{14}$C]glucose in rat epididymal adipocytes. With the exception of biotinyl-A2- and dethiobiotinyl-A2-insulins, which required higher concentrations to achieve the same degree of stimulation, the ligands were as active as insulin. It was of no consequence, as concerns stimulation of glucose oxidation, whether the ligands contained biotin or dethiobiotin. Succinoylavidin, avidin, and streptavidin did not interfere with the ability of insulin to promote glucose oxidation in adipocytes, but the stimulatory activity of the biotinylated insulins was significantly inhibited by avidins. The degree of avidin inhibition appears to provide a measure for the capacity of the ligands to bind simultaneously to both the avidins and the insulin receptors. A systematic investigation of these effects showed that biotinylinsulin stimulation of adipocytes is totally inhibited at an avidin to ligand molar ratio of 50:1. Ligands containing spacer arms retained significant activity (20–30%) even at ratios as high as 100:1. We interpret this to mean that these ligands can react simultaneously with avidins and insulin receptors and that they are promising tools for receptor isolation studies.

In recent papers (Hofmann et al., 1977, 1982, 1984; Finn et al., 1981), we have described the synthesis, biological activity, and avidin binding properties of insulin derivatives containing biotin or dethiobiotin attached either directly or via a spacer arm to the terminal amino group of the B chain. The ultimate goal of these studies is the development of bifunctional reagents to be used for insulin receptor isolation by avidin–Sepharose affinity chromatography. They are intended to serve as a model for the development of a general method for isolating peptide and protein hormone receptors.

The results of these experiments have contributed to a better understanding of the interaction of avidin with the various biotinyl- and dethiobiotinylinsulin derivatives).[1] Dissociation rate measurements with Suc-avidin–biotinylinsulin complexes (Finn et al., 1980; Hofmann et al., 1982) have shown that attachment of the insulin molecule via its $N^{\alpha,B1}$-amino group to the carboxyl group of biotin increases the rate of the off reaction (as compared with the Suc-avidin–biotin complex) more than 1000-fold. This rate change indicates that the insulin molecule interferes with binding of the biotin residue to avidin. Introducing a spacer arm (6-aminohexanoic acid) between the biotin and the insulin residues improves the stability of the ensuing Suc-avidin complexes.

Although we have shown that some of these ligands bind to Suc-avidin, we have not assessed their ability to bind to insulin receptors. The success of the avidin–biotin approach to receptor isolation depends critically on the ability of the ligands to bind simultaneously to avidins and receptor. In order to address this point, we have systematically investigated the receptor binding properties, i.e., the ability to stimulate glucose oxidation, in rat epididymal adipocytes of biotinyl and dethiobiotinyl derivatives of insulin containing spacer arms varying in length. Furthermore, we have measured the ability of the ligands to interact with the receptors in the presence of excesses of avidin, Suc-avidin, and streptavidin.

The aim of the experiments presented in this paper is to attempt to determine the optimum length of spacer arm that will permit reasonably strong binding of the biotinyl portion of the molecule to "avidins" on the one hand and maximal interaction between the insulin portion of the molecule and its receptor on the other.

## Experimental Procedures

Collagenase CLS was purchased from Worthington, [1-$^{14}$C]glucose (sp act. 2–4 mCi/mmol) and [$^{14}$C]biotin (sp act. 48 mCi/mmol) were from Amersham, and bovine serum al-

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[1] Abbreviations: X, spacer; Suc-avidin, succinoylavidin; biotinyl-insulin, $N^{\alpha,B1}$-biotinylinsulin; dethiobiotinylinsulin, $N^{\alpha,B1}$-dethiobiotinyl-insulin; biotinyl-A1-insulin, $N^{\alpha,B1}$-[6-(biotinylamido)hexyl]insulin; biotinyl-A2-insulin, $N^{\alpha,B1}$-[6-[[(biotinylamido)hexanoyl]amido]hexyl]insulin; dethiobiotinyl-A1-insulin, $N^{\alpha,B1}$-[6-(dethiobiotinylamido)hexyl]insulin; dethiobiotinyl-A2-insulin, $N^{\alpha,B1}$-[6-[[[(dethiobiotinyl)amido]hexanoyl]-amido]hexyl]insulin; dethiobiotinyl-A1-DPA-insulin, $N^{\alpha,B1}$-[*N*-[3-[[6-[6-(5-methyl-2-oxo-4-imidazolidinyl)hexanamido]hexanamido]-propyl]amino]propyl]succinamoyl]insulin; Tris, tris(hydroxymethyl)-aminomethane. For structures and syntheses of these compounds, see Hofmann et al. (1977, 1982, 1984).

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bumin fraction V and avidin were from Sigma. Streptavidin was a gift from Bethesda Research Labs, and Suc-avidin was prepared as described previously (Finn et al., 1980). Biotin was a gift from Dr. W. E. Scott of Hoffmann-La Roche Inc. Sephadex G-50 was from Pharmacia. Scintillation cocktail (3a70B), 2,5-diphenyloxazole (PPO), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) were from Research Products International.

*Ligand Displacement Studies.* Complexes were prepared by mixing Suc-avidin or streptavidin (10 nmol) in 50 mM Tris-HCl, pH 7.6, with the desired ligand (80 nmol, a 2-fold excess) in 0.01 N HCl (0.6 mL). Excess [$^{14}$C]biotin (2 $\mu$mol, sp act. 3.75 mCi/mmol) in Tris buffer was added, and the solution was incubated at 25 °C (total volume 3.0 mL). For experiments lasting longer than 24 h, sodium azide (0.05%) was added. At specified intervals, aliquots (0.1 mL) were subjected to gel filtration on Sephadex G-50 columns (0.9 × 55 cm) equilibrated with Tris buffer (Wei & Wright, 1964). Fractions (1 mL each) were collected in polyethylene minivials, scintillation cocktail (3a70B, 5 mL) was added, and the radioactivity was measured. The radioactivity in the fractions corresponding to high molecular weight material provided a measure of the rate of dissociation. Recovery of radioactivity from the column was quantitative. The total [$^{14}$C]biotin binding capacities of the various preparations of Suc-avidin or streptavidin were evaluated in the same manner. Rates of dissociation were determined from the slope of the curve obtained (least-squares fitting) by plotting log of the percent complex remaining vs. time (Hofmann et al., 1982).

*Adipocyte Assays.* Sprague-Dawley rats (up to 200 g) were sacrificed by decapitation; the epididymal fat pads were removed and placed in 0.9% saline. The tissue was minced, incubated for 1 h in Krebs–Ringer bicarbonate buffer (37 °C) containing 4% bovine serum albumin and collagenase, 1 mg/100 mg of tissue, and processed as described by Gliemann (1967). Isolated adipocytes (less than 5 × $10^4$ cells/mL) were incubated in the same medium containing insulin, the ligands, or the complexes for 2 h. The reaction was stopped by addition of 1 N sulfuric acid, and $CO_2$ was trapped as described (Rodbell, 1964). Radioactivity was measured in a counting cocktail containing POPOP (100 mg/L) and PPO (4.15 g/L) in toluene.

*Synthesis of Dethiobiotinamide.* To *N*-hydroxysuccinimido dethiobiotinate (500 mg) (Hofmann et al., 1982) was added methanol (60 mL) saturated at 0 °C with ammonia. The suspension was stirred overnight at room temperature, the solvent was removed in vacuo, and the solid was dried and crystallized from water, mp 200–201 °C. A single spot on thin-layer chromatography ($R_f$ 0.3) in the chloroform–methanol–water (8:3:1, upper phase) system was seen. Anal. Calcd for $C_{10}H_{19}N_3O_2$: C, 56.32; H, 8.98; N, 19.70. Found: C, 56.23; H, 8.80; N, 19.43.

## Results

*Dissociation of Biotinyl and Dethiobiotinyl Ligands from Suc-avidin and Streptavidin.* We have determined the dissociation rates for Suc-avidin complexes of a number of biotinyl and dethiobiotinyl derivatives of insulin in which the distance between the carboxyl groups of biotin or dethiobiotin and insulin has been varied from 7 to 20 atoms (Table I). The rate of dissociation of biotin ($K_{-1}$ = 4 × $10^{-8}$ $s^{-1}$) is negligible. Solubility problems with avidin complexes, at the concentrations used, precluded dissociation measurements. It should be mentioned that the dissociation kinetics for most of the complexes are biphasic and the kinetic constants given are those for the slower rate.

Table I: Dissociation Rates for Suc-avidin Complexes[a]

| ligand | $t_{1/2}$ | $k_{-1}$ ($s^{-1}$) |
|---|---|---|
| biotin | 127 days[b] | 6.3 × $10^{-8}$[c] |
| biotinylinsulin | 2.6 h | 7.4 × $10^{-5}$[c] |
| biotinyl-A1-insulin[d] | 76 days | 1.1 × $10^{-7}$ |
| biotinyl-A2-insulin | 70 days | 1.2 × $10^{-7}$ |
| dethiobiotin[e] | 14 h | 1.4 × $10^{-5}$[c] |
| dethiobiotinyl-A1-insulin | 6 h | 3.2 × $10^{-5}$[c] |
| dethiobiotinyl-A2-insulin | 7 h | 2.8 × $10^{-5}$ |
| dethiobiotinyl-A1-DPA-insulin | 14 h | 1.4 × $10^{-5}$ |

[a] The dissociation kinetics for the complexes are biphasic. Green et al. (1971) and Chignell et al. (1975) have shown that avidin possesses two classes of biotin binding sites. The kinetic constants given are those for the slower rate. [b] Finn et al., 1980. [c] Previously reported values (Hofmann et al., 1982) were determined at room temperature. [d] See abbreviations footnote for nomenclature. [e] Within experimental error, the same results have been obtained with a 100-fold excess of [$^{14}$C]biotin (see Experimental Procedures for details).

In general, ligands containing dethiobiotin dissociate more rapidly than the corresponding biotin derivatives. The interposition of a 6-aminohexyl spacer arm between the biotin or dethiobiotin and insulin substantially decreases the rate of dissociation of all the complexes. Curiously, no further change in rate is evident when two 6-aminohexyl groups separate the biotin or dethiobiotin from the insulin residue; however, the rate of dissociation of dethiobiotinyl-A1-DPA-insulin, the derivative with the 20-atom spacer arm, is as slow as that of dethiobiotin itself.

The technique employed to determine the rate of dissociation of Suc-avidin complexes depends on the rapid and essentially irreversible binding of biotin to Suc-avidin. The presence of an excess of biotin prevents the rebinding of dissociated ligands. This technique cannot be employed for the determination of dissociation rate constants for streptavidin complexes because biotin dissociates much more rapidly from streptavidin than from avidin and Suc-avidin. Nevertheless, this approach provides information on the relative rates of dissociation of biotinyl and dethiobiotinyl analogues; i.e., the rate of dissociation of 6-(dethiobiotinylamido)hexanoic acid > dethiobiotinyl-A1-insulin > biotinylinsulin > biotinyl-A1-insulin > biotinyl-A2-insulin.

Streptavidin has been reported not to bind dethiobiotin (Lichstein & Birnbaum, 1965), and we have confirmed this observation. It does, however, bind dethiobiotinyl-A1- and dethiobiotinyl-A2-insulin (see section on inhibition by "avidins"). To ascertain which part of the molecule is responsible for streptavidin binding, we prepared dethiobiotinamide (to evaluate the effect of removing the charge on the carboxyl group) and 6-(dethiobiotinylamido)hexanoic acid (to evaluate the contribution to binding of the hydrophobic spacer arm) and measured their dissociation rates. Dethiobiotinamide formed no complex with streptavidin but 6-(dethiobiotinylamido)hexanoic acid did, and its rate of dissociation was somewhat faster than that of dethiobiotinyl-A1-insulin. Apparently, both the hydrophobic spacer arm and insulin contribute to the unexpected interaction between these dethiobiotin derivatives and streptavidin.

*Stimulation of the Conversion of [1-$^{14}$C]Glucose to $^{14}CO_2$ by Biotinyl-X- and Dethiobiotinyl-X-insulins.* The ability of the biotinyl-X- and dethiobiotinyl-X-insulins to stimulate conversion of [1-$^{14}$C]glucose to $^{14}CO_2$ in isolated rat epididymal adipocytes has been compared to that of insulin. Activity is expressed as percent of the activity obtained with an insulin standard assayed concurrently so that values from different assays can be compared. The results (Figure 1) show

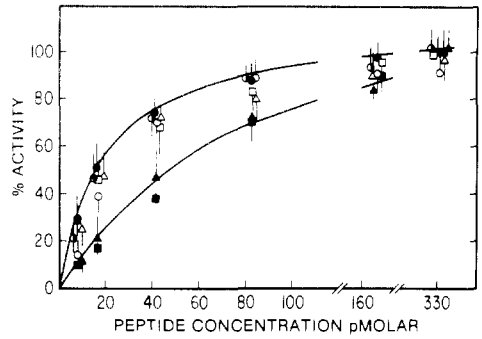


FIGURE 1: Stimulation of glucose oxidation in isolated rat epididymal adipocytes by insulin and insulin derivatives: (●) insulin; (○) biotinylinsulin; (□) biotinyl-A1-insulin; (■) biotinyl-A2-insulin; (△) dethiobiotinyl-A1-insulin; (▲) dethiobiotinyl-A2-insulin; (◑) dethiobiotinyl-A1-DPA-insulin. Upper curve connects the data points for insulin activity. Vertical bars represent SD, $n = 9$.

that all of the derivatives are capable of stimulating $CO_2$ production to the same maximum level as insulin itself, although somewhat higher concentrations of the biotinyl-A2 and dethiobiotinyl-A2 derivatives are required to achieve the same level of activity. The activity of the dethiobiotinyl-A1-DPA-insulin is nearly identical with that of insulin.

*Inhibition of Hormonal Activity of the Ligands by Avidin, Suc-avidin, and Streptavidin.* For inhibition assays, insulin or insulin derivatives (at concentrations sufficient to achieve 50–70% activity) were mixed with avidin, Suc-avidin, or streptavidin in mole ratios (avidin to ligand) varying from 1:1 to 100:1. Since the avidins have four binding sites, a mole ratio of 100:1 is actually a ratio of 400 equiv to 1 (assuming that four ligands can be accommodated simultaneously on each avidin molecule). The same ratios of the three avidins to insulin were tested to determine if addition of the avidins per se had any effect on hormonal activity. None of the avidins inhibited insulin activity.

Large excesses of avidin or Suc-avidin were required to demonstrate inhibition of biotinylinsulin activity (Figure 2A), but adding increasing amounts of either of the avidins decreased hormonal activity progressively. Streptavidin was the most effective inhibitor; hormonal activity was virtually abolished at a 10:1 mole ratio.

The activity of ligands with spacer arms between the insulin and the biotin was also decreased in the presence of excesses of any of the avidins, but in general, inhibition curves reached a plateau between ratios of 10:1 and 100:1 (Figure 2B,C). Somewhat larger ratios of avidin and Suc-avidin were required to reach a plateau with dethiobiotinyl-A1- and dethiobiotinyl-A2-insulins (Figure 2E,F). In the case of either biotinyl or dethiobiotinyl derivatives, streptavidin inhibited activity more effectively than either Suc-avidin or avidin. An exception is dethiobiotinyl-A1-DPA-insulin (Figure 2D), where all the "avidins" were equally effective inhibitors and activity plateaued at a 10:1 ratio.

## Discussion

In order for biotinyl or dethiobiotinyl analogues of insulin to be employed as bifunctional ligands for affinity chromatography of the receptor, they must be capable of binding simultaneously to avidins and the receptor and the bonds must be stable enough to withstand the exhaustive washing procedures necessary to remove contaminants from affinity columns. The problem is one of finding a spacer arm long enough to separate the biotinyl and insulin portions of the ligand so that receptor and avidin binding can occur simultaneously.

In this and previous studies (Hofmann et al., 1977, 1982, 1984; Finn et al., 1981), we have examined the binding of

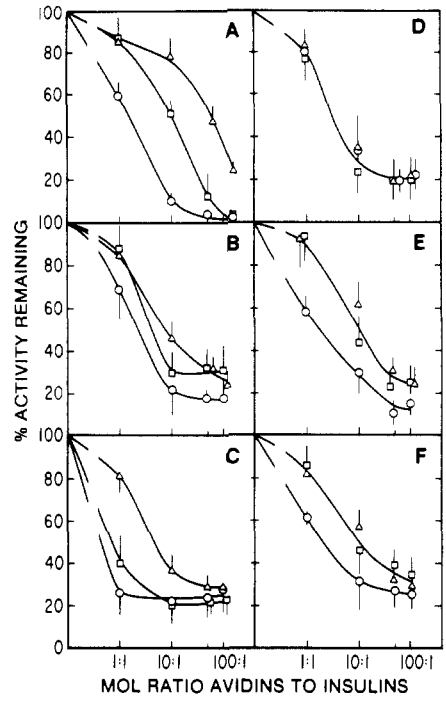


FIGURE 2: Inhibition of biotinyl-X- and dethiobiotinyl-X-insulin-stimulated glucose oxidation in rat epididymal adipocytes by (□) avidin, (△) Suc-avidin, and (○) streptavidin: (panel A) Biotinylinsulin; (panel B) biotinyl-A1-insulin; (panel C) biotinyl-A2-insulin; (panel D) dethiobiotinyl-A1-DPA-insulin; (panel E) dethiobiotinyl-A1-insulin; (panel F) dethiobiotinyl-A2-insulin. Vertical bars represent SD, $n = 9$.

biotinylated and dethiobiotinylated insulins to Suc-avidin and streptavidin. With the exception of dethiobiotinylinsulin, all of the ligands form complexes with both avidins. As is apparent (Table I), the dissociation rates of these complexes vary considerably. It should be mentioned that dissociation is a biphasic process and that only the slower of the two observable rates is given here. From the point of view of constructing affinity resins for receptor isolation, rapidly dissociating ligands will presumably be lost during column preparation.

Interaction of the above ligands with insulin receptors can be inferred from the observation that they are capable of stimulating glucose oxidation in rat epididymal adipocytes (Figure 1). It is of no consequence, as concerns stimulation of glucose oxidation, whether the ligands contain biotin or dethiobiotin. Attachment of a long hydrophobic chain to the $N^{\alpha,B1}$ position of insulin apparently decreases ligand-receptor affinity since larger amounts of both biotinyl-A2- and dethiobiotinyl-A2-insulins are required to achieve stimulation equivalent to that of insulin. The less hydrophobic and longer spacer arm of dethiobiotinyl-A1-DPA-insulin appears to permit better interaction since this ligand is fully active.

In the past the ability of complexes of Suc-avidin and biotinylinsulin to bind to the insulin receptor was determined by assessing their ability to compete with [$^{125}$I]iodoinsulin for binding to rat liver plasma membranes (Finn et al., 1980). A new experimental approach had to be found once it was discovered that biotinylinsulin dissociates rapidly from Suc-avidin. Thus, the possibility that only the dissociated biotinylinsulin molecules and not the complexes were competing with [$^{125}$I]iodoinsulin for receptor binding sites could not be eliminated.

The first step in developing a ligand that would accommodate simultaneous binding was to have an assay that could discriminate between receptor binding of dissociated biotinylinsulin analogues and their respective avidin complexes.

The situation can be viewed as shown in Figure 3 for Suc-avidin. Obviously, the same considerations apply to avidin or streptavidin interactions.

When Suc-avidin, a biotinyl-X-insulin analogue, and receptor are mixed, three possible complexes may be formed: (1) a hormonally active ternary complex, (2) a hormonally active binary complex between the biotinyl-X-insulin and its receptor, and (3) a hormonally inactive binary complex between Suc-avidin and the biotinyl-X-insulin derivative. These are assumed to be in equilibrium. We reasoned that the presence of a large excess of Suc-avidin, by the law of mass action, would favor the formation of avidin–biotinyl-X-insulin complexes even in instances where the dissociation rates of the complexes are high. Furthermore, if the ligand could not interact simultaneously with avidin and the receptor, then the only complex formed would be hormonally inactive. Thus, if the activity of a given insulin derivative were abolished in the presence of a large excess of avidin, it could be assumed that the active ternary complex could not be formed. The ability of insulin to stimulate the conversion of [1-$^{14}$C]glucose to $^{14}CO_2$ by isolated adipocytes was used to measure receptor binding.

When the effects of large excesses of avidin, Suc-avidin, or streptavidin on the ability of biotinyl-X- or dethiobiotinyl-X-insulin derivatives to stimulate glucose oxidation were examined, two types of effects were observed. (1) When a spacer arm was interposed between biotin or dethiobiotin and insulin, activity was retained even at avidin to ligand ratios as high as 100:1. (2) There was a pronounced difference in the ability of streptavidin, as compared with Suc-avidin or avidin, to inhibit the action of certain of the biotinyl- or dethiobiotinylinsulins.

If the effect of excesses of Suc-avidin or avidin on the various derivatives is considered first, one can see that when there is no spacer arm between the biotin and the insulin as in biotinylinsulin (Figure 2A), approximately 50 mol of either of the avidins/mol of biotinylinsulin is needed to affect substantial inhibition. This is not surprising since dissociation rate studies [Table I and Hofmann et al. (1982)] show that the complex between this derivative and avidin or Suc-avidin dissociates readily. We have previously shown that insulin derivatives of the weaker binding analogues of biotin (dethiobiotin and iminobiotin) are incapable of forming complexes with avidin or Suc-avidin. The finding that at high mole ratios, where interaction with avidin or Suc-avidin would be favored, hormonal activity is abolished indicates that the ternary complex cannot exist.

Ligands having a 6-aminohexyl spacer arm, e.g., biotinyl-A1-insulin and dethiobiotinyl-A1-insulin, form more stable complexes with avidin or Suc-avidin, judging from their rates of dissociation (Table I), and consequently, inhibition by either of these avidins occurs at lower mole ratios (Figure 2B,E). Although avidin or Suc-avidin binding is favored, complex formation does not abolish hormonal activity; thus, the complex can still interact with the insulin receptor. A similar situation is found with biotinyl-A2-, dethiobiotinyl-A2-, and dethiobiotinyl-A1-DPA-insulins.

As mentioned above, streptavidin interacts differently from avidin or Suc-avidin with most of the biotinyl and dethiobiotinyl ligands. The finding that greater inhibition is observed at a given mole ratio of streptavidin to biotinylinsulin (Figure 2A) than for the same ratio of either Suc-avidin or avidin suggests that streptavidin is capable of forming a more stable complex with biotinylinsulin. For the reasons discussed previously (Results), only relative dissociation rates of ligands

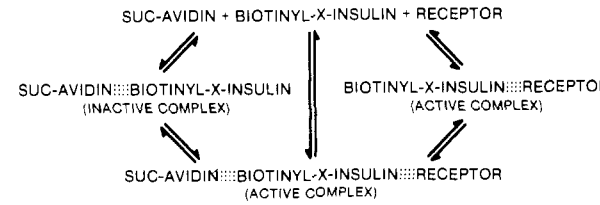


FIGURE 3: Representation of possible interactions of biotinyl-X-insulins with Suc-avidin and insulin receptors. The length of the arrows is not intended to signify relative reaction rates.

from streptavidin complexes could be derived. From these results it could be concluded that factors other than the interaction of streptavidin and biotin play a role in the dissociation behavior of the streptavidin complexes.

In general, the residual adipocyte-stimulating activity of ligands that dissociate slowly from Suc-avidin reaches a plateau at a mole ratio of avidin to ligand of 10:1. Higher mole ratios are required to reach a plateau with rapidly dissociating ligands, e.g., biotinyl-, dethiobiotinyl-A1-, and dethiobiotinyl-A2-insulins. It appears likely that the plateau activity is that corresponding to the ternary complex (Figure 3). One might have anticipated that the ternary complexes involving ligands with long spacer arms would be more active than those in which the distance between the receptor and avidin is not as great. This is, however, not the case. All the complexes reach a plateau at between 20 and 30% residual activity.

Although little information is available on the structure of the biotin binding site of either streptavidin or avidin, Green et al. (1971) attempted to determine the depth of the biotin binding site on avidin by studying the effect of chain length of $\omega$-bis(biotinyldiamines) on avidin polymer formation. Using values of 1.25 Å for the C–C, 1.16 Å for C–N, and 1.18 Å for the CO–N bond distances (assuming a fully extended chain), he concluded that since stable polymers were formed when the chain linking the carboxyl groups of the biotins was 18 Å long, the biotin carboxyl must lie about 8–9 Å beneath the van der Waals surface of the molecule.

Using the same values for bond distances and assuming fully extended chains, we have calculated that the distance between the carboxyl group of dethiobiotin and the N-terminal amino group of the insulin B chain would be 9.77 Å for dethiobiotinyl-A1-insulin, 18.36 Å for dethiobiotinyl-A2-insulin, and 25.52 Å for dethiobiotinyl-A1-DPA-insulin. Thus, any of these ligands should have sufficient space between the dethiobiotinyl and insulin portions to bind normally to avidin. However, only the longest of the three ligands shows the same rate of dissociation from Suc-avidin as dethiobiotin itself. In any event, the findings of Green served as a guide in designing these bifunctional ligands.

Spacer arms are also required for optimizing interaction between the avidin–ligand complexes and the receptor. Although strong binding exists between biotinylinsulin and streptavidin, the complex is unable to interact with insulin receptor. Predictably, insulin derivatives having one or two 6-aminohexanoic acid residues in the spacer arm have more residual activity in the presence of excess avidins.

In a previous paper (Hofmann et al., 1984), it has been shown that dethiobiotin, prepared by Raney nickel desulfurization, consists of a mixture of 86% of the cis and 14% of the rearranged trans compound. It is doubtful that the biological assays of dethiobiotinyl-X-insulins, namely, stimulation of glucose oxidation and inhibition of this activity by avidins, are sufficiently sensitive to be influenced by this contamination. As concerns the rate of dissociation of the Suc-avidin–dethiobiotinyl-X-insulin complexes, the effect of the trans

isomer, a compound that is not available in pure form, is unknown. It appears unlikely that the trans isomer is responsible for the biphasic character of the dissociation curves since the corresponding biotin derivatives behave similarly. In conclusion, ligands that stimulate glucose oxidation in the presence of a large excess of avidins are promising tools for insulin receptor isolation.

**Registry No.** Streptavidin, 9013-20-1; insulin, 9004-10-8; biotinylinsulin, 89889-04-3; biotinyl-A1-insulin, 89889-05-4; biotinyl-A2-insulin, 89889-07-6; dethiobiotinyl-A1-insulin, 89889-06-5; dethiobiotinyl-A2-insulin, 89889-08-7; dethiobiotinyl-A1-DPA-insulin, 89889-09-8; dethiobiotin, 533-48-2; *N*-hydroxysuccinimido dethiobiotinate, 80750-24-9.

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# 18,19-Dihydroxydeoxycorticosterone, a New Metabolite Produced from 18-Hydroxydeoxycorticosterone by Cytochrome P-450$_{11\beta}$. Chemical Synthesis and Structural Analysis by $^1$H NMR†

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ABSTRACT: A new metabolite was produced from 18-hydroxydeoxycorticosterone by the cytochrome P-450$_{11\beta}$ linked hydroxylase system purified from bovine adrenocortical mitochondria. It was identified as 18,19-dihydroxydeoxycorticosterone by chemical synthesis on the basis of high-performance liquid chromatography, gas chromatography–mass spectrometry, and proton nuclear magnetic resonance ($^1$H NMR) spectroscopy, and detailed structural analysis of it was performed by $^1$H NMR spectroscopy. The methylene protons at the C-19 position of the steroid were nonequivalent and coupled with each other, having a coupling constant of 10.6 Hz. These protons had different coupling constants, 6.7 and 3.4 Hz, for the hydroxy proton at the C-19 position. Due to these couplings, the signals of the methylene protons were observed around 3.9 ppm as two double doublets. The methylene protons at the C-21 position were also nonequivalent, having a coupling constant of 11.1 Hz. Coupling constants between these methylene protons and the hydroxy proton at the C-21 position were 8.2 and 4.2 Hz, respectively. These results indicate that both hydroxymethyl groups at the C-19 and C-21 positions do not freely rotate in chloroform solution. The signals of hydroxy protons at the C-19 and C-21 positions were found at 1.25 and 1.87 ppm, respectively, by means of decoupling of the corresponding methylene protons. The hydroxy proton at the C-18 position was found to scarcely couple with any proton. This fact suggests that this hydroxy group is linked to the C-20 position, making a hemiketal bridge between the C-18 and the C-20.

It has been recently reported that the urinary excretion of 19-nordeoxycorticosterone was elevated in adrenal regeneration hypertensive rats (Gomez-Sanchez et al., 1979) and in spontaneously hypertensive rats in connection with the development of hypertension (Dale et al., 1982). This steroid has also been shown to be a potent mineralocorticoid and to have higher sodium-retaining activity than deoxycorticosterone (Kagawa & Van Arman, 1957). It has been speculated that 19-nordeoxycorticosterone was produced from 19-hydroxydeoxycorticosterone (Gomez-Sanchez et al., 1982; Dale et al., 1982). In fact, Gomez-Sanchez et al. (1982) have found that rat adrenal glands have the enzymes required to convert deoxycorticosterone to 19-hydroxydeoxycorticosterone, 19-oxodeoxycorticosterone, and 19-carboxydeoxycorticosterone. These facts, together with the fact that 18-OH-deoxycorticosterone[1] can also produce hypertension (Melby et al.,

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[1] Abbreviations: NMR, nuclear magnetic resonance; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; δ, chemical shift relative to internal chloroform (7.27); 18-OH-deoxycorticosterone, 18,21-dihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 18-OH-corticosterone, 11β,18,21-trihydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; 18,19-(OH)$_2$-deoxycorticosterone, 18,19,21-trihydroxy-4-pregnene-3,20-dione; 18,19-(OH)$_2$-progesterone, 18,19-dihydroxy-4-pregnene-3,20-dione; 18-OH-19-nordeoxycorticosterone, 18,21-dihydroxy-19-nor-4-pregnene-3,20-dione; 19-nordeoxycorticosterone, 21-hydroxy-19-nor-4-pregnene-3,20-dione; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-2558$01.50/0 