Proc. Nat. Acad. Sci. USA
Vol. 69, No. 5, pp. 1277–1281, May 1972

Affinity Chromatography and Purification of the Insulin Receptor of Liver Cell Membranes

(insulin–agarose/detergent extraction)

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Communicated by Albert L. Lehninger, March 16, 1972

ABSTRACT **Relatively simple and rapid procedures are described for the large-scale preparation of liver membranes that contain virtually all of the high affinity insulin-binding activity of liver homogenates. The presumed insulin receptor, which is extracted from these membranes in soluble form with Triton X-100, can be further purified by ammonium sulfate fractionation (3-fold purification) or by diethylaminoethyl-cellulose chromatography (60-fold purification). Several insulin–agarose derivatives have been synthesized that can efficiently extract the insulin-binding protein from the detergent extracts of the membranes. The receptor macromolecule can be eluted from the affinity columns in high (50–80%) yield by use of urea-containing buffers of moderately low pH. The receptor, thus purified by small-scale affinity chromatography experiments, approaches theoretical purity on the basis of its specific activity. This protein is purified about 250,000-fold from the liver homogenate by detergent extraction and affinity chromatography.**

Structures capable of specifically binding insulin have recently been identified and studied in isolated adipose tissue cells (1–5), fat-cell membranes (6), and liver-cell membranes (7–10). The receptor for this peptide hormone appears to be localized exclusively to the surface membranes of cells (11–13).

The recent (13, 14) extraction and solubilization of the binding proteins in intact form from fat- and liver-cell membranes with nonionic detergents, and the availability of a rapid, quantitative assay for the soluble receptors, provide the necessary basis for studies designed to purify these membrane proteins. The extraordinarily small quantity of insulin receptors in mammalian-cell membranes and the absolute requirement for the presence of detergents to maintain their solubility pose special problems in purification studies. The ability of insoluble insulin–agarose derivatives to simulate the biological effects of native insulin in isolated fat cells (12), mammary gland cells (15, 16), and liver cells (17), and the adsorption of fat cell ghosts to insulin–agarose columns in a manner that permits their subsequent elution by insulin-containing buffers (18), suggest that such insoluble hormone derivatives interact effectively with membrane structures and are, thus, potentially useful for purification of the receptors by affinity chromatography (19–22). The selective adsorption of liver-membrane fragments that contain glucagon receptors to glucagon–agarose columns (23) also points toward the promise of these procedures in receptor purification.

The present studies demonstrate that conventional procedures can be used for purification of the solubilized insulin receptor, and that the receptor proteins can be adsorbed to, and subsequently eluted from, affinity columns with an overall purification that probably exceeds 2×10^5-fold.

METHODS

Methods have been described for measurement of specific binding of [^{125}I]insulin to liver-cell membranes (Table 1), detection of water-soluble insulin–receptor complexes (13, 14), and extraction of the insulin-binding proteins with Triton X-100 (13, 14). Protein was determined by the method of Lowry *et al.* (24) with crystalline bovine albumin as the standard. In most affinity chromatography experiments, the concentration of protein in the elution samples was too low to be detectable by standard procedures so that acid hydrolysis amino-acid analysis (Spinco model 120; ref. 25) of the pooled sample was performed; analyses were also performed on bovine albumin for standardization.

TABLE 1. *Specific insulin-binding activity and fractionation of liver homogenates*

Sample	Volume (ml)	Protein (g)	Activity (cpm/mg)†
Crude homogenate	2000	88 [100]*	1,800 [100]
600 × *g* Supernatant	1650	50 [62]	3,900 [122]
12,000 × *g* Supernatant	1400	28 [32]	5,750 [101]
40,000 × *g* Pellet	200	4.6 [5]	37,000 [108]

The liver tissue (370 g total) of 40 male Sprague–Dawley rats (150 g; 2 months old) was suspended in 2 liters of ice-cold 0.25 M sucrose and homogenized with a Polytron PT35ST for 3 min at a setting of 3.5. The homogenate was centrifuged at 600 × *g* for 10 min and the supernatant was centrifuged at 12,000 × *g* for 30 min. This supernatant, after adjusting with NaCl and $MgSO_4$ to achieve concentrations of 0.1 M and 0.2 mM, respectively, was centrifuged at 40,000 × *g* for 40 min. The pellet was suspended in 1200 ml of 0.05 M Tris·HCl buffer (pH 7.4) by homogenizing (Polytron) for 10 sec, and the suspension was centrifuged at 40,000 × *g* for 40 min. This step was repeated, and the pellet was suspended in a total volume of 200 ml with the Tris·HCl buffer. Specific binding of insulin was determined after incubation of diluted samples with [^{125}I]insulin (7×10^5 cpm/ml) for 20 min at 24° (6, 7). No significant insulin-binding activity could be detected in the first 40,000 × *g* supernatant with the polyethylene glycol assay (14).

* In square brackets are percentages of total.

† [^{125}I]Insulin-binding activity.

TABLE 2. *Purification of insulin receptor of liver-cell membranes by ammonium sulfate fractionation*

Fraction	Protein (mg/ml)	Insulin-binding activity: Protein cpm/mg	Insulin-binding activity: Total cpm	Insulin-binding activity: Recovery (%)*	Purification*
Membrane	9.4	38,000	—	—	—
Triton extract	4.8	88,000	2.1×10^6	100	0
$(NH_4)_2SO_4$ fractions					
0–20%	1.8	167,000	3×10^5	14	1.9
20–40%	5.2	260,000	1.4×10^6	67	3
40–60%	3.0	67,000	2×10^5	9	—
40–60% supernatant	—	5,000	—	—	—

Liver membranes were extracted with 1% (v/v) Triton X-100 and centrifuged for 60 min at 44,000 rpm (130,000 × *g*). The supernatant was dialyzed overnight against 0.1 M sodium phosphate buffer (pH 7.4)–0.1% Triton. The ammonium sulfate (at 4°) pellets were dissolved in about 1 ml of the above phosphate buffer and dialyzed overnight before assaying for specific [^{125}I]insulin binding (13, 14).

* Relative to Triton extract of membranes.

Preparation of Affinity Adsorbents. The insulin–agarose derivatives used in these studies are depicted in Fig. 1. Derivatives A and B were prepared by attaching insulin by the N-terminal residue of the B chain (B1phe) or by the single lysyl residue (B29) directly to the polymer backbone, by the cyanogen bromide procedure (12). C, one of the most useful of the derivatives, was prepared by recently described procedures (26) that involve the use of active carboxyl esters of agarose. The *N*-hydroxysuccinimide ester (26) of 3,3′-diaminodipropylaminosuccinyl agarose (20, 26) was reacted at 4° with porcine insulin (5 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.4) containing 6 M urea. After 1 hr, glycine was added and the reaction was continued at 24° for 3 hr. Such derivatives contain 0.3–0.5 mg of insulin per ml of agarose. Derivative D was similarly prepared except that the buffer used for coupling was 0.1 M $NaHCO_3$ (pH 9.2). Derivative E was prepared by reacting the *N*-hydroxysuccinimide ester of diaminodipropylaminosuccinyl-agarose with a large excess of 3,3′-diaminodipropylamine (26), succinylating with succinic anhydride (20), forming the corresponding *N*-hydroxysuccinimide ester, and coupling with insulin as described for D. Derivatives described by F were prepared by procedures similar to those used for preparation of azoglucagon–agarose (23). At pH values near 5.5, the predominant azo linkage was with histidyl residues, whereas at pH values near 7.5–8, a mixture of azohistidyl and azotyrosyl linkages were present. Derivative G, obtained by reacting the bromoacetyl agarose derivative (20) with insulin in 0.1 M $NaHCO_3$ buffer (pH 9.0) for 2 days at 24°, contained a mixture of linkage forms (amino, histidyl, and tyrosyl). Derivative H was obtained by reacting a sulfhydryl agarose (20, 23) with an insulin derivative having both N-terminal residues blocked with acetyl groups (12). Various insulin–glass-bead derivatives were prepared by reacting insulin (as described for C above) with the *N*-hydroxysuccinimide ester of succinylated aminoalkyl glass beads (26) of various porosity [30–120 nm (300–1200 Å)].

RESULTS

The insulin receptor of liver-cell homogenates is purified about 20-fold during preparation of the "membranes" (Table 1). However, these steps were not designed to achieve purity but rather to obtain in high yield, and by rapid and simple procedures, large amounts of material with insulin-binding activity ("membranes") that could then be used as starting material for subsequent purification procedures. The binding activity of these membranes is essentially unchanged during storage at −20° for 3 months. It is notable that during preparation of the membranes the yield of binding activity is very high.

Although ammonium sulfate fractionations of the solubilized insulin receptor (Table 2) can be performed satisfactorily despite the presence of detergents in the buffers, the receptor molecules coprecipitate with the bulk of the protein in the sample and the final purification that is achieved is very modest. Nearly 90% of the starting activity can be recovered during these procedures.

Substantial purification of the insulin receptor can be achieved by chromatography on DEAE cellulose with buffers that contain Triton X-100 (Fig. 2). The insulin-binding protein adsorbs to this ion-exchange resin at pH 6.3; lower pH values could not be used because loss of activity was observed during dialysis with buffers having a pH below 6. During dialysis against the buffer of pH 6.3 a large amount of protein precipitates with only minimal loss of insulin-binding activity so that a 3-fold purification can be achieved by this

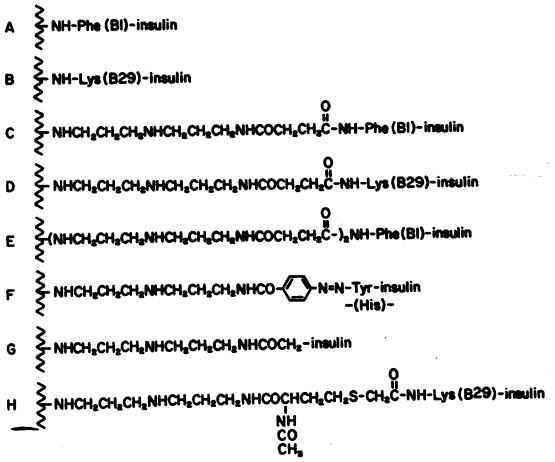

FIG. 1. Various insulin–Sepharose 4B derivatives for affinity chromatography of water-soluble insulin receptors of liver membranes (see *Methods*).

simple procedure. During chromatography, the receptor is further purified by a factor of nearly 20.

Affinity chromatography experiments with the adsorbents depicted in Fig. 1 revealed that those derivatives in which a spacer or "arm" separates the insulin molecule from the matrix backbone were the most effective in extraction of the insulin receptor. The only exception was derivative F, which was quite ineffective in either the predominantly azohistidyl form or in the form containing a mixture of azohistidyl and azotyrosyl bonds. Derivatives C, D, E, and H (Fig. 1) retain a greater proportion of the chromatographed receptor than derivative G, and derivative H appeared to be less stable during storage than derivatives C, D, and E. No clear differences could be discerned in the apparent effectiveness of these last three derivatives. Similarly, essentially the same results were obtained when the same substitutions were performed on agarose beads of various porosity. The various insulin derivatives of porous glass beads that were tested were totally ineffective in chromatography. These derivatives demonstrated almost continual leakage of insulin in the buffers used for equilibration of the columns, presumably a reflection of the lability of the basic silane linkage bonds.

Affinity chromatography of the insulin receptor illustrates that although no loss or retardation of insulin-binding activity is detectable with columns containing unsubstituted agarose, considerable adsorption occurs to columns containing the selective adsorbent. Depending on the conditions of the ex-

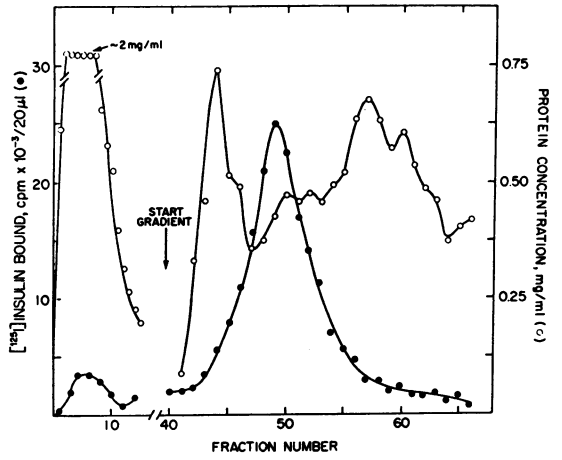

FIG. 2. Purification of insulin receptor of liver-cell membranes that is solubilized by Triton X-100 on DEAE-cellulose column. Liver membranes were extracted with 1% (v/v) Triton X-100 (13, 14) and the high-speed (50,000 rpm, 70 min) supernatant was dialyzed (at 4°) for 18 hr against 0.05 M sodium acetate buffer (pH 6.3)–0.2% (v/v) Triton. The rather large precipitate that formed during dialysis was removed by centrifugation; about 15% of the initial insulin-binding activity was found in this pellet. 12 ml of the supernatant (5 mg of protein per ml) were passed (at 4°) over a column (0.5 × 5 cm) of DEAE-cellulose, in the acetate form, which had been equilibrated with the dialysis buffer described above. The column was washed further with about 40 ml of the same buffer, and elution was achieved with a linear two-component gradient prepared from 25 ml of 0.1 M ammonium acetate (pH 6.3)–0.2% Triton, and 25 ml of 1.0 M ammonium acetate (pH 6.3)–0.2% Triton. Fractions contained about 1.8 ml, and the column flow rate was about 8 ml/hr. [^{125}I]insulin binding was determined by the polyethylene glycol assay (13, 14). Under the conditions used, the sample applied to the column bound 1.5 × 10^5 cpm of [^{125}I]insulin per mg of protein, and the peak elution fraction (tube 49) could bind 2.5 × 10^6 cpm per mg of protein, indicating purification of about 17-fold. Recovery of insulin-binding activity (fractions 45–55) was about 65%.

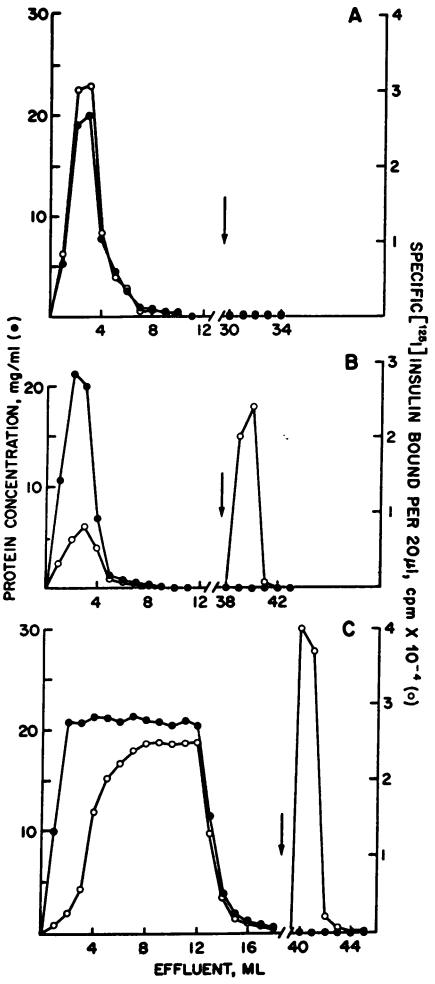

FIG. 3. Affinity chromatography of detergent-solubilized insulin receptor of liver-cell membranes on columns containing unsubstituted agarose (*A*) and derivative C (Fig. 1), diaminodipropylaminosuccinyl-*N*-phenylalanyl-insulin–agarose (*B*, *C*). Liver-cell membranes were homogenized (Polytron), extracted with 2% (v/v) Triton X-100 by shaking at 24° for 40 min, and centrifuged (Spinco 65 rotor) at 50,000 rpm for 70 min (13, 14). The supernatant was dialyzed for 16 hr at 4° against Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.1% (v/v) Triton X-100, and the precipitate that formed during dialysis was removed by centrifugation for 30 min at 35,000 rpm. 2 (*A*, *B*) or 12 (*C*) ml of the supernatant were slowly chromatographed at 24° over affinity columns (V_t 1.3 ml, in Pasteur pipettes) that had been washed for 20 hr with 0.1 M $NaHCO_3$ buffer (pH 8.4) and followed by equilibration (2 hr) with Krebs–Ringer bicarbonate buffer containing 0.1% (v/v) Triton X-100. The columns were washed thoroughly (note break in *abscissa*) before elution (*arrow*) with 0.05 M sodium acetate buffer (pH 6.0) containing 4.5 M urea and 0.1% (v/v) Triton X-100. After application of this buffer to the column, the flow was stopped for 15 min before resumption of chromatography. 1-ml Fractions were collected for determinations of protein (24), and specific binding of [^{125}I]insulin was determined with the polyethylene glycol assay (13, 14); the effluent samples (20 µl) were added directly to the assay mixture (0.2 ml).

TABLE 3. *Purification of soluble insulin receptor of liver membranes on an insulin–agarose affinity column*

Sample	Volume (ml)	Protein concentration (mg/ml)*	Insulin-binding activity: Specific activity (cpm/mg of protein)	Insulin-binding activity: Total activity (cpm)	Insulin-binding activity: % Recovery of activity	Insulin-binding activity: Purification
Sample applied to column (Triton extract)	3.0	26.2	36×10^3	2.8×10^6	—	—
Column effluent, breakthrough	4.5	16.1	7.9×10^3	5.8×10^5	20	—
Elution	1.8	3×10^{-3}	2.9×10^6‡	1.6×10^6	57†	8000‡

The high-speed supernatant of a Triton X-100 extract of liver membranes was chromatographed and eluted on an affinity column [V_t, 1.7 ml of derivative E (Fig. 1)] as described in Fig. 3. Similar results can be obtained with Triton extracts of fat-cell membranes.

* Determined by amino-acid analysis, on the basis of leucine content and by use of crystalline bovine albumin as standard. This was the only way that permitted detection of protein in the elution samples.

† Represents 70% recovery of the activity that was presumably adsorbed to the column.

‡ Because of the difficulty in determination of such low concentrations of protein in the effluent, these figures must be considered provisional.

periment, between 20 and 90% of the binding activity can be extracted with such columns. However, extraction of activity by passage of the sample through the column is not by itself proof that the adsorbent is selectively binding the protein. The binding protein could be degraded or denatured during chromatography or, in solution, it could bind free ligand (insulin) during chromatography that is adsorbed on the gel or that is slowly cleaved during the experiment. The effluents were tested for the presence of native insulin by examination of their ability to displace the binding of [^{125}I]insulin to the unchromatographed receptor. The failure to find displacement, however, does not exclude the presence of a significant amount of insulin–receptor complex. The protein–ligand complex that may emerge in the effluent, because of the very high affinity of the complex, would not be detected by the usual assay procedures. In view of these considerations, it is clear that proof of adsorption to the gel requires that the protein be *recovered* after appropriate elution procedures.

The insulin-binding macromolecule could be eluted from the insulin affinity columns by buffers containing urea and having a relatively low pH (Fig. 3*B* and *C*). It was previously demonstrated (13) that these concentrations of urea dissociate the insulin–receptor complex, and that this effect is completely reversed by reducing the urea concentration by dilution or dialysis. Urea concentrations higher than 4.5 M resulted in much lower recoveries of insulin-binding activity, consistent with the irreversible denaturation observed with high urea concentrations (13). Elution could not be achieved by simply using buffers of low pH, since inactivation of binding activity occurs below pH 5.5. Under the conditions described in Fig. 3, between 50 and 80% of the total activity that was presumably adsorbed to the column could be consistently recovered with virtually all the derivatives tested.

The specificity of the process of adsorption of the insulin-binding macromolecule to the column could be demonstrated directly by experiments in which native insulin was added to the sample before chromatography. The breakthrough protein, after passage through a Sephadex G-100 column to separate free native insulin (13, 14), was examined for binding of [^{125}I]insulin under conditions (40°, 90 min) that promote exchange of free and bound insulin. The same binding activity was present in the samples that had been chromatographed on insulin and on unsubstituted agarose columns. Furthermore, adsorption of the insulin-binding protein to the affinity column could be effectively prevented by first digesting the insulin–agarose with Pronase, or by reducing and alkylating the insulin adsorbent before use.

With the use of these affinity adsorbents, it is possible to purify the insulin-binding macromolecule from the Triton X-100 extract by about 8000-fold (Table 3). This is a substantial degree of purification that may approach the theoretical maximum, as will be described in the *Discussion*. However, it has not been possible to obtain large quantities of the binding protein by these procedures. As demonstrated in Fig. 3*C*, the effective "capacity" of these columns is rather low, since chromatography of large quantities of extract does not lead to correspondingly greater adsorption of binding activity. This low capacity does not result from an insufficient amount of insulin on the adsorbent, since only a very small fraction of the total amount present would be expected to participate in the specific interaction with the binding protein that is adsorbed. It is probable that the low capacity is related to interfering reactions that result from the application of very large volumes of crude, concentrated protein solutions through these small columns. Although proportionately larger quantities of the binding protein can be purified with larger columns, the quantities of protein that are obtained and the size of the columns required make such procedures impractical for large-scale purifications of the present time. The affinity columns appear to have a much greater capacity for the insulin-binding protein of Triton X-100 extracts of fat-cell membranes than for proteins prepared by the same method from liver-cell membranes.

DISCUSSION

The need for the continued presence of detergents to maintain the insulin-binding protein of liver- or fat-cell membranes in a soluble state does not appear to seriously handicap the application of conventional procedures, or of affinity chromatography, to the purification of this macromolecule. The efficacy of the various procedures used in purification is given in Table 4. If it is assumed that one monomeric

TABLE 4. *Résumé of procedures used in the purification of the insulin receptor of liver-cell membranes*

Preparation or procedure	Insulin-binding activity (pmol/mg of protein)	Purification
Crude liver homogenate	0.008	0
Liver membranes	0.15	20*
Triton extract of membranes	0.26	1.7†
$(NH_4)_2SO_4$, fraction 20–40%	0.75	3‡
DEAE-cellulose chromatography	14	60‡§
Affinity chromatography¶	about 2000	about 8,000‡ about 250,000*

Details of these procedures are presented in earlier tables and figures. The Triton extract was used for $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, and affinity chromatography.

* Compared to crude liver homogenate.

† Compared to liver membranes.

‡ Compared to Triton extract of liver membranes.

§ Dialysis of Triton extract, as described in Fig. 2, results in a 3-fold purification; DEAE-chromatography results in a further purification of about 20-fold.

¶ These are tentative figures because of the difficulty in accurately determining the small amounts of protein obtained by these procedures.

insulin molecule (mol. wt. 6000) binds to one molecule of receptor complex of mol. wt. 300,000 (13), the completely purified receptor would theoretically bind 3300 pmol of insulin per mg of receptor protein. To achieve complete purity would then require a 400,000-fold purification of the crude liver homogenate or a 12,000-fold purification of the Triton extract. It is estimated (Tables 3 and 4) that the two steps of detergent extraction followed by affinity chromatography yield a protein that approximates theoretical purity.

To elucidate more precisely the degree of purity, possible subunit structure, stoichiometry of binding, and other molecular parameters, it will of course be necessary to use much larger quantities of the "purified" binding macromolecule than are currently available. The difficulties associated with large-scale purification of this receptor can in part be appreciated by considering that despite the large size of the macromolecule it represents only about $2 \times 10^{-4}\%$ of the protein of the liver homogenate and about $4 \times 10^{-3}\%$ of the membrane protein. For example, a total of about 0.2 mg of receptor protein would be expected to be present in the liver homogenate obtained from 40 rats (Table 1), which contains about 90 g of protein.

Despite the scarcity of the insulin-binding macromolecule in biological tissues, large-scale purification may be possible with affinity chromatography provided that the peculiarly low "capacity" of the columns when crude detergent extracts are used can be resolved. The promise of these techniques is indicated by small-scale experiments (Fig. 3, Table 3) that illustrate a high efficiency of adsorption of the binding protein, a high yield on elution, and an apparently high degree of purity of the eluted material. Preliminary experiments suggest that the "capacity" of the columns may be greatly improved if the Triton extract is partially purified by other procedures before chromatography on affinity columns.

The availability of purified insulin-binding protein would permit detailed studies of structure–function that may shed light on the nature of the interaction of this macromolecule with insulin and with other components of the cell membrane. It will be important to determine if the insulin-binding macromolecular complex purified in this report contains binding structures for other peptide hormones or catecholamines, and whether it contains enzymic activities.

The valuable technical assistance of Miss Lily Wu is gratefully acknowledged. Supported by NIH Grant AM14956 and the Kroc Foundation, Santa Ynez, California. The author is a recipient of a USPHS Research Career Development Award (AM31464).

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