

of the supernatant was processed with the internal standards and analyzed by mass fragmentography (Table 1). Extraction recoveries for each of the indoles were (mean of six determinations): serotonin, 68 percent; 5MT, 53 percent; NAS, 74 percent; and melatonin, 69 percent. As measured by mass fragmentography, rat pineal contains (in micrograms per gram; mean of four assays  $\pm$  standard error of the mean): serotonin,  $54 \pm 4.4$ ; 5MT,  $4.2 \pm 0.38$ ; melatonin,  $3.9 \pm 0.10$ ; and NAS,  $0.13 \pm 0.022$ . Values for melatonin and NAS do not agree with those of Miller and Maickel (5), but our melatonin value does agree with melatonin values reported for bioassay (7). Since the relative fluorescence of melatonin is three times greater than that of NAS (8), it is possible that with the extraction procedures used for the *o*-phthalaldehyde method, there is contamination of melatonin in the NAS sample, thereby giving a higher value for NAS ( $7.8 \mu\text{g/g}$ ) and a lower value for melatonin ( $0.48 \mu\text{g/g}$ ) (7). The pineal 5MT concentrations are comparable to those obtained by the *o*-phthalaldehyde method (7), and serotonin values are the same as those measured by *o*-phthalaldehyde or ninhydrin fluorescence (9).

Absolute identification of the analyzed pineal indoles was by multiple ion detection (Fig. 1) (10). Multiple ion detection of the assayed indoles was done with the fragments listed in Table 1, and the fragment ratio obtained for both the authentic compounds and the pineal extract (Fig. 1) is the same as that listed in Table 1, thereby confirming the specificity of the assay. The pineal extracts processed without internal standards do not have any "biological background."

In summary, we have described a gas chromatographic-mass spectrometric assay for the simultaneous measurement of serotonin, NAS, 5MT, and melatonin with a sensitivity greater than 1 pmole. Routinely, specificity is based on the gas chromatographic retention time of the compound and measurement of the ion density of a specific fragment (*m/e*) at this time. Absolute identification of compounds extracted from pineal gland is obtainable by multiple ion detection.

F. CATTABENI\*

S. H. KOSLOW†

E. COSTA

Laboratory of Preclinical Pharmacology,  
National Institute of Mental Health,  
Saint Elizabeths Hospital,  
Washington, D.C. 20032

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2. With the routine analytical methods presently used there is no differentiation between serotonin and *N*-methylserotonin, whereas with the method presented here, these two compounds are differentiated.
3. Using this method, we have detected significant concentrations of some of these indoles in various brain nuclei. Enzymes for the synthesis of these compounds have also been reported to be present in the rat retina [D. P. Cardinali and J. M. Rosner, *J. Neurochem.* **18**, 1769 (1971)].
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6. Those indole derivatives lacking the *N*-acetyl group also form PFP derivatives in which all the available primary and second-

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\* On leave of absence from the Department of Pharmacology, School of Pharmacy, University of Milan, Italy.

† Address correspondence to S.H.K., Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, WAW Building, Washington, D.C. 20032.

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## Water-Soluble Insulin Receptors from Human Lymphocytes

**Abstract.** Specific insulin receptors from human lymphocytes in culture have been prepared in aqueous solution without use of detergents or related compounds. Receptors prepared in this fashion exhibit characteristics identical to those reported in intact cells.

The solubilization of polypeptide hormone receptors from broken cell preparations (1) has contributed to our knowledge of hormone-receptor interactions. In all of these procedures, detergents or vigorous mechanical techniques are used (1, 2). However, the study of physical and chemical properties of detergent-solubilized protein is subject to uncertainties because of the presence of residual detergent molecules bound to the protein (3). Sim-

ilarly, studies on the "solubilized" hormone receptors prepared by mechanical extraction are limited by the presence of microfine particles (4).

Circulating human lymphocytes have specific membrane insulin receptors that are similar to those in the fat cell and liver cell membranes of rats (5). Further, specific insulin receptors found in cultured human lymphocytes are identical to those in the circulating cells (5). The accessibility of the cultured lymphocytes

Fig. 1. Gel filtration of soluble receptor from lymphocytes. [ $^{125}\text{I}$ ]Insulin ( $2 \times 10^{-11} \text{M}$ ) was incubated with receptor (approximately 0.3 mg of protein) in the presence and absence of  $10^{-6} \text{M}$  unlabeled insulin for 75 minutes at  $23^\circ \text{C}$  in a total volume of 0.25 ml. The mixture was made 3 percent in sucrose and chromatographed on a 1 by 30 cm column of Sephadex G-50 previously equilibrated with PBS (pH 7.6) containing 0.1 percent serum albumin. Flow rate was about 5 ml per hour. Results are shown for (A) [ $^{125}\text{I}$ ]insulin alone; (B) [ $^{125}\text{I}$ ]insulin plus soluble receptor; and (C) [ $^{125}\text{I}$ ]insulin plus receptor in the presence of  $10^{-6} \text{M}$  unlabeled insulin. When material in the unretarded peak (fractions 12 to 16) from (B) was rechromatographed, approximately 85 percent of the radioactivity appeared in the void volume, and the remainder eluted in the position corresponding to [ $^{125}\text{I}$ ]insulin. The arrow marks the position of the void volume, as determined by chromatography of Blue Dextran 2000 (Pharmacia).

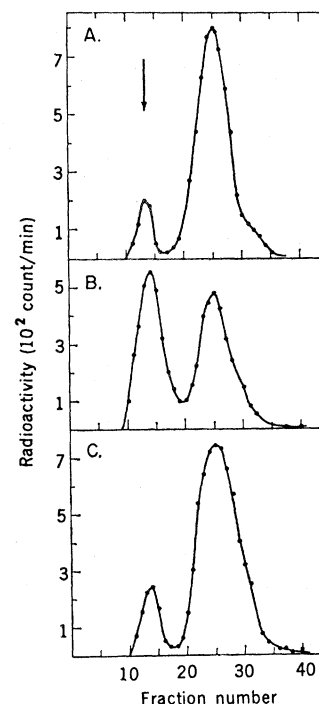
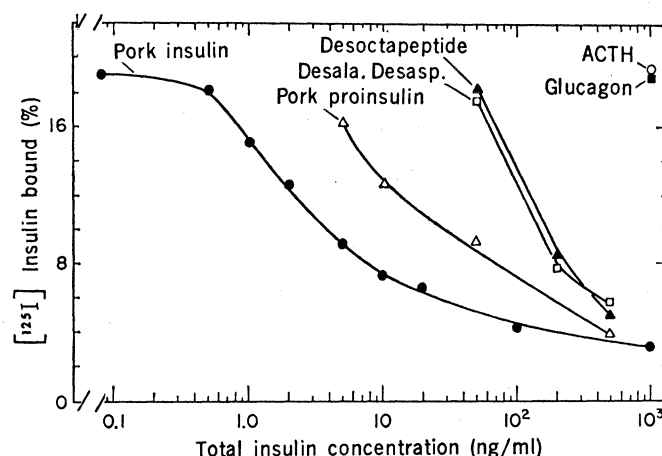


Fig. 2. Effect of insulin, insulin analogs, and other peptides on inhibition of [ $^{125}$ I]insulin binding to soluble receptor. [ $^{125}$ I]Insulin ( $2 \times 10^{-11}M$ ) was incubated with receptor (0.25 to 0.5 mg of protein) in the presence of the peptides indicated for 75 minutes at 23°C (Desala, Desasp., desalanine-desasparagine insulin; ACTH, adrenocorticotrophic hormone). At the end of incubation, hormone-receptor complex was precipitated by polyethylene glycol and separated by filtration through Millipore filters (2).



phocytes makes this a convenient tissue for the isolation of the insulin-specific binding molecule. The unique phenomenon of release of surface proteins from intact cultured lymphocytes has been observed when cells are incubated in buffered, serum-free medium (6). In this report we describe the isolation of specific water-soluble insulin receptors from cultured human lymphocytes by this technique, which does not require detergents or related compounds.

In previous studies of binding of insulin to receptors in intact lymphocytes (7), we observed a decrease in the insulin-binding capacity of these cells after 60 to 90 minutes of incubation at 30°C. This loss of binding capacity was not due to cell death or degradation, and we now report that the insulin-binding activity can be recovered in centrifuged supernatants of these cell suspensions.

Soluble insulin receptor was routinely prepared as follows. Suspensions of cultured lymphocytes (RMPI 4265), containing approximately  $5 \times 10^7$  cells per milliliter of 10 mM phosphate-buffered saline (PBS) at pH 7.6, were incubated for 70 minutes at 30°C with gentle agitation in a volume of 4 to 10 ml; 0.005M iodoacetamide was included to inhibit protease activity that might occur with cell breakage. Cells were counted and viability was determined before and after incubation in each experiment. After incubation the cells were centrifuged at 800 to 1000g at 4°C, and the supernatants were immediately removed and placed into test tubes kept at 1°C. These supernatants were centrifuged at 100,000g for 90 minutes at 4°C before they were assayed for insulin-binding activity (no differences were observed when supernatants were centrifuged at 200,000g for 2 to 4 hours at 4°C). Protein concentrations were determined by the method of Lowry *et al.* (8), with bovine serum albumin as the standard. Protein solutions were concentrated at 4°C by ultrafiltration with Amicon type UM-10 membranes. Soluble receptor solutions were used directly or stored at -20°C until use.

Biologically active [ $^{125}$ I]insulin was

prepared as described (5). To measure binding, soluble receptor in PBS (pH 7.6) was incubated with [ $^{125}$ I]insulin ( $10^{-11}$  to  $10^{-10}M$ ) for 70 to 90 minutes at 23°C. Formation of insulin-receptor complexes was measured by the polyethylene glycol (Carbowax) precipitation method of Desbuquois and Aurbach (9) or by gel filtration on columns of Sephadex G-50.

[ $^{125}$ I]Insulin was incubated with soluble receptor in the presence and absence of unlabeled insulin, and the samples were filtered on a column of Sephadex G-50 (Fig. 1). There was significant [ $^{125}$ I]insulin radioactivity in the void volume, a result that indicated the formation of an insulin-receptor complex (2). The formation of this complex was completely inhibited by the simultaneous presence of excess unlabeled insulin ( $10^{-6}M$ ). When the insulin-receptor complex was dissociated with mild acid treatment (10), the [ $^{125}$ I]insulin recovered was found to be undegraded by the criteria of adsorption to talc, precipitation by trichloroacetic acid, and elution on Sephadex G-50 (10). Further, insulin and insulin derivatives inhibited binding of [ $^{125}$ I]insulin to soluble receptor in direct proportion to their biologic potency (Fig. 2). These results were identical to those reported for the intact cells (5).

The water-soluble insulin receptor described in this report did not pass through dialysis membranes, did not sediment at 200,000g in 4 hours at 4°C, was not retained on Millipore filters with 0.2- $\mu$ m diameter pores, and was not retarded on columns of Sephadex G-200. Binding activity was abolished by tryptic digestion, but was unaffected by digestion with deoxyribonuclease or ribonuclease, results suggesting that the soluble receptor is a protein (11). The insulin receptor of the cultured lympho-

cyte is unique inasmuch as it can be obtained from intact cells in water-soluble form without the use of detergents. It is hoped that characterization of the material will contribute to the understanding of the specific interaction of insulin with its receptor under conditions that most nearly approximate physiologic states.

JAMES R. GAVIN III

Section on Diabetes and Intermediary Metabolism, Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20014

DONALD N. BUELL

Immunology Branch, National Cancer Institute, Bethesda, Maryland 20014

JESSE ROTH

Section on Diabetes and Intermediary Metabolism, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20014

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