ΙΑΤΡΙΚΗ.— **Metabolism of exogenous insulin into high molecular weight forms** *in vivo*, *by Harry N. Antoniades, James D. Simon and Dimitri Stathakos* *. Ἀνεκοινώθη ὑπὸ τοῦ Ἀκαδημαϊκοῦ κ. Β. Μαλάμου.

It has been suggested that endogenous insulin is metabolized in extrapancreatic sites (**1**) into a high molecular weight form which was called bound insulin or insulin complex (**2**, **3**). The *in vivo* and *in vitro* biologic activities of partially purified blood serum bound insulin preparations of various species were shown to be similar to those of crystalline insulin (**3**). However, bound insulin was shown to be unreactive with insulin antisera as judged by *in vitro* radioimmunoassay techniques.

The present studies were undertaken in order to investigate whether exogenous insulin injected into rats is metabolized into a high molecular weight form *in vivo*.

## MATERIALS AND METHODS

Porcine [$^{125}$I] insulin (specific activity: 45.8 to 58.1 mCi/mg insulin) was purchased from Abbott Laboratories. Crystalline porcine insulin (25 units/mg, lot PJ-5682) was obtained from Eli Lilly and Company and guinea pig anti-insulin antiserum (GPAS) from Peter Wright (Indianapolis); neutralizing potency was 1.11 units porcine insulin per ml antiserum. Rabbit anti-guinea pig antisera were purchased from Sylvana Corporation. The guinea pig anti-insulin antiserum was diluted 220-fold with 5% bovine serum albumin in borate buffer, pH 8.0. The neutralizing capacity of the diluted GPAS was about 500 microunits insulin per 0.1 ml. The diluted preparations were stored at -15°C and were used for the immunoprecipitation studies of [$^{125}$I] insulin described below.

Dithiothreitol (Cleland's reagent) was obtained from Calbiochem; coomassie brilliant blue R-250, bovine serum albumin, ovalbumin, chymotrypsinogen A (beef pancreas) and cytochrome C from Schwartz-Mann; β- galactosidase from Boehringer-Mannheim; sodium dodecyl sulfate from

---

* Χ. Ν. ΑΝΤΩΝΙΑΔΟΥ, J. D. SIMON καὶ Δ. ΣΤΑΘΑΚΟΥ, **Μεταβολισμὸς τῆς ἐξωγενοῦς ἰνσουλίνης εἰς μεγαλομοριακὰς μορφὰς in vivo.**

Matheson; pyronin Y from Fisher, N, N, N′, N′ -tetramethylethylendiamine from Canalco; Acrylamide and N, N′ - methylenebisacrylamide from Eastman. The mixture of acrylamide-bisacrylamide used for gel electrophoresis was deionized on a column of Rexyn-300 (Fisher).

Gel Filtration. Sephadex G-100 or Biogel P-150 (100 - 200 mesh) was swollen in borate buffer (pH 8.0) or in 8 M urea-1 M acetic acid (pH 2.4). Sephadex G-100 was poured into a 4.4 cm × 90 cm glass column with a wet bed volume of 930 ml and Biogel into a 1 cm × 60 cm plastic column with a wet bed volume of 34 ml 10 ml fractions were collected from the Sephadex column and 1.2 ml from the Biogel. Gel filtration was performed at 2°- 5° C with the exception of runs with 8 M urea-1 M acetic acid which were performed at room temperature. 0.25 ml serum samples were applied on the Biogel column and 7.0 ml serum samples on the Sephadex column. In studies with 8 M urea-1 M acetic acid the samples were incubated for 24 hours at room temperature before chromatography.

Immunoprecipitation of the [$^{125}$I] insulin fractions collected in borate buffer during gel filtration was performed as follows: 0,5 ml of each fraction were added to glass rimmed tubes (13 × 100 mm) containing 0.4 ml 5 % bovine serum albumin (BSA) in borate buffer (pH 8.0). 0.1 ml of diluted GPAS was added to each tube and stored at 2° - 5°C for 48 hours. Following the incubation with GPAS, 0.2 ml of undiluted rabbit anti-guinea pig antiserum was added followed by the addition of 0.1 ml normal guinea pig serum diluted 1 : 50 with 5 % BSA in borate buffer (pH 8.0). The tubes were incubated for 72 hours at 2° - 5° C and then centrifuged at 2° C. The supernatant fluid was decanted into rimmed glass tubes (13 × 100 mm) and both supernatant fluids and precipitates were counted for five minutes each in a Nuclear Chicago well-type scintillation counter. The radioactivity in the precipitate is referred to as the immunoprecipitable radioactivity and that in the supernatant fluid as the nonimmunoprecipitable radioactivity.

Insulin Radioimmunoassay. Insulin radioimmunoassays were carried out by the double antibody technique of Morgan and Lazarow (**4**) as modified by Soeldner and Slone (**5**).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS). The procedure used in these

studies is that described by Fairbanks et al (**6**) with the following modifications :

Each sample was incubated for 30 minutes at 95° C with an equal volume of a mixture containing 5 % SDS, 20 mM Tris-HCl (pH 7.9), 4 mM EDTA, 20 % sucrose and 50 μg/ml Pyronin Y. For the reduction of disulfide bonds dithiothreitol was added prior to incubation to a final concentration of 25 mM; β -galactosidase, albumin, ovalbumin, chymotrypsinogen A and cytochrome C, used as markers, were incubated in the presence of the reductant. After cooling to 15° C, the samples were applied on 8 % polyacrylamide gels containing 2 % SDS and electrophoresis was conducted with a voltage gradient of 7 V/cm and a current of 6 mA per tube. When tracking dye (Pyronin Y) had covered a distance of 80 mm (approximately 3 h) the gels were fixed and stained or, alternatively, sectioned in 2 mm intervals; the slices were suspended in 0.5 ml $H_2O$ for counting.

Fixation of the gels at 4° C in 15 % trichloracetic acid-25 % isoprypyl alcohol was followed by staining for 4 hours at 37° C with 0.5 % coomassie blue in 25 % isopropyl alcohol - 10 % acetic acid. Destaining was carried out overnight in 10 % acetic acid in the presence of activated charcoal. The mobilites of the molecular-weight markers in Fig. 4 were corrected (**7**) for the elongation of the gels after staining.

In Vivo Studies. Groups of four Charles River Laboratories male CD fed rats (120 - 130 g) were injected into the jugular vein with 0.5 ml 0.15 M NaCl containing 6 milliunits porcine [$^{125}$I] insulin. The animals were narcotized lightly during injection with 50 % $CO_2$ : 50 % $O_2$. Blood samples (0 2 ml) were obtained from the jugular vein of each rat at 5, 10, 60, 240 and 360 minutes after injection. The sera were separated by centrifugation and pooled. 0.25 ml pooled serum samples obtained at the various intervals after injection were subjected to filtration on Biogel P-150. 7.0 ml pooled serum was collected from rats 60 minutes after the injection of porcine [$^{125}$I] insulin and was also subjected to filtration on Sephadex G-100. All fractions were examined for the presence of immunoprecipitable and nonimmunoprecipitable radioactivity as described above.

In other studies, crystalline insulin (10 mU/rat) was injected into groups of 4 rats and 60 minutes later the animals were bled, the serum

separated by centrifugation and pooled. 7.0 ml of the pooled serum was subjected to Sephadex G-100 chromatography. Fractions corresponding to various molecular sizes were pooled, lyophilized, dissolved in 10 ml distilled water and dialyzed at 2° C against 3 liters 0.15 M NaCl for 48 hours with three changes. Duplicate 0.5 ml samples from each fraction were immunossayed for insulin.

Seperation of High Molecular Weight [$^{125}$I] Insulin Metabolites. Rat sera obtained five minutes after the injection of porcine [$^{125}$] insulin were subjected to Biogel P-150 filtration in borate buffer, pH 8.0 as described above. The tubes of the high molecular weight front fraction were pooled and dialyzed for 24 hours against 1 liter cold 0,15 M NaCl at 2°-5° C. The dialyzed preparations from several runs were pooled, 0.25 ml aliquots were placed in plastic tubes and stored at-15° C.

## RESULTS

Metabolism of Porcine [$^{125}$I] Insulin in Rats Figure 1 shows the metabolism of porcine [$^{125}$I] insulin in rats. Within 5 minutes of injection most of the radioactivity appeared in the high molecular weight front fraction, comprising about 59% of the total immunoprecipitable and 61% of the total nonimmunoprecipitable radioactivity. The immunoprecipitable radioactivity remained predominantly in the high molecular weight front fraction in subsequent blood samples obtained at 10, 60, 240, and 360 minutes after injection (Fig. 1). With progression of time multiple immunoprecipitable fractions appeared between the front and the insulin fraction. Nonimmunoprecipitable radioactivity in the small molecular weigh region following the insulin fraction may represent degradation products of [$^{125}$I] insulin.

Figure 2 shows the distribution of radioactivity in fractions obtained by Sephadex G-100 chromatography of a serum sample obtained 60 minutes after the injection of [$^{125}$I] insulin. There are at least two major high molecular weight fractions of immunoprecipitable radioactivity, corresponding to molecular weights of about 70,000 (b) and to greater than 100,000 (a). These two high molecular weight fractions comprise over 50% of the total immunoprecipitable radioactivity of the serum

sample. The immunoprecipitable radioactivity of the insulin fraction (c) appeared at about the 12.000 molecular weight region as indicated by the presence of cytochrome C in that region. Under these conditions of gel filtration insulin appears as a dimer.

Immunoreactivity of [$^{125}$] Insulin Fractions.

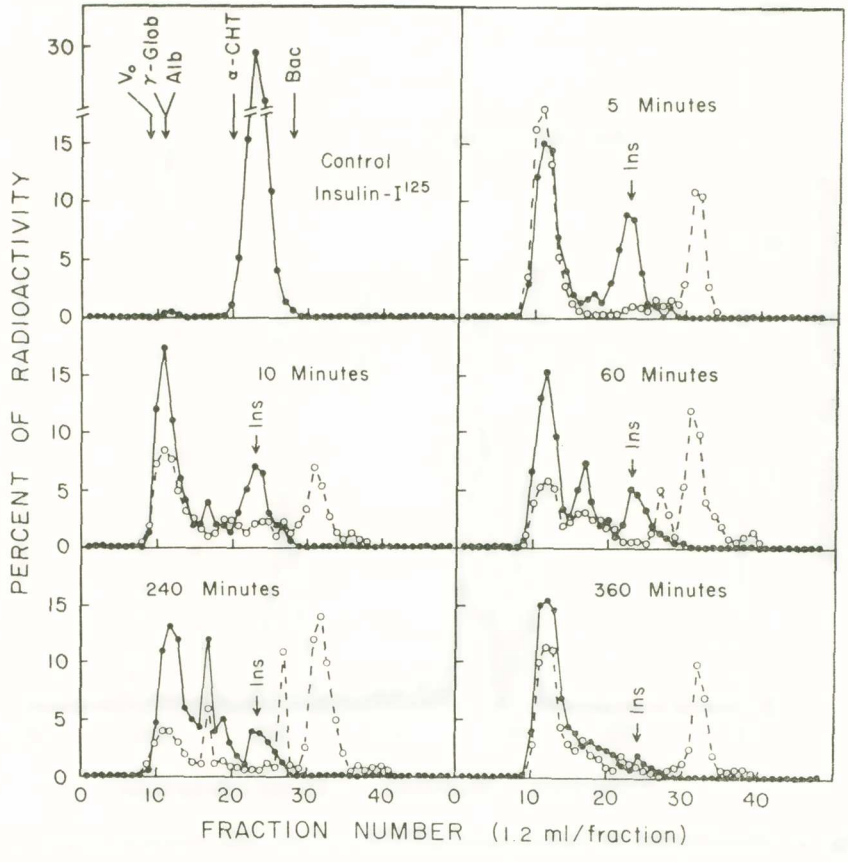


Fig. 1. Distribution of immunoprecipitable (o—o) and nonimmunoprecipitable (o----o) radioactivity in Biogel P-150 fractions of rat sera obtained at various intervals after the injection of porcine insulin-$^{125}$I insulin in rats. Control insulin-$^{125}$I was chromatographed in the presence of 0.25 ml rat serum.

The radioactivity associated with the insulin fraction (Figs. 1 and 2c) was largely immunoprecipitable with guinea pig anti-insulin antisera. Over 90% of the radioactivity of this fraction was recovered in the immunoprecipitate. The high molecular weight fractions exhibited redu-

ced immunoreactivity compared to insulin. Only 50 to 65 % of the radioactivity of these fractions was recovered in the immunoprecipitate.

Metabolism of Nonradioactive Crystalline Porcine Insulin. Sephadex G-100 fractions corresponding to fractions a, b, b-c, c, and c, as shown in Fig. 2, were obtained from

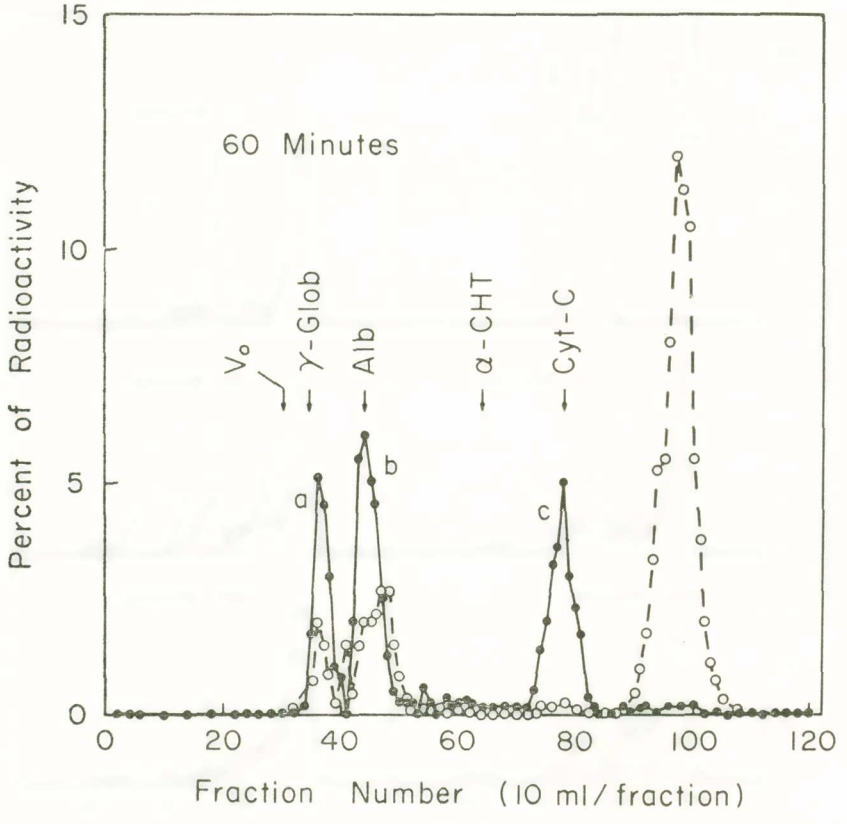


Fig. 2. Distribution of immunoprecipitable (o—o) and nonimmunoprecipitable (o----o) radioactivity in Sephadex G-100 fractions of rat sera obtained 60 minutes after the injection of porcine [$^{125}$I] insulin in rats.

filtration of pooled rat sera collected 60 minutes after the administration of crystalline porcine insulin. The various fractions were subjected to insulin radioimmunoassay and the results are shown in Table 1. Most of the insulin was recovered in the front fraction, indicating that nonradioactive insulin is also metabolized *in vivo* into a high molecular weight form.

TABLE 1.

**Distribution of immunoreactive iusulin in Sephadex G-100 fractions of pooled blood sera 60 minutes after intravenous administration of porcine crystalline insulin.**

| Sephadex Fractions * | Molecular Size (approx.) | Insulin Microunits per ml serum |
|---|---|---|
| a | > 100,000 | 120 |
| b | 70,000 | 20 |
| b to c | 12,000 - 70,000 | 10 |
| c | 12,000 | 50 |
| < c | < 12,000 | 0 |

* As indicated in Figure 2.

Effect of Urea and SDS on the High Molecular Weight [$^{125}$I] Insulin. The high molecular weight [$^{125}$I] insulin metabolite was not affected by rechromatography on Biogel P-150 in borate buffer, pH 8.0 (Fig. 3A) and in 8 M urea - 1 M acetic acid (Fig. 3B) and remained in the high molecular weight region. In the studies presented in Fig. 3, control porcine [$^{125}$I] insulin, used in the *in vivo* studies, was also subjected to gel filtration either alone or after incubation with 0.25 ml rat serum. The *in vitro* incubation of the control [$^{125}$I] insulin with rat serum did not affect its molecular weight. However, as shown in Fig. 3A, commercial preparations of porcine [$^{125}$I] insulin contain about 10% of high molecular weight radioactivity which is diminished following treatment with 8 M urea - 1 M acetic acid (Fig. 3B). This contaminant, which has been assumed to represent «damaged» insulin, is reactive with guinea pig anti-insulin antisera.

Polyacrylamide gel electrophoresis in SDS produced a shift of 45% of the radioactivity into a low molecular weight fraction (Fig. 4) while the rest of the radioactivity remained in the high molecular weight region. A combination of SDS treatment and reduction of the disulfide bonds with dithiothreitol produced a complete transformation of the high

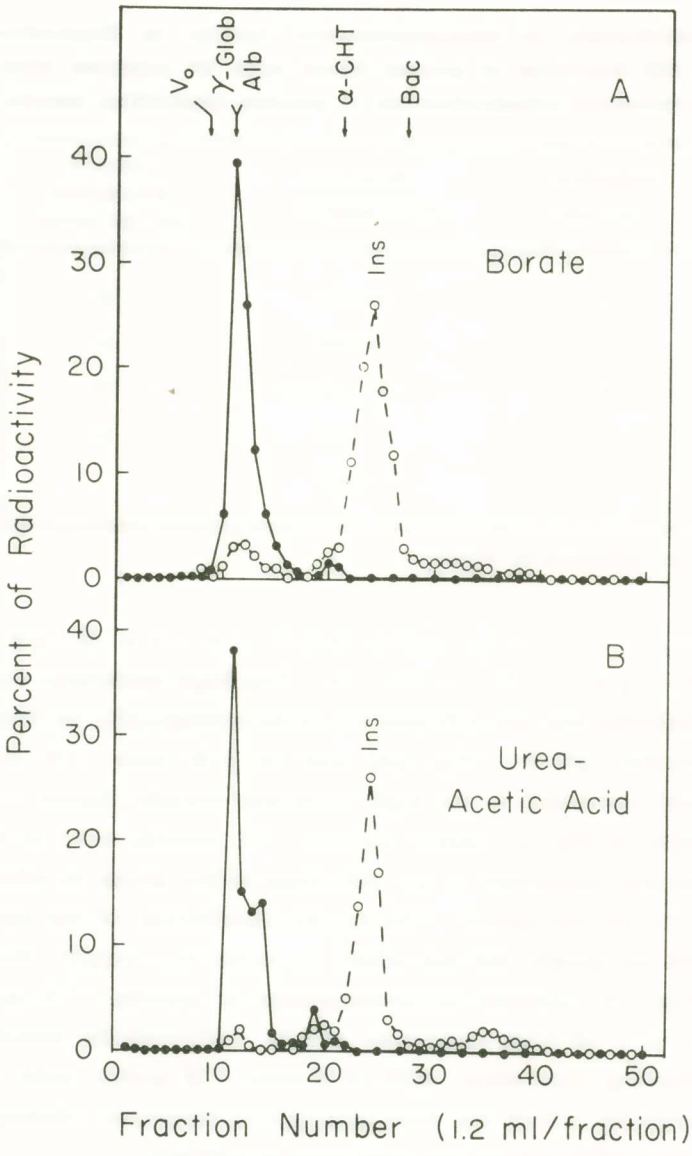


Fig. 3. Rechromatography of the high molecular weight [$^{125}$I] insulin metabolite (o——o) on Biogel P-150 in borate buffer, pH 8.0 and in 8 M urea - 1 M acetic acid. Control porcine [$^{125}$I] insulin (o----o) was chromatographed under the same conditions.

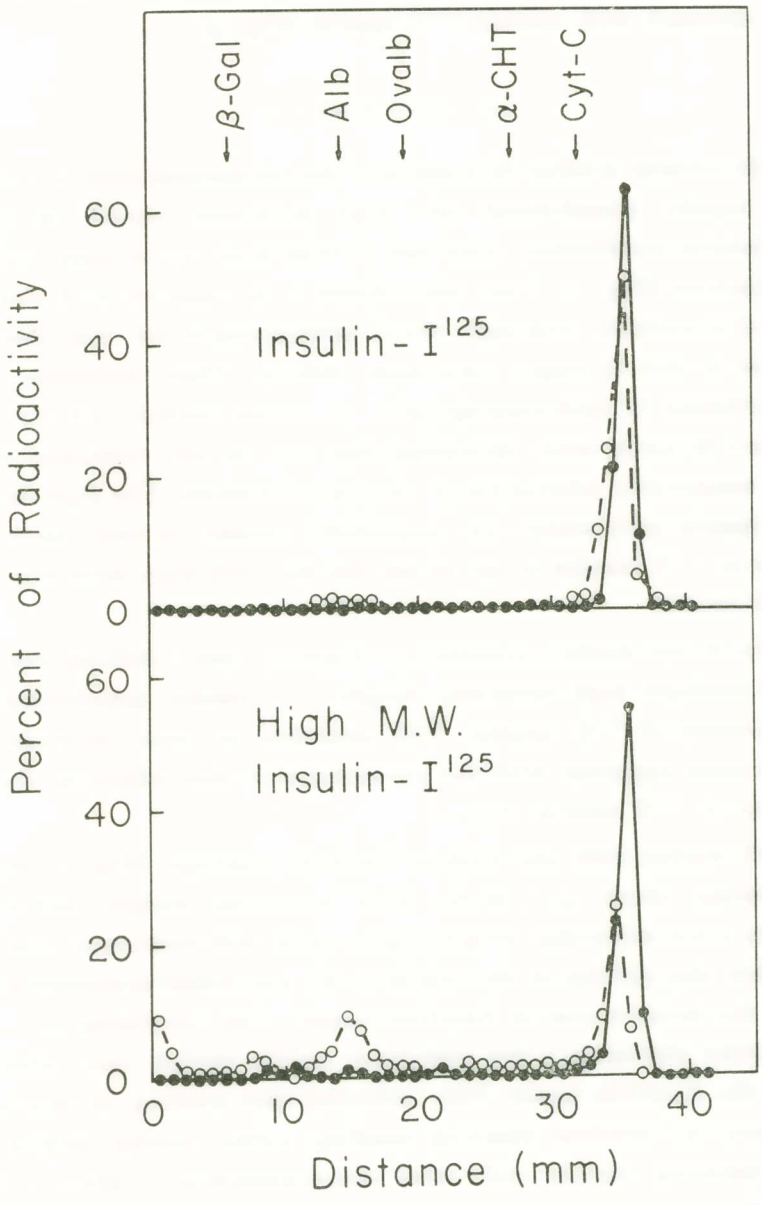


Fig. 4. Polyacrylamide gel electrophoresis of control [$^{125}$I] insulin and the high molecular weight [$^{125}$I] insulin metabolite in sodium dodecyl sulfate (SDS) alone (o----o) and in SDS plus dithiothreitol (o—o).

molecular weight fraction into a low molecular weight fraction, similar to that obtained with control [$^{125}$I] insulin (Fig. 4).

## DISCUSSION

The present studies demonstrate that exogenous insulin injected in rats is rapidly transformed into high molecular weight metabolites. These insulin metabolites remained predominant for at least four hours after injection (Fig. 1). They were shown to be reactive with guinea pig anti-insulin antisera although their immunoreactivity was diminished compared to that of single component insulin. Their molecular weight was unaffected by rechromatography in borate buffer, pH 8.0, and in 8 M urea-1 M acetic acid, indicating that they do not represent the product of nonspecific adsorption of insulin by proteins. The high molecular weight insulin metabolites are apparently formed *in vivo*, since *in vitro* addition of [$^{125}$I] insulin to rat serum did not yield high molecular weight forms of insulin.

The *in vivo* studies presented in Figures 1 and 2 indicate the appearance of multiple high molecular weight [$^{125}$I] insulin metabolites following injection of [$^{125}$I] insulin. The presence of these multiple forms becomes more apparent with the progression of time following the administration of [$^{125}$I] insulin (Fig. 1).

SDS studies with and without disulfide cleavage (Fig. 4) produced the following: about 45% of the high molecular weight radioactivity shifted to a low molecular weight region with SDS treatment alone, indicating that this portion of the radioactivity was bound noncovalently, and without the involvement of disulfide bonds. The remaining 55% of the radioactivity shifted to a low molecular weight region only after reduction of the disulfide bonds. This indicated that binding of this portion of radioactivity involves disulfide bonding, either among insulin molecules or between insulin and other macromolecules. Unlike SDS, 8 M urea - 1 M acetic acid did not produce dissociation of the high molecular weight fraction (Fig. 3).

Table 1 shows the formation of high molecular weight immunoreactive insulin metabolites in rats following injection of nonradioactive

porcine crystalline insulin (Table 1). The presence of high molecular weight endogenous immunoreactive insulin in patients with insulinoma has been reported recently by Yalow and Berson (**8**). The major component of immunoreactive insulin in the sera of these patients exhibited a molecular weight greater than that of albumin. Konijnendijk and Bouman (**9**) have reported the presence of endogenous immunoreactive insulin (IRI) in high molecular weight fractions obtained by gel filtration from peripheral bovine sera. Small amounts of IRI in gel-filtered serum fractions have also been reported by Kajinuma *et al* (**10**). Bouman (**11**) reported the generation of considerable quantities of high molecular weight immunoreactive insulin by incubating normal serum with crystalline insulin in the presence of small amounts of liver homogenates at 37° C, an effect significantly reduced in the absence of serum. Production of a substance similar to that of bound insulin from crystalline insulin has been reported by Gershoff *et al* (**12**) during perfusion of crystalline insulin in isolated rat liver. However, like serum bound insulin, this material obtained from liver perfusion was unreactive with anti-insulin antisera although it exhibited potent insulin-like biologic activities *in vivo*.

The present studies were initiated from our proposal that endogenous insulin in blood circulates primarily as a biologically inactive high molecular weight metabolite termed bound insulin (**1 - 3**). Both endogenous bound insulin and the insulin metabolites produced from the injection of exogenous radioactive insulin in rats exhibited multiple high molecular weight fractions. Like the radioactive metabolites, bound insulin was not generated *in vitro* by the addition of insulin to blood serum, and its molecular weight was not affected by treatment with 7 M urea or 5 M acetic acid (**3**). However, the important difference between bound insulin and the insulin metabolites formed from exogenous insulin is the lack of immunoreactivity of bound insulin with anti-insulin antisera as judged by *in vitro* radioimmunoassay although recent studies have shown that its *in vivo* biologic activity in rats can be neutralized by potent antisera (**13**). Whether this difference in *in vitro* immunoreactivity reflects a difference between metabolites formed from endogenous and exogenous insulin or unrelated substances remains to be shown.

**Acknowledgment**

This work was supported by the John A. Hartford Foundation.

## ΠΕΡΙΛΗΨΙΣ

Χοίρειος κρυσταλλικὴ ἰνσουλίνη, μετὰ ἢ ἄνευ ραδιενεργοῦ σημάνσεως, ἐνιεμένη εἰς ἐπίμυας μεταβολίζεται πρὸς μεγαλομοριακὰς μορφάς. Κατεδείχθη ὅτι οἱ ἐν λόγῳ μεταβολῖται ἀντιδροῦν μετὰ ἀντι-ινσουλινικῶν ἀντιορρῶν ἂν καὶ παρουσιάζουν μειωμένην δραστικότητα ἐν συγκρίσει πρὸς ἰνσουλίνην. Πέντε λεπτὰ μετὰ τὴν ἐνδοφλέβιον ἔνεσιν $^{125}$I - ἰνσουλίνης, ὁ ὀρρὸς ὑπεβλήθη εἰς ἠθμοχρωματογραφίαν κατὰ τὴν ὁποίαν πλέον τοῦ 50 % τῶν σεσημασμένων πρωτεϊνῶν, τῶν παρεχουσῶν ἰζηματινοαντίδρασιν, ἐνεφανίσθη εἰς τὴν περιοχὴν ὑψηλῶν μοριακῶν βαρῶν. Ὁμοία εἰκὼν παρετηρήθη μέχρι καὶ τῆς ἕκτης ὥρας ἀπὸ τῆς ἐνέσεως.

Τὰ μοριακὰ βάρη τῶν μεταβολιτῶν τῆς ἰνσουλίνης δὲν ἀλλοιοῦνται κατὰ τὴν ἀναχρωματογράφησίν των ἐπὶ Biogel P - 150 ἐντὸς βορικοῦ ρυθμιστικοῦ συστήματος (pH 80) ἢ ἐντὸς συστήματος 8 M οὐρίας — 1 M ὀξικοῦ ὀξέος. Κατόπιν ἐπωάσεως καὶ ἠλεκτροφορήσεως παρουσίᾳ δωδεκυλοθειικοῦ νατρίου (SDS), ποσοστὸν 45 % τῶν σεσημασμένων μεταβολιτῶν ἐνεφανίσθη εἰς τὴν περιοχὴν χαμηλῶν μοριακῶν βαρῶν. Ἀφ' ἑτέρου, ἡ ἀνωτέρω ἐπώασις παρουσίᾳ SDS, συνδυασθεῖσα μετὰ συγχρόνου ἀναγωγῆς τῶν δισουλφιδικῶν δεσμῶν, ἐπέφερεν ὁλικὴν μετατόπισιν τῆς ραδιενεργοῦ σημάνσεως πρὸς τὴν περιοχὴν χαμηλῶν μοριακῶν βαρῶν.

## REFERENCES

1. Antoniades, H. N., Bougas, J. A., Pyle, H. M., Camerini, R. and Gundersen, K, 1962.— New Eng. J. Med. **267,** 953 - 957.
2. Antoniades, H. N., Huber, A., Boshell, B. R., Saravis, C. A. and Gershoff, S. N. 1965.— Endocrinology **76**, 709 - 721.
3. Antoniades, H. N. and Simon, J. D. 1970.— in Diabetes, ed. Rodriguez, R. R. and Vallance - Owen, J. (Excerpta Medica, Amsterdam) p.p. 63 - 87.
4. Morgan, C. R. and Lazarow, A. 1963.— Diabetes **12**, 115 - 126.
5. Soeldner, J. S. and Slone, D. 1965.— Diabetes **14**, 771 - 779.
6. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. 1971.— Biochemistry **10**, 2606 - 2717.
7. Weber, K. and Osborn, M. 1969.— J. Biol. Chem. **244**, 4406 - 4412.
8. Yalow, R. S. and Berson, S. A. 1972.— Biochem. Biophys. Res. Comm **48**, 391 - 395.

9. Konijnendijk, W. and Bouman, P. R. 1970.— Diabetologia **6**, 379 - 385.
10. Kajinuma, H., Ide, T., Kuzuya, T. and Kosaka, K, 1969.— Diabetes **18**, 75 - 83.
11. Bouman, P. R. 1968.—International Symposium on Protein and Polypeptide Hormones, Liege, part 3 (Excerpta Medica, Amsterpam), pp 582-584.
12. Gershoff, S. N., Weich, N. L. and Antoniades, H. N. 1970 — Diabetes **19**, 296 - 301.
13. Antoniades, H. N. and Simon, J. D. 1972.— Diabetes **21**, 930-934.

The Center for Blood Research and the Department of Nutrition, Harvard University, School of Public Health, Boston, Massachusetts 02115 ; The Department of Biological Chemistry, Harvard Medical School, and the Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114.