THE FATE OF INJECTED BENCE JONES PROTEIN*

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A number of early workers have shown (1, 2) that Bence Jones protein injected into animals is rapidly excreted in the urine. That such a rapid excretion of newly synthesized Bence Jones protein also occurs in multiple myeloma patients was first demonstrated by Putnam and Hardy (3, 4) with the aid of isotopically labeled amino acids. However, no quantitative studies regarding the fate of injected Bence Jones protein have been reported either with animals or with humans. Such information is necessary to facilitate the analysis of the metabolic relationship of Bence Jones protein with the abnormal globulins which are synthesized in patients afflicted with multiple myeloma. This paper reports on the metabolic fate and the quantitative aspects of the excretion of C^{14}–biosynthetically labeled Bence Jones protein injected intravenously into a single myeloma patient who was synthesizing the protein and subsequent experiments with labeled and unlabeled Bence Jones proteins injected into rabbits. Although the clearance of the protein was not measured, this has recently been done by Migita *et al.* (5), who used I^{131}–conjugated Bence Jones proteins injected into normal human subjects and dogs.[1] The results of our work suggest that in spite of the rapid excretion of part of the injected protein, a significant portion appears to be metabolized by the recipients.

EXPERIMENTAL

Injection of Bence Jones Protein, Measurement of Excretion Rate, and Determination of Radioactivity in the Rabbit.—Three adult female rabbits were injected with 4 ml of physiologi-

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[1] We are indebted to Dr. Shunsuke Migita and collaborators at the Faculty of Medicine, Kyushu University, Fukuoka, Japan, for making available to us in advance of publication their data on the clearance of I^{131}-conjugated Bence Jones proteins injected into normal human subjects and dogs (5).

cal saline and the urine examined for the presence of protein. It was thereby established that no proteins were excreted under the stress of the experimental conditions. Human Bence Jones protein isolated from the urine of two individuals (6–8) was used for further injections. Rabbit 1 received a single injection of 212 mg of unlabeled Bence Jones protein (Bo.) suspended in 2 ml of saline. Rabbit 2 received 2 injections of unlabeled Bence Jones protein (He.), one of 108 mg in 5 ml of saline and a second of 216 mg of the same protein in 5 ml saline 4 days later. The third rabbit was injected daily over a period of 9 days; each injection was made with 230 mg of C^{14}-lysine–labeled Bence Jones protein (Bo.) (4388 counts/minute for infinite thickness) in 5 ml of saline. All injections were by the ear vein.

The urine from all rabbits was collected until the samples no longer showed the presence of protein. Total urine volumes were measured, and the amount of protein injected into the rabbit and recovered from the urine was estimated by biuret method (9). To avoid interference with the biuret reaction by urine pigments, the protein was first precipitated with trichloroacetic acid and then dissolved by boiling in pH 8.6 veronal buffer of 0.1 ionic strength.

For radioactivity determinations, blood samples were drawn from rabbit 3 at 15, 30, 60, 120, 240, and 480 minutes after the first intravenous injection of C^{14}-labeled Bence Jones protein. On subsequent days a single blood sample was taken 8 hours after the daily injection. After the last injection blood samples were taken for 3 additional days. The rabbit was then bled to death by heart puncture and the following organs removed for histological examination and radioactivity determinations: spleen, kidney, bone marrow, appendix, and liver. The serum from the blood obtained by heart puncture was dialyzed against veronal buffer and further fractionated by starch zone electrophoresis. The serum from all blood samples was isolated and placed directly on planchets, as were the various serum fractions obtained by starch zone electrophoresis. The isolated organs were ground in a mortar and pestle and also placed on planchets. All specimens were counted as dry samples for infinite thickness.

Injection of Bence Jones Protein into the Human, Collection of Urine, and Determination of Radioactivity.—In a preceding experiment (6, 8) an adult male had been given 450 μc of DL-glutamic acid-1-C^{14}, and the excreted protein was found to have radioactivity largely confined to its glutamic acid. The experiment had been designed to enable follow-up study of the fate of the reinjected labeled protein in the donor subject. Hence, it was continued although the patient was now uremic and it was recognized that this might affect the experimental result.[2]

After purification by ammonium sulfate precipitation and dialysis two fractions of the Bence Jones protein had been obtained, one of which was insoluble in distilled water. A sample of 1050 mg of the latter was dissolved in Ringer's solution, titrated to pH 9.5 to achieve solution, and readjusted to pH 7.6. The solution was centrifuged to remove insoluble material and passed through a bacterial filter prior to intravenous injection. An aliquot of 200 ml of this solution representing about 0.5 gm protein was injected and the remainder retained for reference.

Except for some nausea with vomiting and a little pyrogenic reaction, the patient suffered no ill effects. He reported he felt well the next day, and there were no obvious changes. Following the injection a total of 29 urine samples were collected over a period of 1 week. The samples were dialyzed extensively against distilled water and lyophilized. No serum samples were taken because of the patient's anemia, but postmortem specimens of the affected tissues were later provided by the pathologist.

About 200 mg of each of the lyophilized specimens, a 25 ml aliquot of the original Bence

[2] A similar experiment had been planned for the other patient (Bo.) whose labeled protein was used in this study because the protein was more radioactive and had the desired physical properties and also because she did not have hyperglobulinemia. However, she returned to the hospital in electrolyte unbalance and expired shortly afterward.

Jones protein used for injection, and bone marrow taken from the sternum and vertebrae of the patient after death were hydrolyzed in 6 N HCl at 105°C for 24 hours in sealed tubes. The hydrolysates were concentrated in a flash evaporator with repeated washings to remove the acid. To the final material (about 10 ml in each case) were added 5 ml of 1.25 M acetate buffer pH 5.1 and 25 mg of acetone-dried crude glutamic acid decarboxylase suspended in 10 ml of distilled water, and the mixture was incubated 2 hours at 37°C. Prior to the addition of the decarboxylase the system was flushed with nitrogen to remove preexisting CO_2. The released CO_2 was collected as $BaCO_3$, washed four times with CO_2-free distilled water, four times with methanol, and deposited on aluminum planchets for measuring radioactivity.

RESULTS AND DISCUSSION

Although the radioactive urinary protein excreted by rabbit 3 was pigmented, it retained the physicochemical properties of the injected Bence Jones protein as well as the capacity to redissolve on boiling. In the analytical ultracentrifuge it sedimented as a single peak with an $s_{20,w}$ of 3.4S, as did the original protein (7). In the Tiselius electrophoresis apparatus the excreted protein was more heterogeneous, for it migrated as a major skewed peak with several minor components. However, the mobility of the main component was unchanged.

The excreted protein was immunologically similar to the injected Bence Jones protein except for a small amount of rabbit serum protein, presumably albumin.[3] In the Ouchterlony immunodiffusion test the urinary protein excreted by rabbit 3 gave a single precipitin line with antiserum to normal human γ-globulin, with antiserum to the injected Bence Jones protein (Bo.), and with antiserum to another Bence Jones protein of the same antigenic type (type A). In all instances the line fused with the major precipitin line for the injected protein, giving a reaction of identity. Although the excreted protein gave a faint precipitin line with anti-rabbit serum, the injected protein did not. Similar results were obtained from immunoelectrophoresis in which both the injected and excreted proteins gave a precipitin line with anti-normal human γ-globulin but only the excreted protein gave a reaction with anti-rabbit serum.

The labeled protein which was injected into the patient migrated as a single boundary in the Tiselius apparatus. In pH 8.6 veronal buffer it had a mobility of -3.4×10^{-5} cm^2 volt^{-1} sec^{-1}. The twentieth sample of the protein excreted after injection had a major component of the same mobility, but it also contained several trace components, the fastest of which had the mobility of serum albumin, (Fig. 1). However, both samples were quite heterogeneous in the analytical ultracentrifuge.

Data presented in Table I show that only a fraction of the Bence Jones proteins injected into the rabbits was recovered in the urine. The amount recovered was characteristic of each protein preparation and reproducible when different experimental animals were used. The fraction which was not excreted (50 per cent or more) must have been disposed of by some other means.

[3] The immunodiffusion study was performed by Dr. Shunsuke Migita.

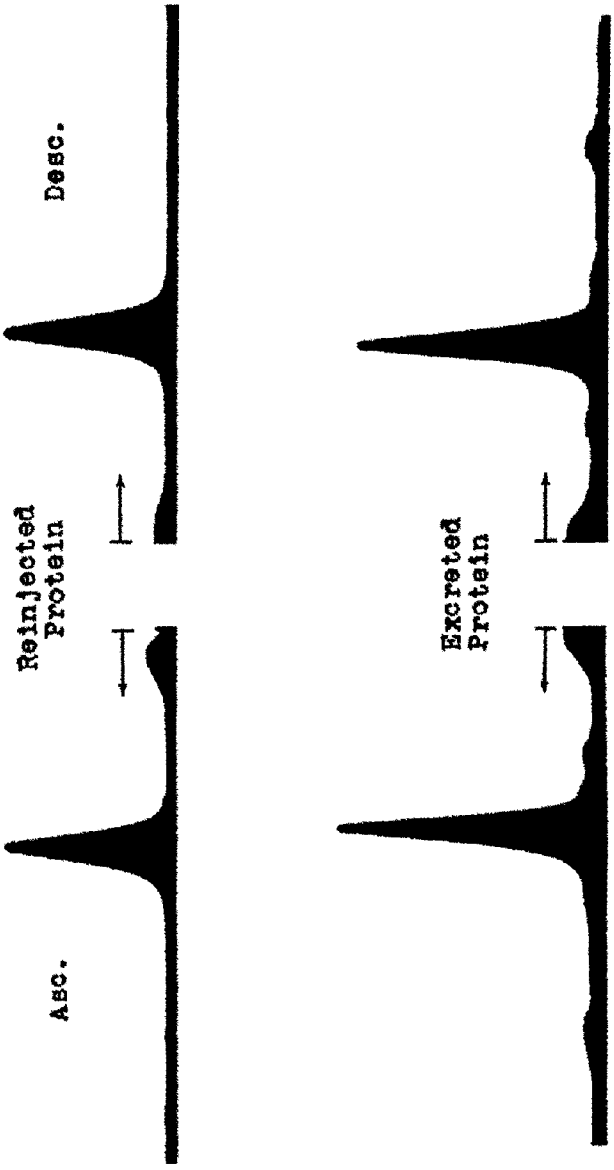

FIG. 1. Electrophoretic pattern at pH 8.6 of C^{14}-labeled Bence Jones protein injected into the donor patient and of the urinary protein subsequently excreted by the subject.

It was the above result, together with the experience with the patient (see later), that prompted investigation of the metabolic fate of C^{14}-labeled Bence Jones protein. As can be seen from Fig. 2, there was a rapid decline of radioactivity in the blood serum of rabbit 3 which can be accounted for in part by the rapid excretion of Bence Jones protein in the urine. However, the extent to which the radioactivity declines cannot be explained by excretion alone since only 50 per cent of the total injected protein followed this pathway. It must, therefore, be assumed that part of the injected Bence Jones protein was metabolized or removed from the serum in some other manner. This view is supported by the findings given in Fig. 3, which show a steady accumulation of radioactivity in the serum during the period of Bence Jones protein injection. That indeed some of the Bence Jones protein was metabolized is shown by the

TABLE I

Urinary Recovery of Bence Jones Protein Injected into Rabbits

Bence Jones protein	Rabbit No.	Excreted protein	
		First injection	Second injection
		per cent	*per cent*
He.	2	31	29
Bo.	1	49	
Bo.	3	49*	

* Represents the cumulative recovery of 1028 mg of the 2070 mg injected. The daily excretion after injection of each 230 mg sample varied from 93 to 147 mg.

appearance of radioactive material in all the serum proteins isolated (Table II). This conclusion is consistent with an observation that the urinary urea also contained measurable C^{14}. Furthermore, the albumin had a higher specific activity than the starch zone fraction which would have contained material with the mobility of the injected Bence Jones protein.

Among the various organs isolated from the rabbit, the kidney had the highest specific activity. Histological examination of this organ did not reveal any pathological changes and seemed to exclude the possibility that the Bence Jones protein was precipitated in the tubules. Since the kidney has an active system for metabolizing proteins, it seems unlikely that the Bence Jones protein was stored intracellularly in this organ in its original form.

Very little radioactivity was found in the urinary protein of the myeloma patient during the 1 week period following injection of his own labeled protein, nor could any radioactivity be detected in the isolated bone marrow. Although the latter might have been predicted, his failure to excrete a substantial fraction of the labeled protein within the first 8 hours after injection was surprising.

Several months earlier after a single injection of glutamic acid-C^{14}, the same patient had excreted more than half the labeled Bence Jones protein in the first 10 hours that he did over the 60 hour period of study. Yet, in the present experiment the radioactivity of the $BaCO_3$ from the Bence Jones protein of the

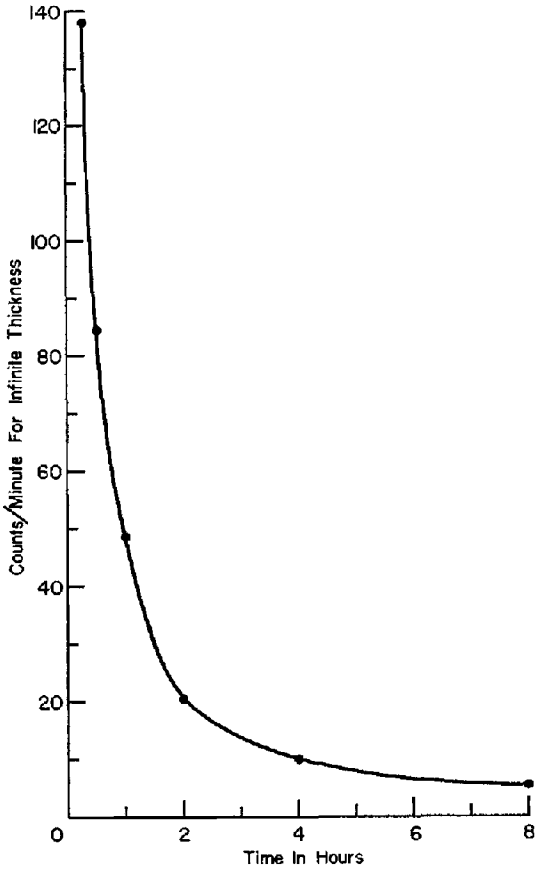

FIG. 2. Decline of serum radioactivity in a rabbit during an 8 hour period following the injection of labeled Bence Jones protein.

first eight specimens of urine was only 50 per cent above background; the thick sample count was only 23 to 33 C. P. M. compared to 2245 for the injected sample. This result could not be explained by simple dilution with newly synthesized unlabeled protein, for the patient would only have excreted about five times the injected amount in a 12 hour period.

Assuming that the injected protein had not been altered during the initial isolation from the patient's urine, the loss of radioactivity could be explained

by the existence of a large body pool of Bence Jones protein, which could mix with and dilute the injected protein. This seems unlikely in view of the preceding experiment with this patient (6, 8) and other isotopic studies (3, 4, 7, 10) which have shown Bence Jones protein to be synthesized and excreted very rapidly. In this case when the patient was uremic and had a low urea clearance, a more plausible explanation is that much of the protein was deposited in the tissues and subsequently metabolized. Earlier study (6, 8) had shown that this

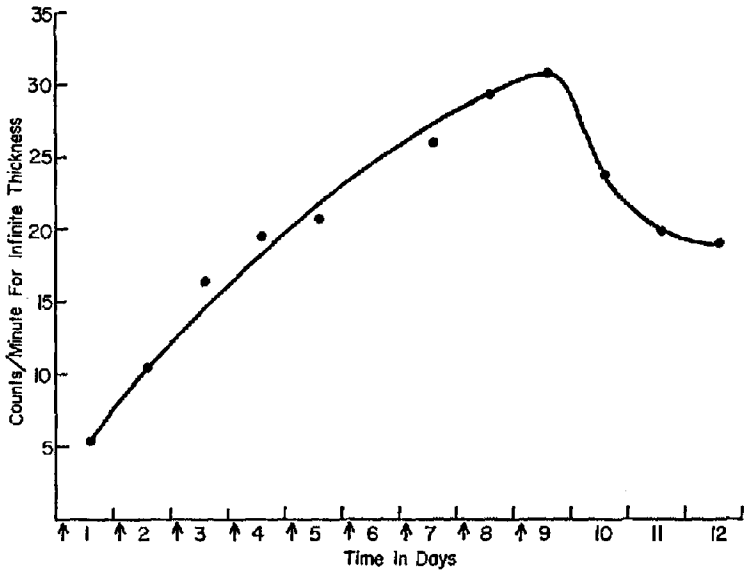

FIG. 3. Serum radioactivity in a rabbit over a 12 day period with repeated injections of labeled Bence Jones protein. Arrows indicate the time of injection.

Bence Jones protein was variable in its physical properties. In fact, the injected sample was the fraction which was insoluble in distilled water and poorly soluble in saline. It may have been denatured during the isolation and in the process of dissolving in Ringer's solution by the titration to pH 9.5. Thus, the poor solubility may have rendered it more susceptible to phagocytosis.[4] It should be borne in mind that only 30 per cent of this Bence Jones protein was excreted when injected into rabbits.

In view of the extensive metabolism of a more soluble Bence Jones protein by these animals, it seems likely that the same process was occurring in the human. In contrast to the rabbit, a myeloma patient requires a large supply of amino acids for the synthesis of excessive quantities of abnormal proteins.

[4] A further portion of the reexcreted labeled protein may have been lost during the reisolation which also involved dialysis against distilled water.

These patients are usually in a wasting condition and in negative nitrogen balance. This subject had an average daily protein intake of only 32 gm before the experiment; yet he was excreting about 5 gm of Bence Jones protein per day. Furthermore, according to the isotope balance data of the earlier experiment, he was synthesizing much more myeloma globulin than Bence Jones protein (8). It is probable he had a particularly active enzyme system for metabolizing proteins, which could account in part for the virtual absence of radioactivity in the urinary protein.

Since rabbits, dogs (5), and normal humans (5) rapidly excrete a substantial fraction of injected Bence Jones protein,[5] it would be valuable to repeat this experiment with other myeloma patients who excrete a more soluble and homogeneous protein and who are not exhibiting signs of renal failure.[2]

TABLE II

Radioactivity of Different Organs and Serum Fractions, 3 Days after the Last Injection of Labeled Bence Jones Protein (Bo.) into Rabbit 3

Organ	Counts/min. for infinite thickness	Serum fraction	Counts/min. for infinite thickness
Kidney	90.1	Albumin	40.9
Spleen	11.9	α-Globulin	19.1
Liver	12.0	β-Globulin	18.0
Appendix	12.0	Fraction having mobility of Bence Jones protein	32.2
Bone marrow	9.8	γ-Globulin	17.0

SUMMARY

Bence Jones protein injected into rabbits was excreted rapidly into the urine. The excreted protein retained the physicochemical and immunochemical properties of the injected Bence Jones protein. In spite of the rapid excretion, a significant portion of the injected Bence Jones protein was found to be metabolized. After reinjection of radioactive Bence Jones protein into the donor patient, only a little radioactivity was recovered in the urinary protein. The implications of these results are discussed.

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[5] In the experiments of Migita *et al.* (5) with dogs and normal human subjects, there was a rapid decrease in plasma radioactivity, as in our study. The radioactivity of the urinary protein reached a maximum about 20 minutes after injection; however, the cumulative recovery of radioactivity in the urine after 3 to 7 hours did not exceed 22 per cent of that injected.

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