Oral administration of amino acids labeled with N\textsuperscript{15} or with C\textsuperscript{13} to patients with multiple myeloma, followed by isolation of the abnormal serum and urinary proteins, has led previously to the conclusion that Bence-Jones protein is not derived by cleavage of circulating serum proteins nor by degradation of tissue proteins (1, 2). In point of fact, no evidence for any kind of precursor relationship between the two types of abnormal proteins was found. The data from these experiments also suggested that the Bence-Jones protein is formed rapidly \textit{de novo} from free amino acids and is excreted rapidly. However, the stable isotopes proved unsuitable for the precise study of the rate of synthesis and excretion of Bence-Jones protein because they are too insensitive for intravenous administration at tracer levels and for measurement of the isotopic concentration of the unhydrolyzed protein. Thus, for the present work lysine, uniformly labeled with C\textsuperscript{14}, was chosen to facilitate the rate study. The lysine was injected into a subject who copiously excreted Bence-Jones protein but lacked an abnormal serum globulin. The urine was collected by urethral catheter, and the specific activity of the purified protein specimens was determined. The activity was maximal at 10 hours and thereafter declined rapidly. This indicates that the urinary protein was synthesized from the free amino acids rather than from preformed tissue proteins and that Bence-Jones protein, once released into the circulation, is treated like other nitrogenous excretory products, despite its size.

**EXPERIMENTAL**

**Methods and Procedures**

\textit{Serum and Urinary Proteins}—The serum and urinary proteins were characterized by analysis in the Tiselius type electrophoresis apparatus and with the analytical ultracentrifuge (3, 4). The Bence-Jones protein was...
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precipitated at 4° by dialysis against 3 m ammonium sulfate at pH 5.2. The heavy precipitate was removed by centrifuging and was washed twice with the salt solution. The precipitate was dissolved in water, and the solution was centrifuged till clear and then dialyzed against 0.9 per cent NaCl until free of sulfate ion. Aliquots were dialyzed against distilled water until free of chloride ion and then were lyophilized. A schlieren effect was noticed after several changes with distilled water in a preparation made prior to the isotopic study. Repeated changes and prolonged dialysis in the cold led to crystallization of the Bence-Jones protein as large sheets of crystals within the dialysis sac. However, the crystals dissolved on warming to room temperature. Although the crystallization was readily repeated, no attempt was made to crystallize the fourteen specimens used in the isotopic study.

Radioactive Measurements—L-Lysine labeled uniformly with C14 was obtained from the Nuclear Instrument and Chemical Corporation. Prior to administration the compound was dissolved in a 0.9 per cent NaCl-5.0 per cent glucose solution and was tested for toxicity and sterility. For determinations of radioactivity, the lyophilized protein was dissolved in 0.02 N NaOH, deposited on oxidized copper planchets, and dried to yield a uniform adhesive film. The planchets were counted with a gas flow counter (Atomic Instrument Company). Counts were made to a minimum of 1000 over background and were corrected for self-absorption. Respiratory CO2 was also collected, and the specific activity and cumulative activity were kindly measured by Dr. George Okita by means of a vibrating reed electrometer. A later attempt to determine the specific activity of the urinary urea CO2 by means of the urease reaction was unsuccessful because the samples available (from 2.5 hours on) had too little activity for accurate measurement.

In order to obtain an estimate of the excretion of the lysine that had been administered, the amino acids in 5 ml. urine samples were decarboxylated by reaction with ninhydrin at 100° in the presence of 15 mg. of glutamic acid as carrier. The CO2 was trapped with Ba(OH)2 and counted as BaCO3 in an aluminum planchet. The data are presented for samples of infinite thickness for a planchet of 3.47 sq. cm. area. Since the lysine concentration of each sample of urine was not determined, correction for the carrier added could not be made.

Subject and Protocol

Subject—The patient M. B. was admitted to the Argonne Cancer Research Hospital for study and terminal care. Relevant biochemical find-
ings were (1) anemia (hemoglobin of 7.4 gm. per cent), (2) hypoproteinemia (total serum protein of 4.8 gm. per cent with 1.7 gm. per cent globulins), and (3) renal insufficiency (urea clearance of 24, as calculated by the square root method, and elevated blood urea nitrogen and uric acid). The patient died of uremia 2 months after admission. Post mortem examination supported the diagnosis of multiple myeloma and revealed an extensive amyloidosis.

Protocol—For the experiment, 300 μc. of L-lysine in 100 ml. of pyrogen-free solution were given intravenously over a 7 minute interval. There was no reaction. A complete urine collection by catheter was maintained for the next 13 hours. The first sample was taken at 30 minutes and the others at progressively increasing times. The catheter was removed, but complete urine collection at 4, 6, or 12 hour intervals was maintained for 90 hours with a total of thirteen specimens. A final specimen was taken on the 13th day. Because of the anemia and hypoproteinemia, the blood activity was not investigated. However, expiratory CO₂ samples were collected by Dr. George Okita over a period of 24 hours. An aliquot of each urine sample was frozen for later study, and the remainder was used immediately for preparation of the Bence-Jones protein.

Owing to the patient’s anorexia, a nitrogen balance study was unsuccessful. During the 4 days of the experiment, the daily protein intake rose from 40 to 99 gm. A week later it had fallen to 10 gm. However, the daily excretion of Bence-Jones protein was almost constant (about 10 gm.).

Results

Serum and Urinary Proteins—The electrophoretic pattern of the patient’s serum in Veronal buffer, pH 8.6 (Fig. 1, A), reveals the absence of a predominant globulin component of the type observed in about two-thirds of the cases of multiple myeloma (3). Area analysis indicated that percentage wise the serum protein distribution was as follows: albumin 53.8, α₁-globulin 5.3, α₂-globulin 17.0, β-globulin 19.4, and γ-globulin 4.6. Thus, α₂- and β-globulin were significantly elevated percentage wise (α₂ by 2-fold), whereas γ-globulin had diminished to one-third of normal on this basis and to one-fifth on an absolute basis.

Since the Bence-Jones protein had a mobility close to that of β-globulin (compare Fig. 1, A and B), the relative increase in this peak may result from the presence of Bence-Jones protein (also from a slight hemolysis).

The Bence-Jones protein appeared to be quite homogeneous by the criteria applied. It moved with a single sharp boundary both in electrophoresis and ultracentrifugation (Fig. 1, B and C) and crystallized readily (Fig. 2). Amino end-group analysis by the dinitrophenyl (DNP) method revealed

2 Dr. Milton Weiner aided in devising the protocol and in the medical management of the patient, under the direction of Dr. Robert Hasterlik and Dr. George V. LeRoy.
only trace amounts of ether-soluble DNP-amino acids, a total of about 0.1 mole per mole of protein. Thus, this protein is one of a group of crystallizable Bence-Jones proteins that are devoid of end-groups detectable by the DNP method (5). Further work on this problem is in progress. In the Veronal buffer, pH 8.6, the Bence-Jones protein had a mobility of $-2.6 \times 10^{-5}$ cm$^2$ volt$^{-1}$ sec.$^{-1}$ and a sedimentation constant of 3.4 Svedberg units, values similar to those reported for proteins D and G (4). It was in the same antigenic group as protein G.$^3$

Fig. 1. Electrophoretic diagrams of the serum proteins (A) of subject M. B. and of a specimen of the radioactive Bence-Jones protein (B) and ultracentrifugal diagrams of the latter protein (C). All experiments were performed in Veronal buffer, pH 8.6, ionic strength 0.1. The arrows in A and B indicate the origin and direction of migration. Since the photographs of A and B were taken after 200 minutes at the same current (16 ma.), the mobilities can be compared by visual inspection. In C, sedimentation was from right to left, with photographs taken at 32 minute intervals at a rotor speed of 59,780 r.p.m.

**Expiratory CO$_2$**—According to current views (6), the first steps in the metabolism of the carbon chain of lysine involve deamination, followed by oxidative decarboxylation yielding CO$_2$. The latter would rapidly appear in the expiratory CO$_2$. These reactions produce glutaric acid, the further metabolism of which is unknown. From Fig. 3, it can be seen that there is a rather rapid initial expiration of C$^{14}$O$_2$ with a maximum at about 2.5 hours. Although the decline in activity for the first 8 hours is almost exponential (half time of about 6 hours), this is followed by a slower non-logarithmic decrease in specific activity. The cumulative activity of exp-

$^3$ Antigenic group A, according to Dr. Leonhard Korngold of the Sloan-Kettering Institute for Cancer Research (private communication).
piratory $^{14}CO_2$ was still rising at 24 hours, by which time 3.9 per cent of the injected activity had been exhaled.

In the choice of $^{14}$-amino acids for studies of protein synthesis, one must consider whether to use specifically or randomly labeled compounds. The choice of an essential versus a non-essential amino acid is also important, for Penn, Mandeles, and Anker (7) have demonstrated that the apparent turnover time varies with the nature of the amino acid selected as tracer. Hence, in an experiment with a different patient, $\alpha$-glutamic acid-$^{1}$C was chosen to investigate the possible precursor relationships of the abnormal serum globulin and of the Bence-Jones protein. Although the fractionation of the serum proteins is not yet complete, data on the specific activity of the expiratory $CO_2$ and the Bence-Jones protein are available for comparison with results obtained after the administration of lysine.

Glutamic acid-$^{1}$C would be deaminated readily to yield $\alpha$-ketoglutarate, which in turn would be decarboxylated rapidly via the citric acid cycle, yielding $^{14}CO_2$ and unlabeled succinic acid (6). In accord with expectation, the specific activity of the expiratory $^{14}CO_2$ was maximal within the first half-hour. Not only did this maximum occur 2 hours earlier than with the uniformly labeled lysine, but it was also almost four times greater, although the two subjects were given about equivalent activity in the L form (compare Fig. 4). (On the other hand, the maximal specific activity of the Bence-Jones protein of the subject given lysine was seven times greater than that of the patient given glutamic acid.)

Since the specific activity-time curves for expiratory $^{14}CO_2$ reflect the blood level of the tracer amino acid, they are a useful index in the absence

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**Figure 2.** Photomicrographs of crystals of Bence-Jones protein from patient M. B. (53 X).
of kinetic data on the latter quantity. It is evident that the blood level of radioactivity is maintained longer in the case of lysine, leading to a more efficient incorporation in the protein, but that glutamic acid $L^{-1}$-C$^{14}$ would give a more instantaneous picture of protein synthesis.

Bence-Jones Protein—The specific activity curve for the Bence-Jones protein (Fig. 5) demonstrates a rapid uptake and decline of isotopic amino acid in the excreted protein. The first specimen of Bence-Jones protein was devoid of radioactivity (obtained at 30 minutes after injection, but

omitted from the graph of Fig. 5). After this lag, the activity rose sharply to a maximum at 9.5 hours. Thereafter, it fell off steeply for a day, and then more slowly. The declining curve was not truly exponential but appeared to have two logarithmic segments. The first, terminating at 36 hours, corresponded to a half time of decline of 15 hours; the second, ending at 90 hours, was equivalent to a half time of about 60 hours. A specimen taken 8 days later had unexpectedly high activity (79 c.p.m. per mg. of protein).

The data of Fig. 5 are alone inadequate to give a complete picture of either the rate or the cumulative balance of the excretion of the labeled Bence-Jones protein. The rate is clearly a function of the urine volume
and protein concentration as well as of the specific activity. The balance is incomplete because urine collection was stopped on the 4th day.

Fig. 4. Comparison of the specific activity-time curves for the expiratory CO$_2$ in two subjects, the one (M. B.) given 300 µc. of L-lysine-C$^{14}$ and the other injected with about 450 µc. of DL-glutamic acid-1-C$^{14}$ (measurements by Dr. George Okita of the Argonne Cancer Research Hospital).

Fig. 5. Specific activity-time curves of the Bence-Jones protein isolated from the urine of subject M. B. and of the BaCO$_3$ obtained by reaction of the urine with ninhydrin. The initial sample of urine, collected by catheter for the first 30 minutes after injection, proved to be inactive and is not represented on the curve.

However, the hourly rate could be calculated for the first 90 hours from the total protein recovered in each specimen and from its activity. A histo-
gram of the activity (total counts) excreted per hour was constructed. The general shape of the curve was similar to that of Fig. 5, with a maximum at the 10th hour. Although there was more fluctuation in the values at the later intervals, no diurnal variation could be discerned. Of the total activity eliminated in the urine via protein in 90 hours, 19 per cent was excreted in the 4 hour period from 9 to 13 hours after injection and 45 per cent in the 12 hour period from 5 to 17 hours after injection. It can be estimated that the latter amount is as great as that excreted in the 12 day period following the time covered by Fig. 5 (estimated from the logarithmic plot of declining activity and the assumption of a daily excretion of 10 gm. of protein).

Urinary Excretion of Lysine-C\textsuperscript{14}—The possibility that an appreciable fraction of the injected lysine was excreted per se in the urine was investigated by ninhydrin decarboxylation of the urinary amino acids in the presence of carrier. Unfortunately, the frozen specimens of the early urine samples were lost. However, the data in Fig. 5 are adequate to reveal the level of excretion and the rate of decline. In comparison with D-glutamic acid, little L-lysine was excreted in the urine.\textsuperscript{4} For example, at 2.5 hours the activity of BaCO\textsubscript{3} obtained from ninhydrin treatment of 5 ml. of urine, with 15 mg. of glutamic acid added as carrier, was 220 c.p.m. for subject M. B., whereas, at the same time and with only 0.3 ml. of urine, and the same weight of carrier, the activity was 12,800 c.p.m. in the subject given DL-glutamic acid-1-C\textsuperscript{14}. Thus, the amino acid activity of the two urines differed by a factor of 870, although the protein was more radioactive in the present case.

Tests for Adsorption—Because the adsorption of lysine by proteins has been represented as leading to spurious results in tracer experiments of protein synthesis (8), several tests were made to assure the absence of this phenomenon. The tests involved (a) dialysis of a mixture of unlabeled protein and lysine-C\textsuperscript{14} of high activity against a series of changes of 0.15 M NaCl until the external solution had negligible activity, (b) dialysis of labeled protein against a solution containing inactive lysine in 0.15 M NaCl, and (c) a comparison of the BaCO\textsubscript{3} activity following the action of ninhydrin on the hydrolyzed and unhydrolyzed labeled protein. The period of dialysis and the number of changes approximated the conditions used in the preparation of the Bence-Jones protein specimens. From the results summarized in Table I, Experiment I, it can be seen that dialysis, even in the absence of lysine outside the sac, effectively reduces the radioactivity inside the sac to a negligible value. Similarly, in Experiment II, there

\textsuperscript{4} Virtually all the urinary amino acid activity of the patient given DL-glutamic acid was in the form of the unnatural isomer; e.g., in a urine sample yielding BaCO\textsubscript{3} with an activity of 6520 c.p.m. by ninhydrin decarboxylation, the activity was only 5 c.p.m. on enzymatic decarboxylation.
was no significant change in the specific activity of the labeled protein upon dialysis against free, inactive lysine. In addition, no activity above background was found in the BaCO\(_3\) obtained from the reaction of ninhydrin in a solution containing labeled protein and inactive carrier amino acid, whereas the specific activity of the BaCO\(_3\) obtained from ninhydrin decarboxylation of the same specimen of protein (and carrier) after hydrolysis was 696 c.p.m. at infinite sample thickness. We conclude that the Bence-Jones protein was labeled only by true incorporation of the isotopic lysine through peptide bonds.\(^6\)

### Table I

**Test for Adsorption of Lysine-C\(^{14}\) by Bence-Jones Protein**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Dialysate</th>
<th>No. of changes</th>
<th>C.p.m. per mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Unlabeled protein and lysine-C(^{14})</td>
<td>0.15 M NaCl</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Change dialysis sac</td>
<td>0.15 &quot; &quot;</td>
<td>4</td>
<td>6.6</td>
</tr>
<tr>
<td>Control</td>
<td>No dialysis</td>
<td></td>
<td>21,500</td>
</tr>
<tr>
<td>II. Labeled protein</td>
<td>10 mg.% lysine in 0.15 M NaCl</td>
<td>3</td>
<td>518</td>
</tr>
<tr>
<td>Control</td>
<td>0.15 M NaCl</td>
<td>2</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>No dialysis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### DISCUSSION

Bence-Jones proteinuria is a characteristic but not an invariable finding in multiple myeloma. Whereas some patients have no detectable Bence-Jones protein in the urine, others excrete up to one-half their daily nitrogen intake via this abnormal metabolic product. Two other aberrations in protein metabolism may occur in this disease, (1) a hyperglobulinemia resulting from the formation of individually characteristic, abnormal serum globulins (3, 9-12) and (2) the deposition of protein in the tissues (amyloidosis). Like the myeloma globulins, the Bence-Jones proteins are not identical; indeed, they appear to differ with each individual (4, 5). Although the response of a given patient is characteristic and consistent, no correlation of the protein abnormalities can yet be made with clinical symptoms, diagnostic findings, plasma cell type, or longevity.\(^6\)

The variety of abnormal plasma and urinary proteins formed by different

\(^6\) A similar conclusion was reached after more extensive tests in the case in which a patient was given \(DL\)-glutamic acid-1-C\(^{14}\). In this instance, in which one-half the activity is excreted via the \(p\) isomer, the dialysis tests and the lack of reaction with ninhydrin were confirmed by the failure of glutamic acid decarboxylase to reduce the activity of the excreted protein.

\(^6\) Kubota, C., Schwartz, S. O., and Putnam, F. W., from data obtained in a survey of 78 cases (in press; see also (13)).
patients with multiple myeloma (3, 4, 9-11) requires that investigation of protein synthesis in this disease be undertaken in several patients with representative aberrations. In preceding experiments (1, 2), two patients were studied with use of glycine labeled with N\textsuperscript{15} or C\textsuperscript{12}. Both subjects had hyperglobulinemia, the one owing to a cryoglobulin of the \(\gamma\) type, the other from an abnormal \(\beta\)-globulin. Each excreted a single Bence-Jones protein, distinguishable from the other. Despite these individually characteristic abnormalities in protein synthesis, the same conclusions were drawn for the two subjects; namely (1) there was no apparent metabolic relationship between the abnormal protein of the plasma and that of the urine, and (2) in each case the Bence-Jones protein was synthesized rapidly and excreted rapidly and appeared to be formed \textit{de novo} from free amino acids rather than by degradation of serum or tissue proteins.

The present investigation extends these observations to a subject of a third type, \textit{i.e.} one who copiously excretes a highly homogeneous Bence-Jones protein but has hypoglobulinemia. The previous experiments, based on 24 hour urine collections, demonstrated that oral administration of a large dose of C\textsuperscript{13}- or N\textsuperscript{15}-labeled amino acid (5 to 20 gm. during an 8 hour period) was followed within 24 to 48 hours by a maximum in the C\textsuperscript{13} or N\textsuperscript{15} content of the urinary protein. In the present case, it is shown that intravenous infusion of a tracer dose of lysine-C\textsuperscript{14} (about 50 mg.) leads to a maximum in the specific activity of the excreted protein within 10 hours. Upon comparison of the activity curves for expiratory CO\textsubscript{2} in the case of lysine and glutamic acid (Fig. 4), one might anticipate a sharper decline in the activity of Bence-Jones protein (and probably an earlier maximum) if the more rapidly metabolized amino acid had been used as the tracer.

Although this experiment gives a more complete picture of the rate of formation and excretion of Bence-Jones protein, the kinetics of these processes cannot be calculated accurately from the data presented. The rate of change in activity of the Bence-Jones protein is a function of at least four factors: (1) the rate of decline in the specific activity of the free lysine, (2) the rate of synthesis of the protein, (3) the rate of its release into the circulation, and (4) the rate of renal excretion. The time required for an amino acid residue to pass from the free amino acid pool to the circulating serum protein has been defined as the "transit time." The transit time is a function of the first three factors listed, and in rabbits, turtles, and the horseshoe crab it is approximately 30 minutes at 37\degree (8). As judged by the first appearance of radioactive protein in the urine, the maximal time lag for synthesis and excretion of Bence-Jones protein is 30 to 60 minutes. This short time lapse was quite unforeseen when the experiments with stable isotopes were planned (1, 2). Although the time lags for the synthesis of serum proteins and the synthesis and elimination of Bence-Jones
protein are comparable, the curves for the decline in activity are not. The serum proteins "turn over" among the most rapidly of all tissue proteins, and, in man, serum proteins have a turnover time of from 5 to 20 days (8, 13). However, it is evident from the even more rapid activity decline illustrated in Fig. 5 that Bence-Jones protein is not derived from serum or from other preformed tissue proteins.

With regard to the first factor above, the specific activity of free lysine is reflected both by the activity of the urinary amino acid (Fig. 5) and by the activity of expiratory CO₂ (Figs. 3 and 4). Neither of these activities declines exponentially, nor does the activity of the Bence-Jones protein (Fig. 5). The similarity in the declining portions of the three curves is evidence, then, that the Bence-Jones protein is formed de novo from the administered amino acid. The data of Fig. 5 also suggest that the Bence-Jones protein is not withheld appreciably more by the kidney than is lysine (or its ninhydrin-reacting metabolites). This finding, though surprising, is compatible with the previous report that N¹⁸ is excreted as rapidly by way of incorporation into Bence-Jones protein as via metabolism to urea (2). Judging by comparison of the rising portions of the activity curves of Figs. 3 and 5 and their respective maxima, one concludes that it takes about 6 hours longer for lysine to be incorporated into Bence-Jones protein and for the latter to be excreted than it does for lysine to be metabolized to CO₂ and the latter exhaled.

Half of the activity excreted by way of labeled protein within the first 90 hours and about one-quarter of that so excreted within the first 2 weeks were voided in the 12 hour interval beginning 5 hours after the injection. Now, since labeled protein must have been made continuously, though with rapidly decreasing activity, it is clear that the bulk of the Bence-Jones protein made and released into the circulation within any given interval is excreted in the ensuing 12 hours. In other words, circulating Bence-Jones protein does not participate actively in the metabolic pool of nitrogen, but is treated like low molecular weight nitrogenous excretory products. The sharp maximum and the rapid decline in the curve of Fig. 5 preclude the presence of a large pool of Bence-Jones protein that undergoes turnover analogous to serum proteins. The question whether some of the Bence-Jones protein is deposited in an inert form in amyloid tissue may be resolved when analyses of tumor masses and amyloid deposits are completed.

Since skin tests with the purified protein yielded no untoward reaction in the subject, it had been planned to measure the renal clearance and rate of excretion by reinjection of the labeled protein. However, the patient's

¹ Under similar conditions serum proteins in man exhibit a similar lag in activity, a slower rise, a much broader maximum, and a considerably slower decline (unpublished observations, F. W. Putnam, F. Meyer, and A. Miyake).
condition did not warrant the experiment. Such an experiment has taken place with the patient given glutamic acid-1-C\textsuperscript{14}, and it was also possible to measure the activity of the L-glutamic acid of serum and of the \( \gamma \)-globulin. The data, which are almost complete, are fully compatible with the conclusions drawn above.

We are greatly indebted to Dr. Robert Hasterlik, Dr. George V. LeRoy, and Dr. Leon Jacobson of the Argonne Cancer Research Hospital for their great interest and unceasing cooperation in this experiment.

**SUMMARY**

The rate of synthesis and excretion of Bence-Jones protein has been studied by intravenous injection of lysine uniformly labeled with C\textsuperscript{14}, followed by measurement of the specific activity of the protein isolated from successive urine samples. Urinary excretion of the labeled amino acid was estimated from the CO\textsubscript{2} obtained by the ninhydrin reaction. The specific activity of expiratory CO\textsubscript{2} was also determined and compared with the activity of expiratory CO\textsubscript{2} after injection of DL-glutamic acid-1-C\textsuperscript{14} in another subject. After a 30 minute lag period, the radioactivity of the urinary protein rose sharply to a maximum at 10 hours and then fell off abruptly in a non-logarithmic fashion. The activity of expiratory CO\textsubscript{2} was maximal at 2.5 hours and also declined rapidly and non-exponentially. The data confirm the earlier conclusion that Bence-Jones protein is rapidly synthesized from the free amino acids rather than from tissue protein precursors. Bence-Jones protein formed in any given interval is excreted for the most part within the ensuing 12 hours.

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