Multiple myeloma is of biochemical interest because the profuse synthesis and the diverse nature of the proteins elaborated by different patients represent one of the most profound alterations in protein metabolism yet encountered. This disease of unknown etiology is generally classified with the tumors. It is believed to affect the plasma cells that are implicated in globulin synthesis and antibody production. Multiple myeloma results in any one or more of three aberrations in protein metabolism: (1) the production of anomalous serum globulins, (2) the excretion of characteristic urinary (Bence-Jones) proteins, and (3) the deposition of protein in the tissues (paramyloidosis) (1). The proteinuria occurs more commonly in the absence of hypergammaglobulinemia than in its presence. The origin of the Bence-Jones protein is uncertain but has been ascribed to renal cleavage of myeloma globulins (2, 3) or degradation of tissue proteins. Physicochemical characterization (4, 5) and end-group analysis (6) have established that there are different types of myeloma globulins and of Bence-Jones proteins, but have failed to elucidate their relationship to each other and to normal serum proteins. Investigation with isotopic tracers of the rate of synthesis and possible precursor relationships of myeloma globulins and Bence-Jones proteins has therefore been undertaken (7, 8).

For the present study labeled glycine, NH₂CH₂Cl³OOH, was given to a patient whose serum contained 5 gm. per cent of an abnormal "β"-globulin, and who excreted 1 to 2 gm. of Bence-Jones protein daily. The abnormal globulin and the urinary protein were isolated from samples taken over a period of 50 days, and the purity of the fractions was ascertained by electrophoretic and ultracentrifugal analyses. The proteins were hydrolyzed, and glycine was separated chromatographically. The Cl³ content of the carboxyl carbon was estimated in the mass spectrometer. From the time course of the Cl³ decline, it is concluded that the rates of synthesis of the two pathological proteins are independent and that the urinary protein is not derived from any known serum or tissue protein. Although the half life of the myeloma globulin was possibly longer than that of normal serum

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proteins, the Bence-Jones protein was apparently synthesized de novo and was excreted almost as rapidly as it was formed.

EXPERIMENTAL

Methods and Procedures—By the method of Sakami et al. (9), NH₂CH₂·C¹³OOH was prepared¹ in a yield of 55 per cent with the use of KC¹³N containing 58.8 atom per cent excess C¹³. The product was tested for toxicity in mice and found to be innocuous at levels up to 25 times the dosage given to the patient (77 mg. per kilo of body weight).² The serum proteins were fractionated and the urinary protein was isolated as later described. Electrophoretic and ultracentrifugal analyses of representative samples were performed as before (4, 5). To avoid dilution of the C¹³, to facilitate comparison of the isotopic abundance of the two pathological proteins irrespective of their glycine content, and to overcome the effect of metabolic labeling of serine,³ the proteins were hydrolyzed and the glycine was separated chromatographically. Duplicate samples were decarboxylated with ninhydrin at pH 6.7 (11). Nitrogen was passed through the solution at 100°, and the CO₂ was collected in Ba(OH)₂. The barium carbonate was converted to CO₂, and the C¹³ concentration was determined in the mass spectrometer.

For the isolation of the glycine, 200 to 250 mg. of the purified Bence-Jones protein or myeloma globulin and up to 500 mg. of the serum albumin were hydrolyzed by autoclaving for 15 hours with 6 N HCl in sealed tubes. The hydrolysates were decolorized with charcoal, filtered, concentrated, and then adsorbed on 2.2 X 60 cm. columns of Dowex 50, 200 to 500 mesh. The amino acids were eluted with 2.5 N HCl, and their concentration was estimated by the calorimetric ninhydrin method (12, 13). Glycine was recovered in the eluate from the fraction between 350 and 380 ml.

The total N of the 24 hour urine samples was measured by Kjeldahl analysis; protein N was determined on the washed precipitate obtained after addition of an equal volume of 10 per cent trichloroacetic acid. The methods for the N-terminal amino acid analysis of the myeloma globulin and the Bence-Jones protein have been indicated (6) and will be published separately.

Case History—The subject (G. M.) was a male, aged 53, who had been

¹ Synthesized by Dr. Charles Gilvarg.
² Toxicity tests were kindly performed by Dr. Robert Feinstein.
³ The Bence-Jones protein and the myeloma globulin both had a glycine content of 3.6 per cent compared to 1.6 per cent and 4.2 per cent reported for human serum albumin and γ-globulin, respectively (10).
⁴ We are greatly indebted to Dr. Steven O. Schwartz and Dr. Robert Lebow of the Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Illinois, for generous cooperation, care of the patient, collection of all samples, and for the patient’s case history.
admitted to Cook County Hospital, Chicago, Illinois, with a brief history of hip and back pain and a weight loss of 40 pounds. The diagnosis of multiple myeloma was based on typical x-ray findings and examination of the bone marrow, and was consistent with the serum protein pattern and the excretion of Bence-Jones protein. The x-rays showed general involvement of the rib cage, the spine, and pelvis, and revealed a pathological fracture of the femur. There were no plasma cells in the peripheral blood, but the marrow was very hypercellular and almost entirely replaced by sheets of plasma cells. The laboratory findings before and after the experiment are summarized in Table I. Throughout hospitalization the

TABLE I

| Blood Findings for Subject G. M. on 1 Day before and 1 Month after Experiment* |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Before          | After           |                 |
| Serum protein, gm. %............| 10.2            |                 |                 |
| Albumin-globulin (Howe)........| 0.42            |                 |                 |
| Electrophoretic distribution of |                |                 |                 |
| serum, %......................| Albumin 36.4, α1-globulin 2.7, α2- 3.8, β- 53.2, γ- 3.8 | Albumin 45.7, α1-globulin 2.4, α2- 3.4, β- 45.0, γ- 5.1 |
| Hemoglobin, % of normal........| 55              | 78              |                 |
| Erythrocytes (per c.mm.)........| 3 × 10⁶         | 4.6 × 10⁶       | 1800            |
| Leucocytes (per c.mm.)..........| 2350            |                 |                 |

* On admission, 2 months prior to the experiment, the hemoglobin was only 25 per cent, the red cells were 1.5 × 10⁶, the white cells were 7350, albumin was 29.2 per cent of total serum protein, and β-globulin was 56.8 per cent. On release, 6 weeks after the experiment, leucocytes had dropped to 850 and urethane was discontinued. 2 months before the experiment the blood non-protein N was 40 mg. per cent.

patient received 4 gm. of urethane daily. The drug produced marked physical improvement and some relief of the anemia, but was eventually withdrawn because it provoked a leucopenia. At the end of the experiment the patient was still ambulatory and was in remarkably good condition, considering the extent of the disease. He survived 2 years.

Protocol of Experiment—G. M. received 5.41 gm. of the labeled glycine. This was given orally in divided doses in gelatin capsules over a period of 11 hours on the 1st day. All blood samples were drawn at 8 a.m., and 24 hour urine collections were taken without catheterization from 8 a.m. of 1 day to 8 a.m. of the next, including the day of administration of the glycine. Because of the anemia, the blood samples had to be taken sparingly. Throughout the experiment the subject's weight remained unchanged at 70.5 kilos.
ORIGIN OF BENCE-JONES PROTEIN

Results

Electrophoretic analyses of the serum before and after the experiment are shown in Fig. 1, and the protein distribution in Table I. The most striking feature of the patterns is the sharp peak migrating with the mobility of β-globulin. Throughout the 1st week of the experiment this component comprised 53 per cent of the serum protein. 2 months later it had declined to 45 per cent, probably as the result of urethane therapy, which is known to depress myeloma protein synthesis (14). Since similar experimental results were obtained with a second patient not on urethane therapy (8), it is believed that the action of this drug does not affect the interpretation of the isotopic data during the first few weeks of the experiment, during which time the serum protein distribution of G. M. remained unchanged. Ultracentrifugal analysis of the whole serum confirmed the albumin-globulin distribution. The sedimentation patterns revealed a sharp component with a sedimentation constant \( s_{20} \) of about 8 Svedberg units (8); this peak is absent in normal serum and is identifiable with the abnormal globulin. Ultracentrifugal analysis by the method of Gofman et al. (15) indicated a low concentration of components in the flotation class \( s_f 20 \) to 30.” This suggests that the myeloma globulin was not a lipoprotein though it migrated electrophoretically in the β position.

Fig. 1. Electrophoretic diagrams of the serum and the globulin fraction of subject G. M. in Veronal buffer, pH 8.6, 0.1 ionic strength. Upper diagrams, serum 1 month before the start of the experiment; middle diagrams, serum 1 month after the experiment; lower diagrams, globulin fraction. A schlieren photograph of the starting boundary is superimposed on the patterns.
After the 1st day the subject excreted an average of 8.2 ± 1.1 gm. of N per 24 hours, of which approximately 3 per cent was protein N, corresponding to a daily output of 1 to 2 gm. of Bence-Jones protein.

For physiochemical characterization and isotopic analysis, the serum proteins were isolated by salt fractionation since electrophoretic separation proved unsuitable. The serum was adjusted to pH 6.5, and the β-globulin was precipitated by slow addition of 2 volumes of 1.8 M (NH₄)₂SO₄ and subsequent dialysis against the latter solution. The precipitate was centrifuged, washed with the salt solution, dialyzed free of sulfate ion, and lyophilized. This rapid but crude procedure yielded a preparation that was 85 per cent β-globulin but contained all the serum γ-globulin. Initially, the latter comprised only 3 per cent of the total serum protein. Electrophoretic analysis also revealed the presence of a small amount of serum albumin (Fig. 1). In the ultracentrifuge the β-globulin split into two major components that had sedimentation constants of 6.2 S and 8.8 S, respectively (uncorrected for concentration). The ratio of the sharp fast peak to the slow peak was 1.6 and thus did not accord with the electrophoretic distribution. This ratio could be altered by further fractionation or by dialysis against water to remove the euglobulin. Previous work (4) has shown that myeloma globulins of the β type may contain two sedimenting components, the one with the s₂₀ of normal γ-globulin (6.6 S), the other with an s₂₀ of about 9 S; yet neither has the mobility of γ-globulin at pH 8.6. To obtain the serum albumin, the supernatant fluid of the 1.8 M ammonium sulfate solution was adjusted to a concentration of 2 M. The precipitate was found free of globulin upon electrophoresis but contained a small amount of a more rapidly sedimenting component in the ultracentrifuge.

The Bence-Jones protein was isolated by adjustment of the urine to pH 5.2 and addition of solid ammonium sulfate with stirring to a concentration of 3 M. The precipitate was dialyzed against water until free of sulfate ion and then was lyophilized. This protein was designated as Protein F in Paper II of this series, in which may be found the electrophoresis diagram at pH 8.6, the mobility curve from pH 3 to pH 10, and the sedimentation data over the same pH range (5). Throughout the pH stability range (pH 5 to 9) the protein migrated with a single component in electrophoresis and ultracentrifugation except for an impurity comprising 3 to 5 per cent. In Veronal buffer, pH 8.6, 0.1 ionic strength, it had an s₂₀ of 3.08 S, and it migrated with a mobility of $-3.37 \times 10^{-5}$ cm² volt⁻¹ sec⁻¹ compared to a mobility of $-3.07 \times 10^{-5}$ cm² volt⁻¹ sec⁻¹ for the myeloma globulin. Despite the similarity in mobilities, the two pathological proteins could be differentiated by their sedimentation behavior and by their N-terminal groups. Like normal, human γ-globulin, but unlike most myeloma globu-
lins so far studied (6), the abnormal serum globulin in this case contained both $N$-terminal aspartic and glutamic acids (1.3 and 1.7 moles, respectively, per 160,000 gm.). On the other hand, the Bence-Jones protein had 2 moles of $N$-terminal aspartic acid per 40,000 gm. with only traces of $N$-terminal glutamic acid, glycine, and threonine.

The isotopic data are given graphically in Fig. 2 in which the vertical bars represent the 24 hour urinary protein samples.

![Fig. 2. Isotope concentration of the glycine carboxyl of Bence-Jones protein, serum albumin, and myeloma (MM) globulin as a function of time. The height of the vertical columns gives the values for the protein obtained from 24 hour specimens.](image)

**DISCUSSION**

The most striking observation in this experiment is the high initial isotopic concentration of the glycine of the Bence-Jones protein and the rapid rate of its decline. On the first 2 days, this glycine had a $^{13}$C concentration one-thirtieth that of the glycine administered. Higher values undoubtedly prevailed for a brief period. We have estimated that the labeled glycine was diluted by about 3 to 4 gm. of unlabeled dietary glycine and by about 20 to 25 gm. synthesized by the body (calculated from data for the rat (16) adjusted for relative body surface area). In contrast to the situation with tissue proteins previously studied, it appears that the glycine of the Bence-Jones protein closely approaches the isotope concentration of the glycine pool. It is noteworthy that the rate of decline of the $^{13}$C concentration of the Bence-Jones glycine was almost identical with the decline in $^{15}$N con-
centration of urinary urea when $N^{15}$-glycine was fed to a normal subject (e.g., half time of about 18 hours) (17, 18).

It is essential to recognize that the Bence-Jones protein is in fact an excretory product and that the body pool of this protein must be small. This is indicated by the high initial isotopic concentration and its rapid decline, the small daily excretion, and the absence of physically detectable Bence-Jones protein in the circulation. Hence, the isotopic decline in the Bence-Jones protein is not a direct measure of turnover, but rather a complex function of synthesis, release into the circulation, renal clearance, and the activity of the glycine pool. The asymptotic shape of the curve after the 1st week is probably related to the return of labeled glycine into the body pool as the result of turnover of plasma and liver proteins (18).

The C$^{13}$ abundance of the Bence-Jones protein initially exceeded that of the serum protein glycine 6-fold but subsequently was lower by one-half. This suggests clearly that, once the myeloma globulin is released into the circulation, it does not break down in the kidney or elsewhere to form Bence-Jones protein. A similar consideration applies to the serum albumin. Although emaciation is characteristic of this disease, the Bence-Jones protein could not have arisen by degradation of normal tissue protein, for the isotopic data indicate de novo synthesis of the urinary protein. If the Bence-Jones protein is not derived by degradation of the serum proteins, neither does it appear to be the sole immediate precursor of the myeloma globulin. Zilversmit et al. (19) and Reiner (20) have shown that in a homogeneous system (a) the isotope concentration of the single immediate precursor will at first be greater than that of the product and subsequently less than that product, and that (b) the specific activity curves of the precursor and product will cross when the specific activity of the product is at a maximum. Contrary to the latter criterion, the globulin curve reaches a maximum at about the same time as does the Bence-Jones curve and some 5 days prior to intersection with the latter. Thus, it would appear that the synthesis of the Bence-Jones protein and the synthesis of the myeloma globulin are independent processes.

The turnover curves for the abnormal globulin and the serum albumin resemble the curves for serum proteins in normal human beings where the half time is approximately 10 days when $N^{15}$-glycine is used as a tracer (21). The myeloma (g) globulin has a half time of 17 to 20 days, some-
what longer than that of the albumin, and more than twice as long as that suggested for $\beta$-globulin by London's data (21). On this basis one might infer that the hyperproteinemia is due to accumulation of the myeloma globulin by virtue of its slow turnover rather than to rapid synthesis. Unfortunately, probably owing to the complex nature of protein "turnover," the data for neither the normal nor pathological state are sufficiently accurate to warrant this conclusion.

This experiment reveals nothing about the site of synthesis or any physiological function of Bence-Jones protein. However, the results indicate that this protein arises by synthesis de novo and is apparently derived directly from the nitrogen pool rather than via any plasma or tissue protein precursor. Although the synthesis of the two abnormal proteins appears to be an independent process, the occurrence of a common precursor, of an additional precursor, or of a diluent of the product has not been excluded. An experiment with a patient who had different abnormal proteins and a graver clinical condition has given further evidence for the direct interaction of Bence-Jones protein with the metabolic pool of nitrogen (22).

SUMMARY

The rate of protein synthesis has been studied by use of isotopic glycine in a patient with multiple myeloma who had an abnormal serum globulin and excreted a Bence-Jones protein. The synthesis of the two abnormal proteins appeared to be an independent process. The Bence-Jones protein is rapidly excreted and is apparently derived directly from the nitrogen pool rather than via any plasma or tissue protein precursor.

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* From the rate of decline of C$^{14}$ concentration in the glycine of the Bence-Jones protein and the myeloma globulin and from the available data on plasma volume, hematocrit, plasma protein distribution, and urinary protein excretion, we have been able to make some calculations about protein synthesis in this subject. We estimate that G. M. synthesized and degraded 8 to 10 gm. of the myeloma globulin daily and that the amount of Bence-Jones protein present in the body at any one time was only about 2 gm. 10 times as much of the labeled administered glycine appeared in the myeloma globulin on the 1st day as was subsequently excreted in the urinary protein.
PROTEINS IN MULTIPLE MYELOMA:
III. ORIGIN OF BENCE-JONES PROTEIN
Frank W. Putnam and Sarah Hardy


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