THE FORMATION OF MYELOMA PROTEIN BY A MOUSE PLASMA CELL TUMOR

BY DANIEL NATHANS,* M.D., JOHN L. FAHEY, M.D., AND
MICHAEL POTTER, M.D.

(*From the Metabolism Service and the Leukemia Studies Section, National Cancer
Institute, National Institutes of Health, Bethesda*)

One of the striking features of multiple myeloma is the frequent occurrence of a homogeneous plasma globulin in high concentration. Although this aspect of the disease has received a great deal of attention, many fundamental questions about the plasma protein abnormalities remain unanswered. For example, it has not yet been established whether the myeloma protein in plasma is produced by the tumor cells themselves or is a response of other tissues to the effect of the tumor. None of the evidence to date clearly differentiates between these two possibilities (1–7).

With the report by Potter, Fahey, and Pilgrim (6) of the mouse plasma cell neoplasm X5563, which is associated with a high concentration of a homogeneous gamma globulin in the plasma and other pathological changes similar to multiple myeloma in man, an experimental approach to the problem of the origin of the myeloma protein became feasible. The present study was undertaken to ascertain by means of *in vivo* labelling whether cells of the tumor form the myeloma protein.

EXPERIMENTAL

Design of the Experiments.—In the event that myeloma protein is synthesized by the tumor cells, it can be assumed that, following the administration of a labelled amino acid, the myeloma protein in the tumor will be more highly labelled and attain its peak radioactivity earlier than that in the plasma; *i.e.*, that a precursor-product relationship will be demonstrated (8).

Uniformly labelled L-lysine-C^{14}[1] in a dose of 1 to 2 microcuries (0.078 micromoles/microcurie) was injected intravenously *via* the tail vein into mice bearing the plasma cell tumor X5563 and the animals were sacrificed at intervals varying from 10 minutes to 16 hours. The plasma and tumor myeloma proteins were isolated from each animal, and the specific activity of the lysine in each sample was measured.

The mice used in these experiments were from C3Hf/Lw, C3Hf/He colonies, maintained

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* Present address: Presbyterian Hospital, New York.
[1] The L-lysine-C^{14} was obtained from Nuclear Chicago, Chicago.

at the National Cancer Institute. The mice were fed Derwood pellets and water *ad libitum* and were housed in plastic cages with 6 to 8 mice per cage. The plasma cell neoplasm X5563 was transferred by the trochar method into the right axillary subcutaneous space, which resulted in a local, well vascularized reddish-purple tumor consisting of discrete masses of typical plasma cells (9). Studies were conducted from about 30 to 60 days after tumor transplantation, when the tumor had attained a size which was distinctly palpable.

Isolation of Plasma and Tumor Myeloma Protein.—Blood obtained from heparinized animals by cardiac puncture was centrifuged and the plasma separated. Total plasma protein was determined by the biuret method, and the percentage of myeloma protein by paper electrophoresis, as described elsewhere (10).

The tumor was excised from the subcutaneous tissue space immediately after the blood specimen was obtained, and was weighed and cooled on ice. It was then quickly minced with scissors and washed five times with cold isotonic saline. (In some instances the tumors were frozen promptly after removal and processed at a later date.) The minced tumor was then frozen rapidly. Approximately 2 to 3 minutes elapsed between excision of the tumor and the freezing of the minced tissue. The frozen mince was homogenized with approximately twice its weight of cold isotonic saline in a Potter-Elvehjem homogenizer for 8 minutes at 4°C., using a power-driven pestle. Smears of the homogenate were examined microscopically and were found to be virtually free of intact cells. The homogenate was then frozen and thawed, resulting in flocculation of considerable cellular material which was removed by centrifugation at 3500 *g* for 30 minutes at 4°C. A clear supernatant solution containing the myeloma protein was retained.

The myeloma proteins from the plasma and the tumor extract were isolated by column chromatography using the anion-exchange adsorbent, diethylaminoethyl cellulose (DEAE-cellulose) (11), employing a modification of procedures described in detail elsewhere (10, 12). The plasma or tissue extracts to be chromatographed were dialyzed against 0.01 M sodium phosphate buffer of pH8 for 12 to 15 hours at 4°C. The resulting solution was centrifuged to remove the small amount of precipitate formed at this low ionic strength. The supernatant protein solution (equivalent to 0.2 to 0.8 ml. of plasma or 1 to 3 gm. of tumor) was applied to a DEAE-cellulose column (9 X 300 mm.) and the proteins eluted at 4°C. with phosphate buffer of pH 8 and continuously increasing ionic strength. The starting buffer of 0.01 M sodium phosphate was contained in a 100 ml. spherical chamber and a solution of 0.1 M sodium phosphate buffer—0.2 M sodium chloride was added continuously from a 50 ml. Erlenmeyer flask reservoir. The protein concentration of the effluent fractions was estimated by optical density at 280 mμ using a Beckman model DU spectrophotometer.

Typical column chromatograms of plasma and tumor extract from X5563-bearing mice are illustrated in Fig. 1. The largest peak in each pattern was found on paper electrophoresis to contain only myeloma protein (homogeneous gamma globulin peak). In three separate determinations the yield of myeloma protein from tumor was 4.7, 5.0, and 5.4 mg. per gm. of tumor.

Specific Activity Measurements.—The specific activities of the purified proteins were determined by measuring the specific activity of lysine isolated from each sample as its ϵ-dinitrophenyl derivative. The protein was precipitated from the column eluate solution with an equal volume of 10 per cent trichloracetic acid. The precipitated protein was reacted with fluorodinitrobenzene using the method described by Porter (13) and hydrolyzed for 16 hours with 5.7 N HCl at 110° C. in a sealed tube. The hydrolysate was taken to dryness in a vacuum desiccator and, after initial extraction of the residue with ether to remove dinitrophenol and N terminal amino acids, the remaining DNP amino acids were taken up in acetone acidified with HCl. ϵ-DNP lysine was isolated from the acetone solution by the one-dimensional paper chromatography system of Blackburn and Lowther (14), and eluted from the paper with an acetone-water mixture. The radioactivity of an aliquot (0.1 to 0.5

micromoles) of the ε-DNP lysine solution was measured as an "infinitely thin" film on a 4 cm. diameter glass disc in a windowless gas flow counter.[2] The probable error of counting
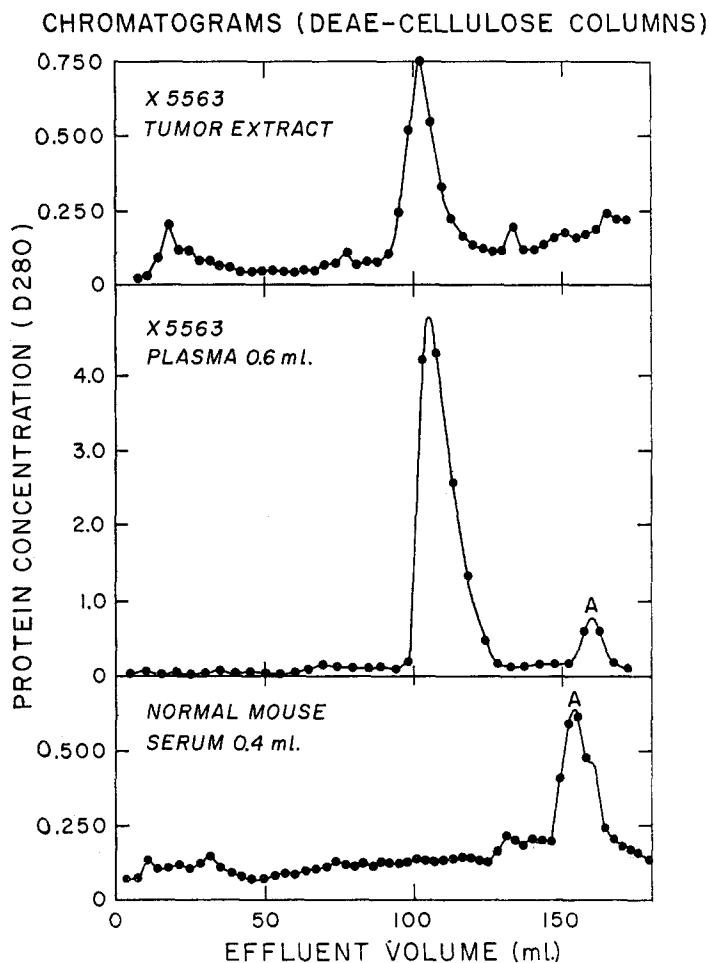
CHROMATOGRAMS (DEAE-CELLULOSE COLUMNS)

FIG. 1. Column chromatograms of X5563 tumor extract, X5563 plasma, and normal mouse serum. The same procedure was employed in each chromatogram and is described in the Experimental section. The label A indicates the site of elution of albumin in the normal serum and X5563 plasma chromatograms.

was generally less than 2 per cent for each sample. The concentration of ε-DNP lysine was determined on another aliquot by measuring the absorption at 360 mμ and utilizing published data for the extinction coefficient (15).

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[2] We are indebted to Dr. James C. Reid, Laboratory of Physiology, National Cancer Institute, for the use of radioactive counting facilities.

For measurement of the specific activity of free lysine in a tumor, a portion of whole tumor homogenate was added without delay to an equal volume of 10 per cent trichloracetic acid and lysine isolated from the supernatant solution as its di-DNP derivative using the two dimensional paper chromatography system of Levy (15). The specific activity of the eluted di-DNP lysine was determined in the same manner as described for the ϵ-DNP derivative, except that absorption at 355 mμ was used to calculate the concentration.

The possibility that adsorbed lysine might contribute to the measured specific activity of the tumor myeloma protein, particularly when the activity of free lysine in the tumor was high, was investigated. When 0.2 microcuries of L-lysine-C^{14} was added to tumor homogenate and the homogenate handled in the manner described above, the ϵ-DNP lysine obtained from the isolated tumor myeloma protein had no radioactivity.

To calculate the actual specific activity (s.a.) of newly formed myeloma protein in the tumor it was necessary to determine the extent to which the tumor myeloma protein preparation contained circulating myeloma protein. This was done in the following manner. Serum containing labelled myeloma protein was obtained from an animal with the X5563 tumor 4 hours after intravenous injection of L-lysine-C^{14}. 0.8 milliliter of this serum was injected intravenously into another X5563 tumor-bearing mouse and the tumor and plasma myeloma proteins were isolated from the recipient 21 hours later. In the recipient animal the specific activity of the tumor myeloma protein was approximately two-thirds (0.68) that of the plasma myeloma protein. Therefore, the measured specific activities of the tumor myeloma protein preparations were corrected as follows: Actual s.a. = (3 $\times$ measured s.a.) − (2 $\times$ plasma myeloma protein s.a. in the same animal). This correction can only be considered approximate, but better representative of the probable difference between the tumor and plasma myeloma protein than the uncorrected data. Such a correction in no way alters the qualitative nature of the results.

Finally, all specific activity values (free lysine, tumor protein, and plasma protein) were corrected to a constant dose of C^{14}-lysine per unit body weight (1.0 μc./30 gm.).

Comparison of the Specific Activities of the Myeloma Protein in Different Animals.—The specific activities of the tumor and plasma myeloma protein were determined at the same time after C^{14}-lysine injection in each animal and hence are directly comparable. However, in order to compare the specific activities of the myeloma proteins in *different* animals without further correction factors, certain assumptions must be made: first, that a constant dose of tracer lysine per unit body weight results in the same specific activity of free lysine in the tumors; second, that the turnover time of the plasma myeloma protein is the same in each animal; third, that the plasma myeloma protein pool size per gram of tumor is approximately constant. The first two assumptions were accepted as being approximately correct. The third assumption is substantiated by the data shown in Fig. 2, which indicate that the pool size of the plasma myeloma protein is approximately proportional to the tumor weight. The fact that the line does not pass through the origin may be due to the presence of necrotic tissue in the larger tumors and to low estimates of extracellular fluid volumes in animals with larger tumors.

Amino Acid Composition of the Myeloma Protein.—The amino acid composition of the serum myeloma protein prepared by column chromatography was determined by two-dimensional paper chromatography of the dinitrophenyl amino acids according to the method of Levy (15). Hydrolysis was carried out with glass-distilled 5.7 N HCl (5 ml. HCl solution per 3.5 mg. of protein) in sealed tubes at 110°C. for 19, 36, and 72 hours. Each hydrolysate was treated with fluorodinitrobenzene and chromatograms of the resulting DNP amino acids were run in triplicate. Tryptophan and tyrosine contents were determined separately by the spectrophotometric method of Goodwin and Morton (16) using the intact protein.

RESULTS

Properties of the Plasma and Tumor Myeloma Proteins.—The tumor and plasma myeloma proteins prepared by column chromatography were shown to be homogeneous and to have identical properties. Both proteins appeared homogeneous on column chromatography and were eluted at the same effluent
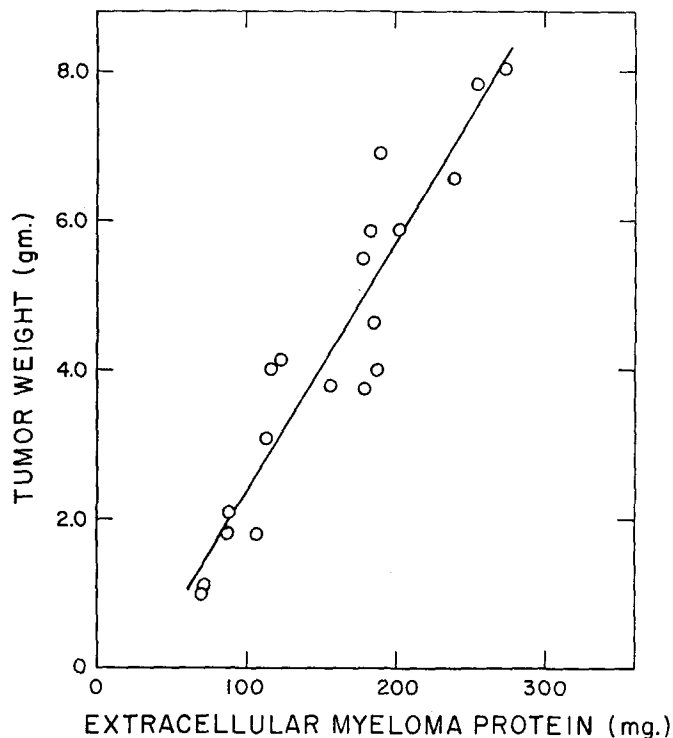

FIG. 2. Relationship of tumor weight to extracellular myeloma protein pool (plasma con centration × estimated volume of distribution of 2 ml./25 gm. total body weight.) The line was determined by the method of least squares.

volume (Fig. 1). Both preparations were homogeneous by paper electrophoresis and had the same electrophoretic mobility (Fig. 3). Ultracentrifugal analyses indicated a homogeneous protein in each case with essentially identical S20 values, 6.4 for the plasma protein and 6.6 for the tumor protein (Fig. 3).[3]

The amino acid composition of the myeloma protein, determined on the serum protein isolated by column chromatography, is presented in Table I.

[3] Ultracentrifugal analyses were carried out by Mr. F. J. Gutter of the Laboratory of Biochemistry, National Cancer Institute.

PAPER   ANALYTICAL
ELECTROPHORESIS   ULTRACENTRIFUGATION

PLASMA
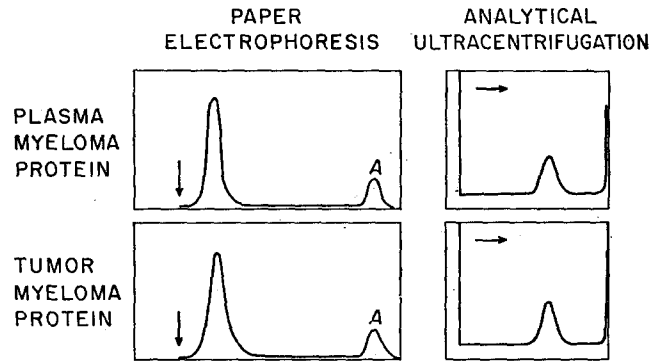MYELOMA
PROTEIN

TUMOR
MYELOMA
PROTEIN

FIG. 3. Electrophoretic and ultracentrifugal analysis of the tumor myeloma protein and the plasma myeloma protein preparations obtained by column chromatography. (On each of the paper electrophoretic densitometer tracings is a peak (A) representing crystalline bovine albumin that was applied prior to the myeloma protein sample in order to detect more readily any minor components present (10). The arrows indicate the point of application. Ultracentrifugation was carried out at 59,780 R.P.M. and these photographs were obtained at 75 minutes. Protein concentrations were approximately 0.6 gm. per cent in the ultracentrifugation samples.)

TABLE I

Amino Acid Composition of the Serum Myeloma Protein Isolated by Column Chromatography

Amino Acid	Optical densities of DNP-amino acids*			Mole fraction‡	Gm. amino acid§ 100 gm. protein
	19 hrs.	36 hrs.	72 hrs.		
Alanine	0.305	0.293	0.356	0.0407	3.34
Arginine	0.257	0.268	0.175	0.0298	4.74
Aspartic + glutamic acid	1.24	1.27	1.48	0.153	19.6
Leucine + isoleucine	0.965	1.07	1.05	0.124	15.0
Cystine	0.095	0.011	0.022	0.0056	1.23
Histidine	0.266	0.236	0.186	0.0452	6.41
Glycine	0.566	0.553	0.607	0.0655	4.50
Lysine	0.723	0.768		0.0514	6.85
Methionine	0.031	0.052	0.021	0.0066	0.91
Phenylalanine	0.332	0.346	0.324	0.0374	5.66
Proline	0.906	0.936	0.870	0.0913	9.63
Serine	0.938	0.969	0.232	0.0984	9.45
Threonine	0.832	0.882	0.485	0.0944	10.3
Tryptophan					3.30‖
Tyrosine					4.73‖
Valine	0.878	0.904	0.963	0.0998	10.6

* Each value is the average of a triplicate determination.
‡ Calculated from the maximal O.D. using Levy's factors (15).
§ Calculated from the mole fractions.
‖ Determined spectrophotometrically on the intact protein (16).

Formation of Myeloma Protein.—Time curves for the specific activities of tumor and plasma myeloma proteins following intravenous C^{14}-lysine injection are illustrated in Fig. 4. The marked differences in specific activity of the tumor and plasma proteins in the same animal clearly demonstrate that the two proteins are in different pools. These curves meet the criteria for a precursor-product relationship as derived by Zilversmit, Entenman, and Fishler (8); that is, the specific activity of the tumor myeloma protein (precursor) exceeds that of the plasma myeloma protein (product) in the early part of the curve,
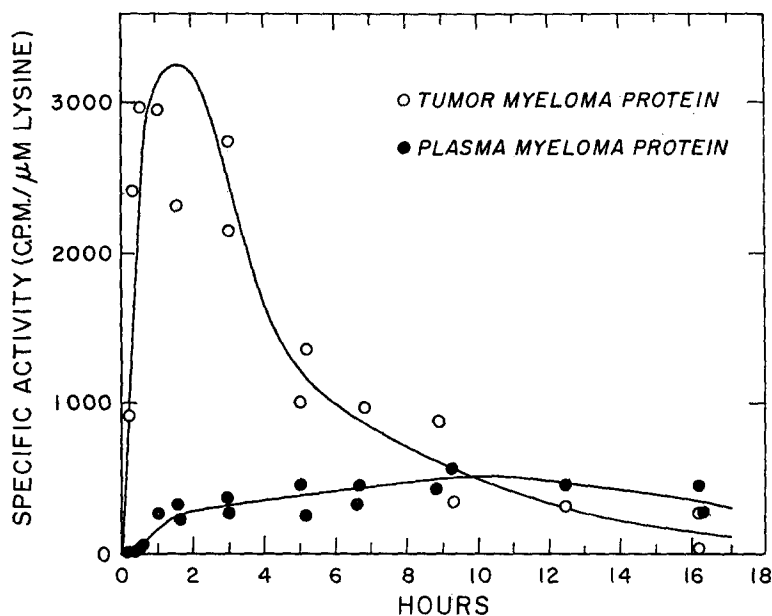

FIG. 4. Specific activity—time curves for the tumor and the plasma myeloma proteins following intravenous injection of L-lysine-C^{14} at 0 time.

is equal to the specific activity of the plasma protein at the point of maximum of the latter, and is less than the specific activity of the plasma protein beyond this. These results indicate that myeloma protein is synthesized in the tumor.

Transit Time of the Myeloma Protein.—The transit time is the interval between intravenous injection of a labelled amino acid and the appearance of labelled plasma protein in the circulation (17). In Fig. 5 the specific activity-time curves of the free lysine in the tumor, the tumor myeloma protein, and the plasma myeloma protein are shown during the first 90 minutes after C^{14}-lysine injection. The transit time of the plasma myeloma protein is approximately 30 minutes. However, there is little or no delay in the appearance of isotope in the free lysine of the tumor and in the tumor myeloma protein.

Hence most of the transit time would seem to represent processes occurring after actual synthesis of the myeloma protein.
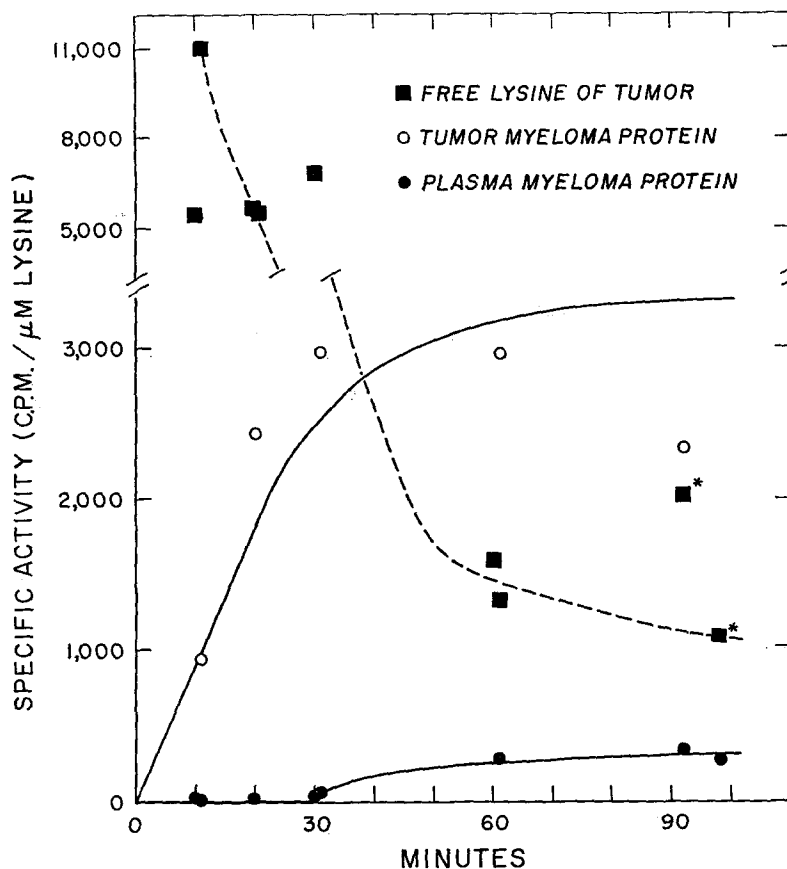

Fig. 5. Specific activity—time curves for free lysine of the tumor, tumor myeloma protein, and plasma myeloma protein during the first 90 minutes following intravenous injection of L-lysine-C^{14}.

The asterisks indicate values with a probable counting error of 15 per cent.

DISCUSSION

Previous data bearing on the origin of the serum myeloma protein are generally consistent with the synthesis of this protein by the tumor cells. One approach has correlated the decline of serum myeloma protein with destruction or extirpation of the neoplasm (2, 3, 7). Although this has been accepted as evidence for myeloma protein synthesis in the tumor, it could equally well be accounted for by the removal of a stimulus for synthesis elsewhere in the body.

Evidence of a second type is based on the demonstration of a protein in extracts of plasma cell tumors (or marrow of patients with multiple myeloma) with characteristics of the serum myeloma globulin (1, 3, 4, 6). However, too few properties (*i.e.* electrophoretic mobility or sedimentation constant) of the protein preparations have been compared to establish identity. Furthermore, the "tumor myeloma protein" may actually have been largely plasma or interstitial fluid protein incompletely washed from the tumor tissue.

In the present study, the protein obtained from the X5563 tumor was identical with the plasma myeloma protein on electrophoretic, chromatographic, and ultracentrifugal analyses. That the "tumor myeloma protein" is not entirely residual plasma protein is clearly shown by the marked difference in specific activities of the two proteins in the same animal 2 hours after C^{14}-lysine injection, indicating that they are in different protein pools.

The specific activity–time curves, which have the characteristic precursor-product relationship, demonstrate that myeloma protein is synthesized in the cells of the plasma cell tumor. Although the data do not exclude the possibility that some of the myeloma protein may be synthesized in tissues other than the tumor, it seems unlikely that this specific protein would be formed both by the neoplastic cells and by other tissues of the host. The correlation between tumor weight and the amount of plasma myeloma protein, and a rough approximation of the rate of synthesis of myeloma protein by the tumor, *viz.* 14 mg. per gm. tumor per day,[4] suggest that the plasma cell tumor is the major site, or perhaps the only site, of myeloma protein formation.

The finding of a transit time of approximately 30 minutes for the myeloma protein of the plasma is in agreement with the values obtained for plasma proteins in several mammalian species under various experimental conditions (17–20). What accounts for the transit time is not clear. It includes the time for entrance of injected labelled amino acid into the cell, intracellular synthesis of the protein, and release of protein into the general circulation. The short delay in the appearance of labelled tumor myeloma protein found in this study would indicate that the time required for entrance of the labelled amino acid into the cell and its appearance in new protein cannot account for more than a fraction of the transit time of myeloma protein. The major portion of the transit time seems to represent processes concerned with release of the newly formed protein from the cell and its entrance into the general circulation, rather than protein synthesis *per se.*

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[4] Calculated from the estimated $t_{1/2}$ of the tumor myeloma protein (2 hours) and the concentration of tumor myeloma protein (1.7 mg. per gm. tumor) as follows:—

$$\frac{24 \text{ hours}}{2 \text{ hours}/0.69} \times 1.7 \text{ mg.}$$

SUMMARY

The origin of the myeloma protein found in mice bearing the plasma cell tumor X5563 has been investigated. Specific activity–time curves of the myeloma proteins isolated from the tumor and from the plasma of these animals were compared following intravenous injection of L-lysine-C^{14}. The results indicate that myeloma protein is synthesized in the plasma cell tumor.

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