A Study on Globulin Formation by Plasma-Cell Neoplasm (5563) Transplantable in Mice

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Antibody and γ -globulin are formed by a number of tissues with highly heterogeneous cell populations such as bone marrow, lymph glands and spleen. Only a small part of the cells in these tissues are plasma-type cells in which antibody protein and at least some of the γ -globulin normally found in serum originate. This makes a study of globulin synthesis difficult. Recently, Dr M. Potter and his collaborators at the National Cancer Institute, Bethesda, have established several lines of plasma-cell tumours transplantable in inbred strains of mice. Each plasma-cell neoplasm is associated with different but characteristic globulins in high concentrations in the serum (Potter & Fahey, 1960). On electrophoresis of the serum at pH 8.6 these globulins migrate in the region of serum γ - and β -globulin. The condition in mice shows great similarity to the human malignant disease, multiple myeloma. The tumour cells are basophilic cells with vacuoles and acentric nuclei characteristic of plasma cells normally found in lymphoid tissue (Dunn, Potter, Fahey & Merwin, 1960). Electron micrographs of thin sections show that the cell cytoplasm has a highly organized endoplasmic reticulum (A. J. Dalton, unpublished work) similar to that found in hepatic or pancreatic cells and normal plasma cells. This organization is characteristic of cells active in protein formation and secretion.

The subcutaneous mouse tumours provide tissue to study the synthesis of globulin *in vitro* and make it possible to follow globulin formation by plasma cells over many transplant generations. One of the plasma-cell neoplasms (5563) has been studied in detail, since the globulin in the serum of animals bearing the 5563 tumour was found to be very similar to normal mouse γ -globulin in its immunological properties. It is referred to below as γ -globulin or γ -myeloma protein. Dr B. Balfour at this Institute stained sections of the 5563 tumour with fluorescein-labelled rabbit antibody against purified myeloma protein. Almost all cells showed a highly fluorescent cytoplasm and therefore contained myeloma protein. The tumour tissue thus provides us with a fairly homogeneous population of plasma cells. In the work described in this

paper the protein products were analysed by starch-gel electrophoresis and were found to be heterogeneous and to consist of at least five components. These stayed constant over many transplant generations.

The origin of serum-myeloma proteins has not been certain. Nathans, Fahey & Potter (1958) have studied formation of myeloma protein *in vivo* by injecting radioactive amino acids into mice carrying the 5563 neoplasm. The authors found that the myeloma protein extracted from the tumour had a higher specific radioactivity than the myeloma protein in the serum showing a precursor-product relationship. In the present study it could be shown that slices of tumour tissue form the myeloma protein *in vitro* at a considerable rate. The effect of various amino acid and nucleic acid analogues on synthesis of the globulins and general tissue protein *in vitro* was investigated. Globulin synthesis by subcellular particles was also investigated; although it was possible to obtain a tumour-microsome fraction which incorporated radioactive amino acid into insoluble protein, incorporation into specific globulin could not be demonstrated.

MATERIALS AND METHODS

^{14}C -Labelled amino acids. Biosynthetic [^{14}C]glycine, L-[^{14}C]valine, L-[^{14}C]leucine and algal [^{14}C]protein were obtained from The Radiochemical Centre, Amersham, Bucks., with specific radioactivities of 40, 100, 100 and 250 $\mu\text{C}/\text{mg}$. respectively. The algal protein was hydrolysed overnight at 105° in 6N-HCl, freed from HCl by evaporation and neutralized; the [^{14}C]amino acids were purified by absorption on a column of Zeo-Karb 225 and subsequent elution with aq. 2M-NH₃ soln. DL- β -p-Fluorophenyl[β - ^{14}C]alanine (2.35 $\mu\text{C}/\mu\text{mole}$) was obtained from Dr M. H. Richmond, at this Institute.

Nucleic acid analogues. These were mostly obtained from the California Corporation for Biochemical Research. A few substances which were personal gifts will be indicated in the text.

Transplantation of tumours into mice. Breeding pairs of the C₃H/He strain of mice and animals bearing the 5563 plasma-cell neoplasm were sent to us through the kindness of Dr M. Potter, National Cancer Institute, Bethesda. The tumour was transplanted into the F₁ generation by implanting three or four pieces of tissue (about 1 mm. in

size) subcutaneously with a trocar. The tumour developed to a size of 2–4 g. in 6–8 weeks.

Chromatography of myeloma protein (5563 neoplasm). The myeloma protein was purified and fractionated by chromatography on diethylaminoethyl (DEAE)-cellulose (Peterson & Sober, 1956) with conditions of gradient elution and packing described by Fahey & Horbett (1959). For purification of myeloma protein, 1 g. of DEAE-cellulose was used/ml. of tumour-bearing mouse serum dialysed against 0.01 M- K_2HPO_4 - KH_2PO_4 buffer, pH 6.5. The starting buffer (90 ml.) was put into a cylindrical vessel which was connected by means of a siphon to a conical flask (nominally 50 ml.) containing 55 ml. of more concentrated buffer, 0.2 M- K_2HPO_4 - KH_2PO_4 , pH 6.5. Mixing in the cylindrical vessel was done with a magnetic stirrer. Fractions (3 ml.) were collected and the protein eluted in the fractions was followed by measuring its extinction at 280 μ .

The fractions were concentrated by ultrafiltration (Sober, Gutter, Wyckoff & Peterson, 1956) and characterized by electrophoretic and immunological analyses.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1955) with a discontinuous buffer system. Glycine-NaOH buffer, pH 8.9 (0.05 M), was used on the gel. Electrophoresis was continued for 6 hr. at 12 ma for a block of starch gel 25 cm. $\times$ 5 cm. $\times$ 0.5 cm.

Production of antisera for specific precipitation of myeloma globulin. Rabbits were immunized with 15–20 mg. of 5563 myeloma protein purified by chromatography on anion-exchange cellulose (see Fig. 2). Two injections were given intramuscularly with Freund's adjuvant (Freund & McDermott, 1942) 1 week apart and after 5–6 weeks six intravenous injections of alum-precipitated myeloma protein were administered over 2 weeks. The animals were bled 1 week later.

Incubation of tumour tissue. The tumour was excised after anaesthetizing the mouse with ether and bleeding it by heart puncture. The tumour tissue was rinsed in Hanks's (1948) balanced salt solution and minced with scissors. Known weights of tissue (110–250 mg. wet wt.) were incubated in conical flasks (previously treated with a silicone) in 4 ml. of medium with gentle shaking. The medium was made up of Hanks's solution containing 100 units of streptomycin and penicillin/ml., acid-hydrolysed rabbit globin (5 mg. of N/100 ml.), DL-tryptophan (4 mg./100 ml.) and 0.1 g. of gelatin/100 ml. [^{14}C]Amino acids (1–2 μ C) were added to each flask; the flasks were gassed with CO_2/O_2 (5:95) and incubated for various lengths of time. A separate flask was used for each time point.

Isolation of myeloma protein and general tissue protein. After incubation several milligrams of non-radioactive amino acid were added to each flask to minimize adsorption of [^{14}C]amino acids on proteins. The samples were frozen and thawed, homogenized in a Potter-type homogenizer for 1 min. and the sediment was removed by centrifuging for 10 min. at 12 000 g at 3°.

The myeloma protein was isolated from the supernatant by specific precipitation with antiserum prepared by immunizing rabbits with purified myeloma protein (as described above). Since it was important to precipitate the myeloma protein with antibody at equivalence point, the globulin content of tumour extract had to be titrated in a control sample (0.1 g. of tumour/ml. of medium extracted in the same way as the incubated tissue). Measured samples of tumour extract were set up in a series of tubes

and various amounts of antiserum were added to determine the equivalence point for the antibody-antigen precipitation. (This is the point where all of the antigen is precipitated by the antibody with neither antibody nor antigen present in excess in the supernatant.) Tumour tissue was found to contain 4–5 mg. of myeloma protein/g. wet wt., and the antibody-antigen ratio at the equivalence zone was around 7:1. On this basis the myeloma globulin in the incubation medium was precipitated with a measured sample of the supernatant to yield 4–5 mg. of antibody-antigen precipitate. Supernatant equivalent to about 100 mg. of tumour tissue was generally used. The antibody-antigen precipitates were washed twice with 0.9% NaCl and then successively with hot and cold ethanol and dried with ether.

The tissue debris which had been sedimented by spinning for 10 min. at 12 000 g was precipitated with trichloroacetic acid at a final concentration of 5% (w/v). The precipitate was washed successively with boiling 5% (w/v) trichloroacetic acid, with hot and cold ethanol and dried with ether.

Preparation of microsomal particles. Mice were killed by a blow on the head and drained of blood. The tumour tissue was excised quickly, chilled in 0.39 M-sucrose, minced and pushed through stainless-steel wire netting (60 mesh). An equal weight of buffered sucrose (0.35 M-sucrose, 35 mM- $KHCO_3$, 8 mM- $MgCl_2$, 25 mM-2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer, pH 7.8, 25 mM-KCl) was added and the cells were further dispersed by homogenizing in a Potter-Elvehjem-type homogenizer with a loosely fitting Teflon pestle at 1000 rev./min. for 20 sec. Since it is difficult to rupture the tumour cells efficiently by homogenizing they were lysed with 2.5 vol. of 0.01 M- $MgCl_2$ for 2 min. and then the sucrose concentration was adjusted to 0.3 M with fivefold-concentrated buffered sucrose medium. The suspension was spun for 15 min. at 8000 g to remove all cells, nuclei and mitochondria and the supernatant represented 'microsomal supernatant' used for the incubation. All manipulations were carried out in the cold or in an ice bath at -3°.

Incubation of microsomes. 'Microsomal supernatant' equivalent to 1 g. of tissue was incubated with [^{14}C]amino acids, 10 μ moles of potassium phosphoenolpyruvate (Boehringer und Söhne, Mannheim, Germany), 1 μ mole of adenosine triphosphate (ATP, Sigma Chemical Co.) and 20–40 μ g. of phosphopyruvate kinase (Boehringer und Söhne). Incubation for 10, 20 and 40 min. was carried out at 37° in an atmosphere of CO_2/N_2 (5:95). After incubation 10 mg. of non-radioactive amino acid was added, the microsomes were spun down in a Spinco model L ultracentrifuge for 50 min. at 100 000 g, the pellet was washed once with sucrose and proteins of the microsomes and the supernatant were precipitated by addition of trichloroacetic acid to a concentration of 5% (w/v) and washed and dried for counting as described by Simkin & Work (1957).

Determination of radioactivity. The finely dried protein powder was plated at infinite thickness on 0.3 cm.² Perspex disks. The radioactivity was determined on an automatic recording Geiger counter with a thin end window to an accuracy of at least $\pm 5\%$. Counts were converted into counts/min./cm.² and corrected to infinite thickness where necessary. A ^{14}C standard 1 cm.² disk (10⁻³ μ C/mg.) gave 850 counts/min. at infinite thickness in this counter.

RESULTS

Globulins produced by 5563 neoplasm

Potter, Fahey & Pilgrim (1957) have shown that animals bearing the 5563 plasma-cell tumour had high levels of a γ -globulin component in the serum which on paper electrophoresis migrated in the region of fast serum γ -globulins. The concentration of the serum globulin was roughly proportional to the weight of tumour mass (Nathans *et al.* 1958) and exceeded the concentration of serum albumin when the tumour was above 2–3 g. in size.

Prolonged free electrophoresis of the tumour serum in veronal buffer (0.1 M), pH 8.6, showed an asymmetric globulin boundary which suggested heterogeneity of the myeloma globulin. The serum was analysed by starch-gel electrophoresis (Smithies, 1955) and the γ -myeloma protein separated into four to five definite bands; the concentration of the

bands decreasing as the mobility of the bands increased. Fig. 1 illustrates starch-gel electrophoresis of normal mouse serum (C_3H/He strain) compared with results for serum from a mouse bearing the 5563 tumour (diluted fourfold to obtain best separation of the globulin components). It can be seen that the globulins in the tumour serum form four definite bands (and probably a fifth band present in small amounts). The γ -myeloma protein may be purified from serum by chromatography on DEAE anion-exchange cellulose (Peterson & Sober, 1956) as described in the Materials and Methods section. The elution pattern is shown in Fig. 2. The components can be partially separated by chromatography on DEAE-cellulose by using a slower gradient (B. A. Askonas & J. L. Fahey, unpublished work). The γ -myeloma globulin then spreads considerably and various fractions can be obtained which on starch gel showed different

Fig. 1. Characteristics of γ -myeloma protein in serum of mice bearing 5563 plasma-cell neoplasm on starch-gel electrophoresis. 6, Normal mouse serum (C_3H/He). 5, Serum from mouse bearing 5563 plasma-cell neoplasm, diluted fourfold to obtain optimum separation of globulin molecules. 1–4, γ -Myeloma-protein fractions purified by chromatography on DEAE anion-exchange cellulose (Peterson & Sober, 1956) by a slow gradient column (B. A. Askonas & J. L. Fahey, unpublished work). Fraction 1 is eluted from the column first, fraction 4 last. Starch-gel electrophoresis was carried out by the method of Smithies (1955), with a discontinuous buffer system and 0.05 M-glycine-NaOH buffer, pH 8.9, in the gel.

mobilities (Fig. 1). It was almost impossible to obtain in this way fractions containing only one component.

The pattern on starch gel is reminiscent of the banding seen with haptoglobin polymers. The molecular weight of two extreme fractions of myeloma proteins separated by chromatography was therefore kindly determined by Dr P. Charlwood [fractions 1 and 4 (see Fig. 1)]. The two fractions had sedimentation constants of 6.5 and 6.6 respectively and thus no difference in molecular weight could be detected between the components. This S_{20} is indicative of a molecular weight of approximately 160 000 and is the same as that of normal mouse γ -globulin.

No difference in immunological reactivity could be detected between the components. The fractions were tested by immune diffusion in agar gel with antiserum against (1) pure normal mouse γ -globulin and (2) 5563 myeloma globulin, and gave reactions of identity. This suggests that the globulin components have very similar structure and antigenic groupings. At present it is not

known what the difference in electric charge is due to.

The serum globulin pattern was followed over many transplant generations (15–20) throughout a period of 2 years. Fig. 3 shows a comparison of sera collected from tumour-bearing animals over a period of 2 years. It can be seen that the globulin components have the same mobility in all the sera. Since it is difficult always to get identical loading of the starch gel it is hard to judge whether minor quantitative changes have occurred or not. The chromatographic behaviour of the γ -globulin components on DEAE-cellulose has also remained unaltered over the years.

Formation of myeloma protein by plasma-cell tumour in vitro

The 5563 plasma-cell tumour was sliced and incubated in the presence of [14 C]amino acids as described in the Materials and Methods section. Myeloma protein was isolated by specific precipitation with antiserum. The slices actively incorporated radioactive amino acid in myeloma protein and general tissue protein insoluble in 0.9% sodium chloride (see Fig. 4 for a typical time curve). By knowing the specific radioactivity and the amount of the proteins isolated the amounts of radioactivity incorporated into tissue protein and myeloma protein may be compared. Table 1 shows several typical experiments. It can be seen that 34–52% of the total protein synthesis by the cell results in formation of the specific γ -myeloma proteins which are secreted by the cell while the cell population is dividing rapidly. Similar ratios between antibody and tissue-protein formation are found in lymph glands and spleen from hyperimmunized animals. Since these tissues contain a lot of cells forming cellular proteins other than the antibody-forming plasma cells, it suggests that the

Fig. 2. Purification of γ -myeloma protein in serum of mice carrying 5563 plasma-cell neoplasm. Chromatography was carried out on a 4 g. column of DEAE ion-exchange cellulose (Peterson & Sober, 1956). Serum (3 ml.) from mice carrying the 5563 neoplasm was dialysed against the starting buffer, 0.01 M-potassium phosphate, pH 6.5. Gradient elution was carried out with 0.2 M-potassium phosphate buffer, pH 6.5, as described in the Materials and Methods section. Fractions (3 ml.) were collected and the protein elution was followed by measuring extinction of the fractions at 280 m μ . The elution pattern is presented in the diagram. Bracket (—) indicates the fractions pooled to prepare γ -myeloma protein.

Table 1. *Relative amounts of radioactive amino acids incorporated into general tissue protein and γ -globulin by tumour slices in vitro*

Expt.	Radioactivity (counts/min./mg.)		Ratio of total radioactivity incorporated into tissue protein and γ -globulin
	γ -Globulin- antibody ppt.	Tissue protein	
A	527	580	1.9
B	774	600	0.9
C	725	760	1.3
D	1530	1459	1.4

tumour cells are not quite as active in protein secretion as normal plasma cells.

An approximate figure for the amount of γ -globulin secreted by the tumour plasma cells in the system *in vitro* can be calculated. Since our counter registered 850 counts/min. for a ^{14}C standard of

$10^{-3} \mu\text{C}/\text{mg.}$, the amount of added radioisotope taken up can be calculated $\{\mu\text{C of } [^{14}\text{C}]\text{amino acid taken up} = 10^{-3}/850 \times \text{radioactivity of protein (counts/min./cm.}^2) \times \text{mg. of protein isolated}\}$. In a number of experiments with $[^{14}\text{C}]\text{glycine}$ and algal $[^{14}\text{C}]\text{protein hydrolysate}$ about 0.7–1 % of the

Fig. 3. Comparison by starch-gel electrophoresis of sera from 5563 tumour-bearing animals over a period of 2 years. 1, Normal mouse serum ($\text{C}_3\text{H}/\text{He}$), diluted fivefold. 2, Sera from mice bearing 5563 plasma-cell neoplasms (different transplantation generations). Date of killing animals: A, June 1960; B, January 1960, small tumour (1–2 g.); C, February 1959; D, August 1958. The γ -myeloma protein concentration in the sera was estimated by determining the total protein concentration with the biuret reagent and the percentage of myeloma protein by densitometry readings of stained paper-electrophoretic strips. The sera were diluted three- to seven-fold to give similar myeloma-protein concentrations for starch-gel electrophoresis. 2B, Animal had a small tumour and therefore serum was diluted only threefold; hence the normal serum components show up more strongly.

added isotope is incorporated into the γ -globulin in 5 hr. by 100 mg. of tissue. With [^{14}C]valine the values are as high as 1.7 %. This higher uptake may be explained by the fact that the 5563 myeloma protein contains twice as much valine as glycine (10.6 % of valine, 4.5 % of glycine; Nathans *et al.* 1958). In these experiments a mixture of amino acids of known composition was added to the medium so that the specific radioactivity of the [^{14}C]amino acid was known. With glycine, the medium contained $1\ \mu\text{C}$ of [^{14}C]glycine and a total of $120\ \mu\text{g}$. of glycine. If we assume that the specific radioactivity of free amino acid within the cells is half that in the medium on basis of work with other tissues (Askonas & Humphrey, 1958), it may be calculated that about 1.5–2.0 mg. of myeloma protein is produced by the plasma-cell tumour *in vitro*/g. wet wt./24 hr.

Effect of nucleic acid and amino acid analogues on protein synthesis by tumour tissue

Various analogues were added to the flasks at the beginning of the incubation and incorporation of [^{14}C]amino acid into myeloma protein and tissue protein was determined at various time intervals and compared with results in control flasks to which no analogue had been added. Fig. 4 shows results of a typical time curve obtained when tumour tissue is incubated in the presence of 3.0 mM-6-azauracilriboside [I am indebted to Professor F. Šorm (Prague) for a

sample of azauracilriboside]. There is very little effect for the first 2 hr.; after that the rate of synthesis of both γ -globulin and tissue protein falls off in the presence of 6-azauracilriboside; at 3 hr. there is about 30 % inhibition and at 6 hr. there is about 50 % inhibition of protein synthesis. Both the tissue-protein and myeloma-protein formation are equally affected, which suggests that protein synthesis of the cell generally is poisoned by ribonucleic acid analogue and continues only at a slow rate. 6-Azauracil was less effective (Table 2) but also caused some inhibition. Fig. 5 shows the effect of 8-azaguanine on the system. It also inhibits the formation of γ -myeloma protein and tissue protein, although it appears to take somewhat longer to have an effect. At 4 hr. there is only 15 % inhibition of protein synthesis, but thereafter the rate of synthesis falls off rapidly. Another ribonucleic acid analogue, 6-mercaptopurine, had barely any effect on protein synthesis during the time period tested (Table 2). Various amino acid analogues showed very little if any effect (Table 2). This may be due to the fact that they are incorporated into the γ -globulin. A small amount of *p*-fluoro-[^{14}C]phenylalanine could be shown to be incorporated into the proteins by the plasma-cell tumour. Incubation of tumour slices was carried out in the usual way, except that the medium contained a synthetic L-amino acid mixture free of phenylalanine. *p*-Fluoro-[^{14}C]phenylalanine ($1.3\ \mu\text{C}$) was added to the incubation

Table 2. *Effect of various nucleic acid and amino acid analogues on formation of myeloma protein and general tissue protein by tumour slices in vitro*

Control flasks at each time point were incubated in the usual medium (see Materials and Methods section). The analogues tested and [^{14}C]amino acids were added to the flasks at the beginning of the incubation period (at 0 hr.). N.D., Not done.

Addition	Concn. (mM)	Incubation time (hr.)	Inhibition of incorporation of [^{14}C]amino acid into protein (%)	
			Myeloma protein- antibody ppt.	Tissue protein
Trifluoroleucine	4.5	7.5	4	5
<i>p</i> -Fluorophenylalanine	2.7	6	13	N.D.
		8	20	N.D.
Thienylalanine	3	6	10	N.D.
6-Azauracilriboside	3.3	3	32	29
	3.3	6	50	50
	3	1	0	0
	3	2.5	15	0
	3	4	30	20
6-Azauracil	3	1	0	0
	3	2.5	17	0
	3	4	13	15
8-Azaguanine	3	4	15	15
	3	6.25	40	34
6-Mercaptopurine	3	6	10	N.D.
Bromouracil	3	6	10	10

Fig. 4

Fig. 4. Effect of 6-azauracilriboside on protein synthesis by tumour slices *in vitro*. ●, Radioactivity of γ -myeloma protein-antibody ppt. ○, Radioactivity of myeloma protein after addition of 3 mm-6-azauracilriboside to incubation mixture at zero time. ▲, Radioactivity of tissue protein insoluble in 0.9% NaCl soln. △, Tissue protein after addition of 3 mm-6-azauracilriboside at zero time. Sliced tumour tissue (100 mg.) was incubated in the presence of $1 \mu\text{C}$ of $[^{14}\text{C}]$ glycine. Myeloma protein (3.5 mg.) and tissue protein (4 mg.) were isolated as described in the Materials and Methods section.

Fig. 5

Fig. 5. Effect of 8-azaguanine on protein synthesis by tumour slices *in vitro*. ●, Radioactivity of γ -myeloma protein-antibody ppt. ○, Radioactivity of myeloma protein if incubation was carried out in the presence of 3 mm-8-azaguanine. ▲, Radioactivity of tissue protein insoluble in 0.9% NaCl soln. △, Tissue protein, when incubated in the presence of 3 mm-8-azaguanine. □, Myeloma protein; incubation was carried out in the presence of 3 mm-8-azaguanine, but 10 mm-guanine was added after incubation for 3 hr. Conditions of incubation and isolation of proteins as described in the Materials and Methods section. Sliced tumour (110 mg.) was incubated in the presence of $1 \mu\text{C}$ of $[^{14}\text{C}]$ valine.

flasks and the radioactivity of the γ -globulin-antibody precipitate was determined. A heterologous antibody-ovalbumin precipitate was first made to obtain values for non-specific coprecipitation. The heterologous precipitate had only 4-5 counts/min. After incubation for 6 hr. both the tissue protein (4 mg.) and the γ -globulin precipitate (4 mg.) had about 70 counts/min./mg. This is equivalent to about 0.04% uptake of p -fluoro-L- $[^{14}\text{C}]$ phenylalanine in 6 hr. by 100 mg. of tumour tissue into both γ -globulin and tissue protein.

Studies with microsomal fraction of tumour tissue

It is of considerable theoretical importance to find out which intracellular sites form γ -globulin and antibody and to determine its characteristics. It was attempted to study this problem with subcellular preparations of tumour tissue. Tumour tissue (5563) was chosen for this since it provides a fairly homogeneous population of plasma cells whereas tissues which usually form γ -globulin and antibody are composed of many cell types.

The microsomal particles were fairly active in incorporating $[^{14}\text{C}]$ valine or $[^{14}\text{C}]$ leucine or both into

trichloroacetic acid-precipitable protein (Table 3), the system described by Zamecnik & Keller (1954) being used as energy source. There was no incorporation in the absence of ATP and phosphoenolpyruvate, and phosphoenolpyruvatekinase was necessary for optimum incorporation. Incorporation did not continue beyond 15-20 min.; at this time 0.1% of the added isotope had been incorporated. This compares favourably with the liver system, but the microsomal particles appear to be more unstable than these obtained from liver by homogenization. It was not possible to spin down the microsomal pellet at 100 000 g and then recombine it with supernatant as described for liver by Campbell, Greengard & Kernot (1960). After ultracentrifuging, the microsomal pellet from the 5563 neoplasm did not incorporate any radioactivity into its proteins.

It was not possible to demonstrate formation of γ -globulin by the subcellular particles. After incubation, the microsomal pellet was extracted with 0.1 M-sodium carbonate buffer, pH 9.5, treated with ultrasonics and incubated for a few minutes with 100 μg . of ribonuclease. Carrier myeloma

Table 3. Incorporation of [^{14}C]amino acids into protein by the microsomal fraction of 5563 neoplasm

Microsomal supernatant was prepared as described in the Materials and Methods section. Each incubation flask contained microsomal supernatant (2.6 ml.) derived from 0.6 g. of tumour tissue (5563), and 1 μC of L-[^{14}C]valine and L-[^{14}C]leucine. The yield of washed and dried protein from the microsomal pellet and the supernatant was 6 and 15 mg. respectively. In 20 min. 0.1% of the isotope was taken up into the microsomal protein.

Tube no.	Addition	Incubation time (min.)	Radioactivity of protein (counts/min./mg.)	
			Microsomal	Supernatant
1	—	20	22	35
2	10 μmoles of phosphoenolpyruvate, 1 μmole of ATP, 20 $\mu\text{g.}$ of phosphoenolpyruvatekinase	20	376	35
3	10 μmoles of phosphoenolpyruvate, 1 μmole of ATP, 20 $\mu\text{g.}$ of phosphoenolpyruvatekinase	35	386	35

Table 4. Relative incorporation of [^{14}C]amino acids into various protein fractions of microsomes

Incubation: Microsomal supernatant (from 0.8 g. of 5563 neoplasm) was incubated for 20 min. at 37° in the presence of 1.5 μC of L-[^{14}C]valine. Control flasks had no further additions. Flasks with full medium also contained 10 μmoles of phosphoenolpyruvate, 2 μmoles of ATP and 30 $\mu\text{g.}$ of phosphoenolpyruvatekinase. The microsome pellet was spun down at 100 000 g for 60 min. after incubation.

Microsome extract: Carrier 5563-myeloma protein (500 $\mu\text{g.}$) was added for eventual precipitation with antiserum to a pool of four incubation flasks. The microsomal pellet was treated with 0.1M-sodium carbonate buffer, pH 9.5, and ultrasonic vibration (25 keyc., 500 w) and 100 $\mu\text{g.}$ of ribonuclease. Before precipitation of the γ -globulin with antiserum, nucleoprotein was removed by precipitation at pH 5.6 to avoid its coprecipitation.

	Radioactivity of protein (counts/min./mg.)	
	Control 48	Full medium 210
Total microsomal protein (trichloroacetic acid ppt.)		
Microsome extract		
Ovalbumin-antibody ppt.	0	1.2
γ -Globulin-antibody ppt.	0	2.6
0.1M-Sodium pyrophosphate, pH 8.3	4	52
Residual protein (trichloroacetic acid ppt.)	38	227

protein was then added (500 $\mu\text{g.}$) and the pH was adjusted to pH 5.6 to remove by precipitation the bulk of the nucleoprotein, which tends to coprecipitate in antibody-antigen reactions. This procedure removes only about 5% of the γ -myeloma protein, when tested in the presence of ^{131}I -labelled myeloma protein. After spinning for 10 min. at 10 000 g the supernatant is clear. A heterologous antibody precipitate was first made as a control (rabbit antiovalbumin and ovalbumin were used). The mouse myeloma protein was then precipitated with specific rabbit antiserum, but no radioactivity was

associated with this precipitate. A small amount of radioactivity could be extracted from the pellet with 0.1M-pyrophosphate buffer, pH 8.3, but most of the radioactivity was associated with protein insoluble in 0.9% sodium chloride and particles (Table 4). The failure to demonstrate γ -globulin synthesis by the microsome may be due to the instability of the particles prepared from the tumour tissue, so that only some stages of protein synthesis are being carried out, and it should not be concluded from such experiments that γ -globulin is not formed in the microsomal particles. It is hoped that more stable preparations will be achieved eventually.

'Bound' 5563 myeloma protein in the microsomal fraction

A small amount of myeloma protein is released when the microsomal fraction is treated with sodium carbonate buffer, pH 9.5, ultrasonics and ribonuclease or with 0.5% deoxycholate. The microsomal fraction from the tumour looks very similar to that of liver in electron micrographs. It consists of ribosome particles and some membranous vesicles which probably contain the protein to be secreted by the cell. Many substances such as alkali, tris, ethylenediaminetetra-acetic acid, deoxycholate, which break the structure of membranes and release nucleic acid from ribosomes, may release the myeloma protein and it is therefore not possible to say whether it is truly 'bound' by any linkage or simply enveloped by the membranous vesicles. On starch-gel electrophoresis the bound myeloma protein gives the same pattern as serum myeloma protein, which suggests that the different components are formed as such by the cell and that the heterogeneity is not due to enzymic action or formation of complexes in the extracellular fluid or in the circulation.

The amount of 'bound' myeloma protein in the microsomal fraction could be determined by titration with specific rabbit antiserum and was found

to be 80 μ g. of myeloma protein/g. wet wt. of tumour tissue, representing 1% of the total microsomal protein. Similar values were found for albumin released from microsomal fractions of normal liver by ultrasonics (Campbell *et al.* 1960). By deoxycholate treatment Peters (1957) obtained 170 μ g. of albumin from chick-liver microsomes. About 50 times this amount of myeloma protein is found free in the cytoplasm and extra-cellular fluid per g. wet wt. of tumour tissue.

DISCUSSION

The globulins secreted by the 5563 plasma-cell neoplasm were found to be heterogeneous and to consist of five definite protein bands on starch-gel electrophoresis. In human myeloma it has generally been thought that myeloma proteins migrating as γ -globulin molecules are homogeneous. Most myeloma sera have, however, been analysed by paper electrophoresis or free electrophoresis, where such small molecular differences would not be detected. One human myeloma protein migrating in the γ -globulin region has been reported to be heterogeneous on starch-gel electrophoresis, giving a pattern very similar to the 5563 protein (Owen, Got & Silberman, 1958). It is not clear what causes the difference in electric charge of the 5563 components. The fractions were found to have the same molecular weight as normal mouse γ -globulin (S_{20} 6.5) and no antigenic differences were detected by immunological techniques. It is possible that there are minor differences in amino acid sequence or in non-peptide molecules forming complexes with the protein. Enzymic degradation of the components as described by Porter (1959) for rabbit antibody is being studied to investigate any structural differences.

The observation that the physicochemical properties of the globulin molecules secreted by the 5563 plasma-cell tumour remain constant over many transplant generations throughout a period of 2 years is of special interest. The other lines of plasma-cell tumours investigated over many transplant generations also are associated with constant and characteristic serum-protein patterns on electrophoresis even when transplanted into hybrid hosts (Potter & Fahey, 1960). One is dealing with lines of differentiated plasma cells which divide rapidly. Various possibilities have been put forward to explain the wide heterogeneity of physical and biological properties of γ -globulin and antibody in normal animals. It has been suggested, for example, that γ -globulin molecules can take on numerous different configurations or that γ -globulin-forming cells are hypermutable, resulting in variability of globulin molecules. The findings with the transplantable plasma-cell neoplasms

indicate that the differentiated plasma cell is stable in its genotype and produces a constant and stable pattern of globulin molecules with no evidence for unstable molecular configurations.

The experiments *in vitro* have shown that the γ -globulin is produced by the tumour tissue and is not formed as a reaction of the host against the tumour graft. Although the plasma-cell tumour is growing rapidly it is simultaneously secreting considerable amounts of globulins into the serum so that the serum-globulin concentration is higher than the albumin concentration when the tumour weight is above 2–3 g. Tissue slices of the 5563 neoplasm incubated *in vitro* in the presence of [14 C]amino acid formed 1.5–2.0 mg. of γ -globulin protein/g. of tissue/24 hr.; 34–52% of the total protein synthesized by the plasma-cell tumour is represented by the γ -myeloma protein secreted into the serum (see Table 1).

The γ -globulin production is slightly lower than albumin formation by normal liver in similar experimental systems. Peters (1957) and Campbell & Stone (1957) reported that liver slices (from chicken and rat) form 3–5 mg. of albumin/24 hr./g. wet wt. of tissue. The plasma-cell tumour shows similar protein synthetic activity to liver hepatoma, which was less active in albumin synthesis (20–50%) than is normal liver (Campbell & Stone, 1957).

Since nucleic acid and amino acid analogues are being used frequently in cancer therapy in cases of myeloma and leukaemia, it was of interest to study the effect of these analogues on protein synthesis by the tumour tissue *in vitro*. This provides an easy method for testing the toxicity of these substances for tumour tissue, whereas in tests *in vivo* inhibition of tumour growth or tumour regression has to be investigated on a large number of animals.

Table 2 and Figs. 4 and 5 have shown that the ribonucleic acid analogues 8-azaguanine- and 6-azauracil-riboside inhibited both globulin formation and general protein synthesis after the first 2–3 hr. of incubation. Similar inhibition was observed when tests for incorporation of [14 C]valine, [14 C]glycine or algal [14 C]protein hydrolysate were made.

The mammalian system differs from the bacterial system, in which Richmond (1959) was able to show inhibition of formation of lytic enzymes in *Bacillus subtilis* within 5 min. of the addition of a low concentration of 8-azaguanine, before incorporation of the analogue into ribonucleic acid could be demonstrated. It is possible that in the slower growing tumour there are bigger pools of nucleic acid precursors than in bacteria so that protein synthesis can proceed for some time. In mammalian systems far higher (10–20-fold) con-

centrations of azaguanine than in the bacterial system have to be used to give any inhibition. As in the bacterial system, azaguanine leads to poisoning of general protein synthesis by the cell.

6-Azauracilriboside was found to have anti-tumour activity in mice with ascites tumours, whereas 6-azauracil was less effective (Šorm & Keilova, 1958). This was similar to results *in vitro* in this study presumably because the azauracil has to be converted into the riboside before utilization. It has been shown by Schindler & Welch (1957) that only the riboside inhibits growth of sarcoma 180 cells in tissue culture, and by Brockman, Sparks & Simpson (1957) that mouse leukaemic cells resistant to 8-azaguanine were not able to convert it into the riboside, whereas sensitive strains formed 8-azaguanineriboside. It is possible that the 6-mercaptapurine had no effect on protein synthesis by the 5563 tumour because the tumour was unable to form the riboside. The riboside was not available for testing.

Dutton, Dutton & George (1958) have reported inhibition (35–50%) of antibody formation by spleen cells from immunized animals with 8-azaguanine and *p*-fluorophenylalanine (about 3 mm) after incubation for 24 hr. Hence the 5563 neoplasm appears to have similar sensitivity to azaguanine, as do normal globulin-forming cells, but no effect on protein synthesis was detected by any of the amino acid analogues tested on the 5563 tumour slices during incubation for 6 hr. (Table 2). It could be demonstrated that small amounts of *p*-fluoro-[¹⁴C]phenylalanine are incorporated into the γ -myeloma protein by the 5563 plasma-cell tumour, and hence γ -globulin continued to be formed in the presence of *p*-fluorophenylalanine. Vaughan & Steinberg (1960) have had similar results and have been able to isolate peptides containing tritiated *p*-fluorophenylalanine from ovalbumin and lysozyme formed by hen's oviduct.

SUMMARY

1. The 5563 plasma-cell neoplasm, transplantable in mice, has been shown to form heterogeneous globulins separating into five definite bands on starch-gel electrophoresis. The different components can be partly separated by chromatography on anion-exchange cellulose and were found to have the same sedimentation constant (S_{20} 6.5) and the same immunological groupings.

2. The protein production of this plasma-cell tumour could be followed over many transplant generations (15–20). Globulin components with the same mobilities were produced by the neoplastic tissue of tumour-bearing mice over a period of two years. This suggested that the genotypes of differentiated plasma cells is stable, with no

evidence for mutation in protein synthetic capacity although there is rapid cell division.

3. By incubating slices of tumour tissue and isolating the myeloma protein by specific precipitation with antisera, it could be shown that the plasma cells form the myeloma protein at a considerable rate (1.5–2.0 mg./g. wet wt. of tissue/day).

4. Amino acid analogues had no significant effect on protein synthesis by the plasma cells during incubation for 6–7 hr. *in vitro*. *p*-Fluoro-[¹⁴C]phenylalanine was incorporated into the myeloma globulin at a very low rate. Ribonucleic acid analogues (3 mm-azauracilriboside and -azaguanineriboside) had no effect for the first 2–3 hr. but after that inhibited both the formation of tissue protein and myeloma protein to a similar extent, causing a 40–50% inhibition of protein synthesis after 6 hr. The tumour tissue appears to behave similarly to bacterial systems and normal tissue.

5. A microsomal fraction could be prepared by lysis of cells with dilute magnesium chloride that incorporated [¹⁴C]amino acids into protein insoluble in 0.9% sodium chloride, but no incorporation into the globulin could be demonstrated. The microsomal fraction contained 'bound' myeloma protein released by extraction with alkali and ultrasonic vibration.

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Comparison of the Aerobic and Anaerobic Reduction of Oxidized Glutathione in Liver Homogenates

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Since the glutathione of animal tissues, other than blood, is predominantly in the reduced form (Bhattacharya, Robson & Stewart, 1955; Martin & McIlwain, 1959), the rate of reduction of oxidized glutathione in respiring tissues must be rapid enough to counteract the enzymic (Ames & Elvehjem, 1945, 1946) and non-enzymic (Barron, 1951) oxidation of reduced glutathione. In most studies of reduction of oxidized glutathione, non-respiring glutathione-reductase preparation (Rall & Lehninger, 1952; Van Heyningen & Pirie, 1953; McIlwain & Tresize, 1957) or anaerobic conditions (cf. Vennesland & Conn, 1954) have been employed. Hitherto few investigators have compared the aerobic and anaerobic reduction of oxidized glutathione in tissue preparations capable of respiration. Martin & McIlwain (1959) found little difference in the aerobic and anaerobic rates of reduction of endogenous oxidized glutathione in slices of guinea-pig cerebral cortex. In avocado mitochondria supplemented with citrate and triphosphopyridine nucleotide (cf. Vennesland & Conn, 1954) the aerobic and anaerobic rates of reduction of oxidized glutathione were approximately equal. In the present investigation the aerobic and anaerobic rates of reduction of added oxidized glutathione in liver homogenates have been compared under a variety of conditions.

MATERIALS AND METHODS

Glutathione. Reduced glutathione (GSH) was obtained from The Distillers Co. Ltd. Oxidized glutathione (GSSG) was prepared from GSH by a modification of the method of

Pirie (1931) without added Cu^{++} ions. To a solution of GSH (2%, w/v) was added an equivalent amount of 3% (w/v) H_2O_2 , and the solution kept in a refrigerator at 2° for 12 hr. Oxidation at this stage was incomplete and the residual SH-groups were estimated potentiometrically. More H_2O_2 was added, a little less than was necessary to complete the reaction, and the solution was stored for a further 12 hr. at 2°. The purpose of this procedure was to avoid side reactions. The final product always contained a small percentage of GSH. It was considered preferable to use a solution of GSSG containing a small percentage of SH-groups rather than one containing side products of oxidation. With this method of preparation the total (GSSG + GSH) present in the final solution was equivalent to not less than 95% of the original GSH.

Sodium glucose 6-phosphate. This was prepared from the barium salt (British Drug Houses Ltd.) by ion exchange with Dowex 50.

Suspension media. 'Phosphate saline' contained 100 vol. of 0.155M-KCl, 10 vol. of 0.1M-potassium phosphate buffer, pH 7.4, and 1 vol. of 0.1M-MgCl₂. 'Bicarbonate saline' was a modification of the saline of Krebs & Henseleit (1932). The stock solution consisted of 100 ml. of 18% (w/v) KCl, 80 ml. of 1.15% (w/v) KCl, 15.4 ml. of 0.2M-KH₂PO₄, 20 ml. of 3.82% (w/v) MgSO₄·7H₂O, 60 ml. of 1.3% (w/v) NaHCO₃ and 1960 ml. of water. Before use 16 vol. of 1.3% (w/v) NaHCO₃ were added to 100 vol. of the stock solution, and the mixture was gassed with N₂+CO₂ (95:5) at 0° for 15 min. Phosphate saline was used in all experiments unless stated otherwise.

Preparation of homogenates. Immediately after removal from the animal the liver was cooled in 0.9% KCl at 0°, dried with filter paper and minced in a chilled Fischer mincer (Jouan, Paris). The minced tissue was weighed and homogenized with 8-30 vol. of ice-cold suspension medium in a chilled stainless Potter-Elvehjem homogenizer.

Incubations. These were carried out in Warburg apparatus at 30°. Vessels containing phosphate saline were gassed with O₂ or N₂ and those containing bicarbonate saline were gassed with O₂+CO₂ (95:5) or N₂+CO₂ (95:5).

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