The *in vitro* Synthesis of Specific Secretory Protein by an Ascites Plasma-Cell Tumor

E. L. KUFF, M. POTTER, K. R. MCINTIRE, AND N. E. ROBERTS

From the Laboratories of Biochemistry and Biology, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

Received June 23, 1964

The transplantable mouse plasma-cell tumor RPC-20 has as its secretory product a single immunologically distinctive protein with a sedimentation rate of 2.8 S and a molecular weight near 24,000. This protein appears in the urine of tumor-bearing mice largely in the monomeric state, but also to a lesser extent in a disulfide-linked dimeric form. The RPC-20 tumor has been converted to an ascites form and maintained by free-cell transplant for thirty-nine generations. With the use of antisera prepared against the urinary protein, it has been shown that the ascites-tumor cells can form the specific secretory product during *in vitro* incubation. Specific synthesis accounted for about 10% of the total protein synthesis carried on by the cells under this condition. The relative rate of synthesis of secretory protein seemed to be roughly equivalent to that of the solid tumor growing *in vivo*. The secretory protein represented 2% of the total protein in the ascites cells.

Plasma-cell neoplasms can be induced with high frequency in mice of the inbred BALB/c strain (Potter and Boyce, 1962). The neoplastic cells elaborate proteins (serum-myeloma globulins and/or urinary Bence-Jones proteins) that are structurally related to the immunoglobulins produced by normal plasma cells (Potter and Kuff, 1961; Fahey, 1961; Potter *et al.*, 1964). Any individual tumor line, arising from a single primary neoplastic focus, continues to secrete its own characteristic protein(s) through many transplant generations. The precise structure of each specific secretory product is thus under genetic control in the tumor cells.

Clearly these tumors represent potentially valuable systems in which to study the mechanisms that regulate the synthesis of specific secretory proteins in mammalian cells, particularly as this specific synthesis may be related to the reduplication of the general cell proteins. The formation of myeloma globulin and Bence-Jones protein has been demonstrated both *in vivo* (Nathans *et al.*, 1958) and in slices of solid tumor incubated *in vitro* (Askonas, 1961a,b; Askonas and Fahey, 1961). It has seemed to us that a system of free cells capable of specific secretory synthesis *in vitro* would offer considerable advantages over the solid tumor in terms of amenability to experimental manipulation. As reported in the present paper, the plasma-cell tumor line RPC-20, which produces a single low-molecular-weight urinary protein, has been converted from solid to ascites form. Using specific antisera prepared against the urinary protein, it has been shown that the ascites-tumor cells can synthesize this protein during *in vitro* incubation.

MATERIALS AND METHODS

The RPC-20 neoplasm originated in 1960 (Potter and Boyce, 1962) and has been maintained as a solid tumor by subcutaneous transplantation in BALB/c mice at intervals of 4–5 weeks. Development of an *ascites line* was begun three years ago by transplanting minced solid tumor intraperitoneally and subsequently passaging ascites fluid containing tumor cells to new hosts. The ascites tumor is presently in its 39th generation. In each generation a certain proportion of the cells continually implant in the peritoneal surfaces and grow as solid nodules. For this reason, a true growth curve cannot be obtained. The total free-cell population reaches a maximum of about 5×10^7 between 3 and 4 weeks after transplantation and includes from 10 to 20% lymphocytes and macrophages. Shortly thereafter, the ascites fluid usually becomes excessively bloody. Female mice were used as hosts in the present study because they excrete relatively small amounts of the normal mouse urinary proteins (Finlayson *et al.*, 1963).

Serum and urine samples were collected as previously described (Fahey and Potter, 1959). *Urinary proteins* were concentrated by slow freezing or by precipitation at 5° with 24% (w/w) ammonium sulfate. The protein solutions were dialyzed at 5° against phosphate-buffered saline, *p*H 7.1 (0.105 M sodium chloride plus 0.02 M sodium phosphate), clarified by centrifugation for 15 minutes at 105,000 × *g* (Spinco Rotor no. 40), and stored at −28° in sealed nitrogen-flushed ampoules.

Three rabbits were immunized by injection of the RPC-20 urinary-protein preparations in complete Freund's adjuvant (Potter and Kuff, 1961). All three *antisera* reacted strongly with the RPC-20 protein as well as showing slight reactions with normal mouse urinary protein and mouse serum albumin. Titration of one antiserum, A64, showed that it contained 320 μg of antibody protein per ml, precipitating 23 μg of urinary protein at equivalence.

Agar-gel immunoelectrophoresis was carried out in Tris-acetate buffer of *p*H 8.2 and ionic strength 0.05 μ (Potter and Kuff, 1961). For *starch-gel electrophoresis*, the vertical system of Smithies (1959) was used, with a gel buffer consisting of 0.045 M glycine–sodium hydroxide, *p*H 8.9 (Askonas, 1961a). Samples were applied on filter-paper strips cut to fill the wells, thus eliminating any irregular migration of protein along the surfaces of the gels.

Urinary protein was *reduced and alkylated* as follows. A solution containing 1–3% protein in phosphate-buffered saline, *p*H 7.1, was made 0.1 M in mercaptoethanol. After 4 hours at room temperature, the solution was dialyzed overnight at 5° against 0.005 M sodium phosphate, *p*H 7.1, containing 0.01 M mercaptoethanol. To the sac contents was added 0.1 volume of a freshly prepared solution of 1.1 M iodoacetamide in 1.1 M Tris. The mixture, which had a final *p*H of 8–8.2, was allowed to remain at room temperature for 30 minutes and then dialyzed exhaustively against phosphate-buffered saline, *p*H 7.1, at 5°.

Free-sulfhydryl groups were estimated by three different methods: (1) spectrophotometrically, by

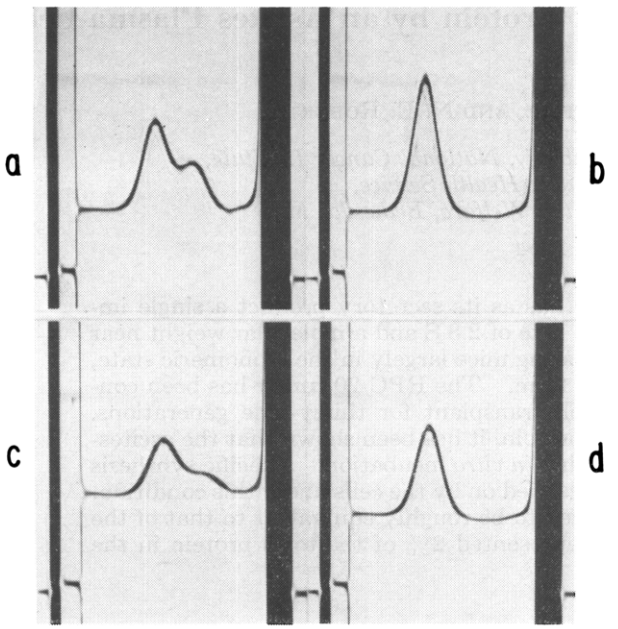

FIG. 1.—Ultracentrifuge patterns of RPC-20 urinary protein. (a) The original preparation; (b) after reduction with 0.1 M mercaptoethanol at *p*H 7.1 for 4 hours; (c) reduced preparation after removal of mercaptoethanol by dialysis under air; (d) reduced and alkylated sample, dialyzed under air. Exposures were made after 134 minutes at 59,780 rpm. The solvent was phosphate-buffered saline, *p*H 7.1; 0.1 M mercaptoethanol was also present in (b). The small base-line deviation near the bottom of the cell did not represent a sedimenting component.

reaction with *p*-mercuribenzoate (Boyer, 1954); (2) by amperometric titration with silver ion (Hartley *et al.*, 1962); and (3) by reaction with [1-^{14}C]iodoacetate of known specific radioactivity (Haber and Anfinsen, 1961). *Total nitrogen* was determined by Nesserlization after acid digestion. *Analytical ultracentrifugation* was carried out in a Spinco Model E ultracentrifuge. *Molecular weights* were estimated by sedimentation equilibrium in fluid columns of approximately 1 mm length (Yphantis, 1960).

For *in vitro incubations*, ascites fluids were removed under sterile conditions and diluted with 2 volumes of chemically defined culture medium NCTC-109 (McQuilkin *et al.*, 1957), modified to contain a total of 3 g of glucose per liter, and pre-equilibrated with air–5% CO_2. The suspensions, containing 1–3 $\times$ 10^6 cells/ml, were incubated at 37° with shaking under the same atmosphere. ^{14}C-labeled amino acids (algal protein hydrolysate, 1.7 c/mg, obtained from New England Nuclear Corp.) were added to a final concentration of 1.5 μc/ml. Aliquots of suspension were removed and cooled rapidly to 0°, and the cells were pelletized by centrifugation for 3 minutes at 500 $\times$ *g*.

Tissue proteins were assayed for radioactivity after nucleic acids and lipids had been extracted by the method of Schneider (1957). The air-dried protein pellets were dissolved in measured amounts of glacial formic acid (Eastman, 97+%, practical grade), and aliquots of the solutions were pipetted onto filter paper disks (cut from electrophoresis wicks, Spinco part no. 300–029) which had previously been inserted into liquid-scintillation vials. Additional aliquots were taken at the same time for determination of protein nitrogen. The disks were dried overnight at room temperature and for 2 hours at 60°. After addition of scintillation fluid (4 ml of toluene containing 4 g of PPO and 0.1 g of POPOP per liter),[1] radioactivity was measured in a Packard liquid scintillation spectrometer. The instrument was adjusted so that the ratio of counts appearing simultaneously in two different channels was linearly related to the counting efficiency for any given sample (Bruno and Christian, 1961). Reductions in efficiency (quenching) owing to the presence of colored-tissue proteins were monitored by reference to a standard quenching series prepared from mixtures of [^{14}C]-leucine and liver-soluble proteins plated on paper disks.

For quantitative determinations, *the specific RPC-20 secretory protein* was extracted from ascites cells with the use of deoxycholate (Peters, 1962). Packed cells were resuspended at 0° in 20–30 volumes of 0.1 M ammonium bicarbonate, *p*H 8.3, containing 5 mM mercaptoethanol. Sodium deoxycholate (1% solution) was added to a final concentration of 0.1%. The lysed cells were held at 0° for 1 hour, after which magnesium chloride (1M) was added to a final concentration of 0.04 M. The suspensions were then adjusted to *p*H 6.5–7.0 with acetic acid added during vigorous stirring. The resultant precipitates ("*p*H 7 precipitates"), which contained from 45 to 60% of the total cell proteins together with most of the deoxycholate, were allowed to aggregate for 30 minutes and were then removed by centrifugation for 20 minutes at 700 $\times$ *g*. Finally the clear orange supernatant fluids ("*soluble extracts*") were dialyzed overnight at 5° against 100 volumes of phosphate-buffered saline, *p*H 7.1, containing 5 mM mercaptoethanol.

Immune precipitations were carried out by mixing two different aliquots of each extract, containing from 1 to 4 μg of the specific protein, with 0.25 ml of antiserum A64 (conditions of antibody excess) in a total volume of 0.5 ml. The mixture contained 2.5 mM mercaptoethanol. Mixtures of urinary protein and A64 representing two points on a predetermined standard precipitation curve were also prepared, as well as controls including mixtures of the soluble extracts with nonspecific rabbit serum. The tubes were incubated at 37° for 1 hour and at 5° for 23 hours. Control tubes showed no visible precipitates but were carried with the immune precipitates through the following washes: 3 times with cold phosphate-buffered saline, *p*H 7.1; once with 5% trichloroacetic acid at 90° (15 minutes); once each with 5% trichloroacetic acid and absolute ethanol at room temperature; and once with ethanol-ether (3:1) at 60°. The tube contents were air-dried and dissolved in formic acid for analysis of nitrogen and radioactivity. The content of specific protein was determined by reference to the standards after correction for the appropriate controls.

RESULTS

Properties of the RPC-20 Secretory Protein.—Abnormal protein appeared in the urine of tumor-bearing animals as two ultracentrifugal components (Fig. 1a) with sedimentation rates ($s_{20w,}$) of 2.8 S and approximately 4.3 S. After reduction with 0.1 M mercaptoethanol at *p*H 7.1 and dialysis against 0.01 M mercaptoethanol, a single symmetrical boundary was observed (Fig. 1b). The sedimentation rate of this component, also 2.8 S, was independent of protein concentration between 0.25 and 1.75%. No more than 4% of the total nitrogen was lost during reduction and dialysis. It was therefore clear that this treatment had resulted in dissociation of the more rapid component to 2.8-S material. Exposure to ethanol under the same conditions had no discernible effect. Removal of mercaptoethanol by dialysis under air for 48 hours was attended by reappearance of the 4.3-S component (Fig. 1c).

[1] Abbreviations used in this work: PPO, 2,5-diphenyloxazole; POPOP, 1,4-2-(5-phenyloxazolyl)benzene.

However, alkylation of the reduced protein prevented reaggregation and resulted in a stable, apparently monodisperse 2.8-S preparation (Fig. 1d).

The untreated urinary protein formed two major bands on starch-gel electrophoresis, whereas only one component was observed after reduction and alkylation (Fig. 2, left). When tested in double-diffusion systems against rabbit antisera, the separated bands of the untreated protein and the reduced-alkylated material all gave single precipitin lines that fused with one another and with that of the original urinary preparation (Fig. 2, right). Quantitative-precipitation tests of the untreated and the reduced proteins were carried out. At equivalence, one μg of each type of protein precipitated 14 μg of antibody protein, showing that reduction had been attended by no loss of antigenic reactivity.

The weight-average molecular weights of the reduced and reduced-alkylated RPC-20 urinary protein are shown in Table I. The value of 23,000–24,000 must be regarded as approximate until the true partial specific volume of the protein has been determined. The frictional coefficient near unity indicates a compact, nearly spherical molecule.

TABLE I
SEDIMENTATION PROPERTIES AND FREE-SULFHYDRYL CONTENT OF RPC-20 URINARY PROTEIN

	Original	Reduced	Reduced and Alkylated
$s_{20,w}$	2.8 and 4.3 S	2.8 S	2.8 S
Molecular weight[a]		23,000	24,000
f/f_0		0.99	1.02
Free sulfhydryl[b]			
[1-^{14}C]Iodoacetate			
35 min[c]		0.95	
65 min[c]		1.06	
p-Mercuribenzoate	0.10	0.85	
Silver ion	0.06	0.90	

[a] Weight-average, assuming a partial specific volume of 0.74. [b] Moles per mole of protein with molecular weight 24,000, as determined with indicated reagents. [c] Time of reaction at *p*H 8.0 and 25°.

The free-sulfhydryl content of the untreated protein was low (Table I). However, reduced preparations contained one reactive-sulfhydryl group per molecule, calculated on the basis of a molecular weight of 24,000. No carbohydrate was detected when 3 mg of the urinary protein was tested by either an anthrone (Seifter *et al.*, 1950) or thymol–sulfuric acid (Shetlar and Masters, 1957) procedure. Based on the sensitivity of these tests, carbohydrate was estimated to be less than one hexose residue per molecule of protein.

The sedimentation patterns of sera from tumor-bearing mice "(tumor sera") were indistinguishable from those of normal mouse serum. However, as will be described in detail elsewhere, starch-gel electrophoresis and immunoelectrophoresis revealed that the RPC-20 protein was present in tumor sera, partly in forms resembling those found in the urine, and partly as a mercaptoethanol-dissociable complex with serum albumin. No evidence was found that the tumor secreted a higher-molecular-weight serum-myeloma globulin.

In extensive serological studies (Potter *et al.*, 1964; McIntire and Potter, 1964), antisera prepared against the RPC-20 protein have failed to react with the immunoglobulins of the normal BALB/c mouse, with a variety of γ2 and β2A myeloma globulins and with

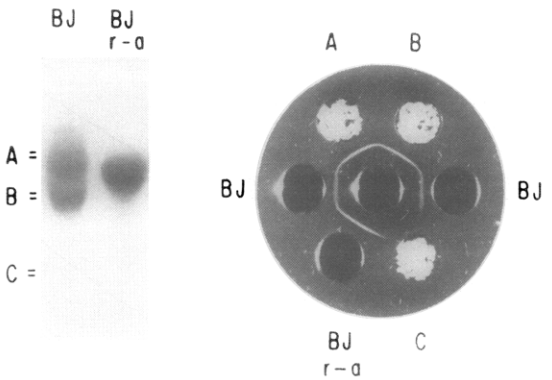

FIG. 2.—Combined starch-gel electrophoresis (left) and agar double-diffusion experiment (right) with RPC-20 urinary protein (BJ). A reduced and alkylated sample (BJ,r-a) was also run in both systems. *A*, *B*, and *C* indicate where regions were cut from the unstained halves of the electrophoretic patterns by reference to holes punched in the gel before slicing. These starch-gel strips were minced and transferred to the indicated antigen wells of the agar plate. The central well contained rabbit antiserum against the untreated RPC-20 urinary protein. Equal quantities of BJ and BJ,r-a were applied to the starch gel.

twenty-seven out of thirty urinary proteins produced by other transplantable mouse plasma-cell tumors. The structural distinctiveness of the RPC-20 protein was also reflected in its tryptic-peptide map ("fingerprint") (Potter *et al.*, 1964; McIntire and Potter, 1964).

The association of RPC-20 secretory protein with microsome fractions prepared from the solid tumor has been briefly reported (Kuff *et al.*, 1962). This protein was apparently contained by the lipoprotein membrane of the microsomal vesicles and could be released in soluble form with the use of the nonionic detergent Triton X-100. The microsomal protein was electrophoretically and immunologically identical with the protein recovered from the urine of the tumor-bearing mice.

It would appear, then, that the RPC-20 tumor secretes a single antigenically distinction protein with a molecular weight near 24,000. The data are consistent with the hypothesis that this protein initially possesses one reactive-sulfhydryl group per molecule by virtue of which it is capable of forming a disulfide dimer and of reacting with serum albumin.

In vitro Synthesis of Specific Secretory Protein by RPC-20-Ascites-Tumor Cells. RPC-20 ascites cells actively incorporated ^{14}C-labeled amino acids into protein when incubated in the NCTC-109 medium. Figure 3 shows a linear time course for the labeling of total cell protein over a 1-hour period. Radioactive protein appeared in the medium at a very low rate during the first 30 minutes but more rapidly thereafter. More than 98% of the total incorporated counts were found to be cell-associated at times up to 30 minutes; however, by 1 hour 10% of the labeled protein was extracellular.

Soluble extracts prepared with Triton X-100 from cells labeled for varying periods of time were enriched with nonradioactive RPC-20 urinary protein and subjected to combined immunoelectrophoresis and autoradiography (Fig. 4). The proteins of the incubation medium were similarly analyzed. Antiserum against the RPC-20 urinary protein gave the precipitin arcs shown in Figure 4a. In the case of the cells, labeling of the arc was only faintly detectable after a 5-minute incorporation period, but increased rapidly thereafter (Fig. 4b). The specific secretory protein in the

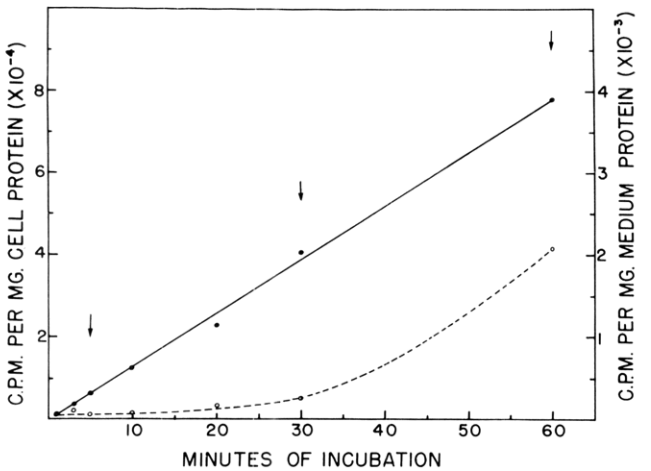

FIG. 3.—The *in vitro* incorporation of ^{14}C-labeled amino acid into protein by RPC-20-ascites cells. The original ascites suspension was diluted with 2 volumes of NCTC-109 medium, and ^{14}C-labeled algal-protein hydrolysate was added at zero time. The solid line refers to total cell protein, the broken line to proteins in the incubation medium. Arrows indicate times at which additional samples were taken for analysis of radioactivity in the specific secretory protein (see Fig. 4).

extracellular medium was clearly labeled at 1 hour but not at 30 minutes. Other labeled proteins were seen in the medium at 1 hour. Their presence was consistent either with a nonspecific leakage of cell protein or with the operation of a secretory mechanism involving some cytoplasmic shedding (Ortega and Mellors, 1957; Thiéry, 1959; Bessis, 1961).

Quantitative Aspects of Specific Synthesis in RPC-20-Ascites-Tumor Cells.—Antisera developed against the RPC-20 urinary proteins were used to isolate the specific secretory protein from ascites cells labeled with [^{14}C]amino acids. Triton extracts of the cells were not desirable for this purpose, since they contained in dispersed form material (possibly lipoprotein in nature) that tended to precipitate nonspecifically when incubated with rabbit sera. After preliminary experiments had demonstrated that deoxycholate was as efficient as Triton X-100 in the extraction of the specific RPC-20 protein, this reagent was routinely used in the preparation of soluble extracts from the tumor cells (see Materials and Methods). With regard to the procedure itself, it was found that magnesium ion was useful in reducing the loss of secretory protein into the *p*H 7 precipitate, and that precipitates formed at *p*H 6 or below carried with them prohibitive amounts of this protein. Mercaptoethanol was added to maintain the protein in reduced form and thus prevent possible spurious association of radioactivity through disulfide linkage. The extracts gave only single precipitin lines with specific antiserum when tested by double diffusion in agar, and no precipitates when incubated with nonspecific rabbit serum.

The entire extraction and immune-precipitation procedure was tested for the recovery of specific protein and for possible contamination of the immune precipitates with other cellular proteins. Use was made of ascites cells derived from another BALB/c mouse plasma-cell tumor line, designated AdjPC-6A. This tumor produces a myeloma globulin (Potter and Kuff, 1961) that does not react with antiserum directed against the RPC-20 urinary protein. Soluble extracts were prepared from AdjPC-6A cells that had been labeled *in vitro* for 30 minutes with [^{14}C]amino acids. Nonradioactive RPC-20 urinary protein was added either at the beginning or at the end of the extraction

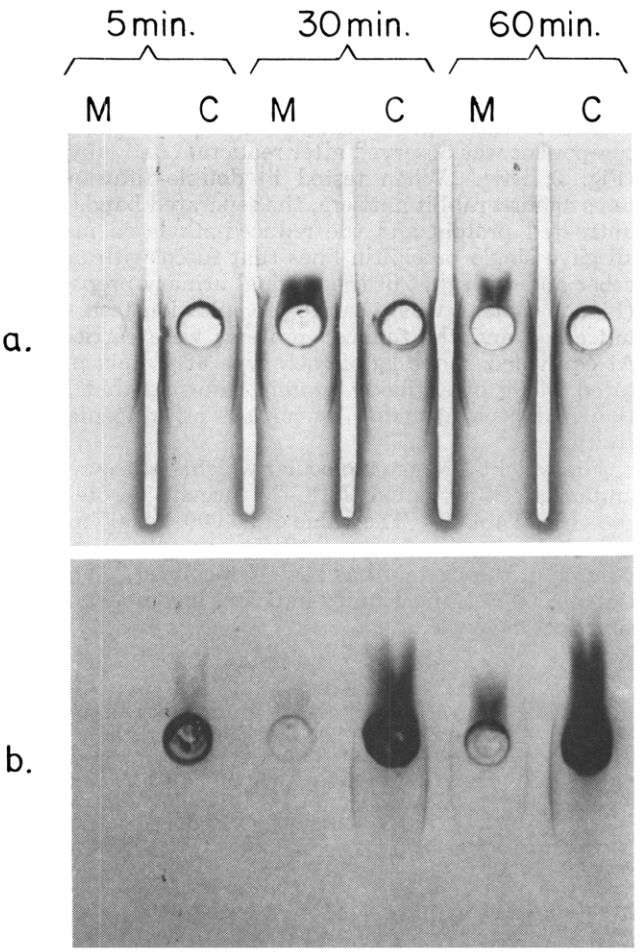

FIG. 4.—Demonstration of specific-secretory-protein synthesis by RPC-20-ascites-tumor cells. After 5, 30, and 60 minutes' incubation with ^{14}C-labeled amino acids, cells were sedimented from medium and lysed in 0.1 M ammonium bicarbonate, *p*H 8.3, containing 0.25% Triton X-100 and 0.1 M mercaptoethanol. Carrier RPC-20 urinary protein was added to the lysates at the level of 200 μg/ml. After 30 minutes, a 2-fold excess of iodacetamide was added and the samples were then passed through columns of Sephadex G-50 equilibrated with phosphate-buffered saline, *p*H 7.1. The corresponding incubation media were similarly treated except that no Triton was added. The protein fractions, designated M (medium) and C (cells), from the Sephadex columns were subjected to immunoelectrophoresis employing antiserum specific for the RPC-20 urinary protein. The resultant patterns were washed with isotonic sodium chloride, stained, and dried. Autoradiographs were prepared by exposing the patterns to Ansco nonscreen X-ray film (Ansco, Binghamton, N. Y.) for a period of 4 weeks.

procedure and was subsequently precipitated from the soluble extracts with specific antiserum. Recovery of the protein added to the final extract was found to be essentially quantitative and no radioactivity was associated with the washed immune precipitates. When RPC-20 protein was added to the initial cell lysates, the overall recovery was 80%, the major loss presumably occurring at the *p*H 7 precipitation step. This recovery rate was the same as that obtained by Peters (1962), in the isolation of serum albumin from rat liver by a somewhat similar method. Less than 0.3% of the total incorporated radioactivity was associated with the immune precipitates, an amount far below that subsequently encountered in actual analysis of the RPC-20 cells.

Table II shows the results of several experiments in which RPC-20-ascites cells were labeled *in vitro* with [^{14}C]amino acids and the radioactivity of total cell

TABLE II
INCORPORATION OF ^{14}C-LABELED AMINO ACIDS INTO WHOLE-CELL PROTEIN AND SPECIFIC SECRETORY PROTEIN BY RPC-20 ASCITES-TUMOR CELLS *in vitro*[a]

			Protein Weight			Protein Radioactivity		
Expt	Incorporation Period (min)	Time *in vitro*[b] (min)	Whole Cells (μg)	Secretory Total (μg)	Secretory Per Cent of Whole Cell (%)	Whole Cells (cpm)	Secretory Total (cpm)	Secretory Per Cent of Whole Cell (%)
1	20	120	1080	11.7	1.0	24,200	1,680	6.9
2	30	10	937	17.5	1.9	22,830	1,625	7.1
	30	110	789	15.2	1.9	20,540	1,320	6.4
3	30	10	800	14.4	1.8	21,325	1,580	7.4
4	30	10	770	16.7	2.2	31,650	2,805	8.9

[a] Approximately 10^6 cells/ml were incubated in modified NCTC-109 culture medium to which a [^{14}C]amino acid mixture was added to levels of 1 or 1.5 μc/ml at the beginning of the incorporation period. Values refer to cells recovered from 1 ml of incubation mixture. [b] To beginning of incorporation period.

proteins and specific secretory protein was then determined. Incorporation periods were limited to 30 minutes or less in order to ensure that labeled protein would still be cell-associated. Between 6.4 and 8.9% of the total incorporated counts (average, 7.3%) were recovered as the specific protein. In view of the incomplete recovery of specific protein in the extraction procedure and the presence of normal cells in the ascites population, it is reasonable to suppose that the relative rate of specific synthesis in the tumor cells themselves approximated 10%. The content of secretory protein varied between 1 and 2% of the total cell protein. A major factor in this variation was the number of erythrocytes present in the different ascites preparations. For example, erythrocytes were particularly numerous in the ascites used in expt 1, Table II. Again, it is felt that the higher values, about 2%, more accurately reflect the true content of the tumor cells.

In evaluating the synthetic capacities of the ascites cells, it seemed desirable to obtain a quantitative index of specific secretory synthesis that would be independent of the immune-precipitation reaction. In experiments not illustrated here, conditions were established for the adsorption of reduced (monomeric) RPC-20 urinary protein on columns of DEAE-cellulose and for its elution as a single peak in a shallow sodium chloride gradient at *p*H 7.4. When a soluble extract of ^{14}C-labeled RPC-20-ascites cells (expt 4, Table II) was chromatographed under the same conditions, a major peak of protein radioactivity was found to emerge in the position characteristic of the urinary protein. The identity of the peak was confirmed by immunological analysis as well as by cochromatography of the soluble extract with carrier urinary protein. The radioactivity included in this peak corresponded precisely with that obtained by direct immune precipitation of the specific secretory protein from the original soluble extract; i.e., by both methods of analysis the secretory protein accounted for 24% of the total protein radioactivity in the extract.

DISCUSSION

Evidence has been presented elsewhere (Potter and Kuff, 1964) that the neoplastic transformation may interrupt or distort the normal course of plasma-cell differentiation in such a way as to fix the cells irreversibly at intermediate levels in the differentiation of a program of immunoglobulin synthesis. The development of antibody-producing capacity is normally accompanied by a progressive restriction in the mitotic activity of the maturing plasma cells (Sainte-Marie and Coons, 1964); however, antibody synthesis is initiated in immature cells which are still capable of cell division (Nossal, 1962). There is direct precedent, then, for conceiving that certain plasma-cell tumors might represent relatively homogeneous populations in the sense that all of the neoplastic cells are capable of both secretion and cell division (although not necessarily simultaneously). Compatible with this hypothesis are the results of Osserman *et al.* (1964), who studied ascites cells obtained from mice carrying intraperitoneal implants of the solid-RPC-20-tumor line. They found that cells in mitosis could be stained with fluorescent-labeled antiserum against the specific secretory protein, although less strongly than the nondividing tumor cells. Similar observations were also made with the cells of another urinary protein-producing tumor. However, these findings do not eliminate the possibility that tumor growth may depend primarily upon cells with relatively little secretory function, and that, of these "stem-line" cells, a certain proportion may continually undergo maturation (see Rifkind *et al.*, 1962; Osserman *et al.*, 1964) to a mitotically quiescent protein-secreting state. The latter situation would imply that at least certain aspects of the normal relationship between maturation and cell division were retained by the neoplastic cells. The capacity for specific synthesis having been established for the RPC-20 ascites-cell population as a whole, it will be of interest to extend the investigation to the level of the individual tumor cells, particularly in connection with their capacity for DNA synthesis.

Askonas (1961a) has reported that between 34 and 52% of the total protein synthesized by slices of plasma-cell tumor X5563 incubated *in vitro* was represented by the γ-myeloma-secretory protein of this tissue. The relative rate of specific synthesis observed in the RPC-20-ascites population was considerably less, probably of the order of 10% in the tumor cells themselves. It is interesting, however, to consider that the molecular weight of the RPC-20 protein is approximately one-sixth that of the 7S myeloma globulin produced by the X5563 tumor. Therefore the relative rates of specific synthesis in the two types of tumor cells may be rather similar when expressed in terms of the *number* of secretory molecules formed during an equivalent amount of cell growth. In fact, if one takes values of 40% and 10% for the relative rate of specific synthesis in the X5563- and RPC-20-tumor cells, respectively, and assumes an average molecular weight of 50,000 for the remainder of the cell proteins, one arrives at the conclusion that one molecule of secretory protein is synthesized for every four to five molecules of cell protein in each tumor.

This estimate, while admittedly rough, serves to emphasize a central problem with regard to the physiol-

ogy of the neoplastic plasma cells, namely, the means by which genetic information for the synthesis of a single protein species is so greatly amplified relative to the information for synthesis of the many individual proteins involved in cell growth. The type of mechanism that might be invoked is clearly related to the earlier question of whether secretory synthesis is in fact a property of the replicating cells themselves. For example, in a system involving a process of constant differentiation, amplification might be based on the preferential survival and accumulation of specific messenger ribonucleic acid for the secretory protein. We are presently comparing the formation of secretory and general cell protein by RPC-20-ascites cells in which RNA synthesis has been blocked by actinomycin-D.

On the basis of the data in Table II, it may be calculated that 8–9 mg of the RPC-20-secretory protein would be synthesized during the replication of 1 g wet wt of tumor cells containing 120 mg of total protein, and that approximately 30 mg of this protein would be formed during the life history of a tumor growing to a final size of 3.5 g. The latter value is in reasonable accord with actual experience in which urine was collected during the day by direct pressure on the bladders of 40 mice bearing solid RPC-20 tumors, and subsequently analyzed for the RPC-20 protein. An average total yield of 15 mg per mouse was obtained over a 12-day period in which the tumors grew from pea-sized nodules to terminal weights between 3.5 and 4 g, and of course this recovery did not include the urinary protein excreted during the night hours. This correspondence suggests that the relative rate of specific synthesis observed in the ascites cells *in vitro* was roughly equivalent to that in the solid tumor. The output of secretory protein per unit time would depend upon the doubling-time of the tumor cells, which is not yet known. For example, a doubling-time of 72 hours, which may be appropriate for a relatively slow-growing tumor such as RPC-20, would result in a calculated production of about 2 mg specific protein/g tissue/24 hours. A value of 1.5–2.0 mg of myeloma globulin/g tissue/24 hours was estimated for the X5563 plasma-cell tumor (Askonas, 1961a), which in our experience grows even more slowly than the RPC-20 neoplasm.

Delays of approximately 30 minutes have been observed in the appearance of newly synthesized antibody and γ-globulin in the extracellular fluid when spleen slices (Askonas and Humphrey, 1958) and lymph-node cells (Helmreich *et al.* 1961) were incubated *in vitro*. This delay is thought to be the consequence of a time-ordered secretory process in the antibody-producing cells (Helmreich *et al.*, 1962). The secretion of γ-myeloma globulin by the X5563 plasmacytoma growing *in vivo* was also characterized by a 30-minute transit time for the newly formed protein (Nathans *et al.*, 1958). The present results (Figs. 3 and 4) were consistent with a similar secretory delay in the RPC-20-ascites cells and suggest that the secretory mechanism in these cells may be essentially the same as that which is operative in the more highly differentiated forms.

A limitation of the RPC-20-ascites-tumor system at present is our inability to maintain the cells *in vitro* for prolonged periods. Evidence has been obtained for specific synthesis in cells cultured in NCTC-109 medium for 24 hours, but not beyond. Until conditions for prolonged culture have been defined, it has seemed advisable to confine experimentation to the first 2–3 hours of *in vitro* incubation, during which period protein synthesis in the cells is relatively unimpaired.

Finally, it should be mentioned that other ascites plasma-cell tumor lines are currently available or under development. Preliminary studies have shown that at least one of them, the Adj PC-6A line mentioned briefly in the present report, elaborates a β2A myeloma globulin *in vitro*.

References

Askonas, B. A. (1961a), *Biochem. J. 79*, 33.

Askonas, B. A. (1961b), *Protein Biosyn. Symp. Wassernaar, Neth. 1960*, 363.

Askonas, B. A., and Fahey, J. L. (1961), *Biochem. J. 80*, 261.

Askonas, B. A., and Humphrey, J. H. (1958), *Biochem. J. 68*, 252.

Bessis, M. C. (1961), *Lab. Invest. 10*, 1040.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Bruno, G. A., and Christian, J. E. (1961), *Anal. Chem. 33*, 650.

Fahey, J. L. (1961), *J. Exptl. Med. 114*, 385.

Fahey, J. L., and Potter, M. (1959), *Nature 184*, 654.

Finlayson, J. S., Potter, M., and Runner, C. R. (1963), *J. Natl. Cancer Inst. 31*, 91.

Haber, E., and Anfinsen, C. B. (1961), *J. Biol. Chem. 236*, 422.

Hartley, R. W., Jr., Peterson, E. A., and Sober, H. A. (1962), *Biochemistry 1*, 60.

Helmreich, E., Kern, M., and Eisen, H. N. (1961), *J. Biol. Chem. 236*, 464.

Helmreich, E., Kern, M., and Eisen, H. N. (1962), *J. Biol. Chem. 237*, 1925.

Kuff, E. L., Potter, M., McIntire, K. R., and Roberts, N. E. (1962), *Federation Proc. 21*, 104.

McIntire, K. R., and Potter, M. (1964), *J. Natl. Cancer Inst. 33*, 631.

McQuilkin, W. T., Evans, V. J., and Earle, W. R. (1957), *J. Natl. Cancer Inst. 19*, 885.

Nathans, D., Fahey, J. L., and Potter, M. (1958), *J. Exptl. Med. 108*, 121.

Nossal, G. J. V. (1962), *Advan. Immunol. 2*, 163.

Ortega, L. G., and Mellors, R. C. (1957), *J. Exptl. Med. 106*, 627.

Osserman, E. F., Rifkind, R. A., Takatsuki, K., and Lawlor, D. P. (1964), *Ann. N. Y. Acad. Sci. 113*, 627.

Peters, T., Jr. (1962), *J. Biol. Chem. 237*, 1186.

Potter, M., and Boyce, C. R. (1962), *Nature 193*, 1086.

Potter, M., Dreyer, W. J., Kuff, E. L., and McIntire, K. R. (1964), *J. Mol. Biol. 8*, 814.

Potter, M., and Kuff, E. L. (1961), *J. Natl. Cancer Inst. 26*, 1109.

Potter, M., and Kuff, E. L. (1964), *J. Mol. Biol. 9*, 537.

Rifkind, R. A., Osserman, E. F., Hsu, K. C., and Morgan C. (1962), *J. Exptl. Med. 116*, 423.

Sainte-Marie, G., and Coons, A. H. (1964), *J. Exptl. Med. 119*, 743.

Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.

Seifter, S., Dayton, D., Novic, B., and Muntwyler, E. (1950), *Arch. Biochem.* (now *Arch. Biochem. Biophys.*) *25*, 191.

Shetlar, M. R., and Masters, Y. F. (1957), *Anal. Chem. 29*, 402.

Smithies, O. (1959), *Biochem. J. 71*, 585.

Thiéry, J. P. (1959), *Ciba Found. Symp. Cellular Aspects Immunity 1960*, 59.

Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.