Specific Protein Synthesis in Isolated Epithelium of Guinea-Pig Seminal Vesicle

GENERAL FEATURES

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Four intrinsic soluble proteins are synthesized and secreted by sexually mature guinea-pig seminal-vesicle mucosa, which comprises a monolayer of a homogeneous columnar epithelial cell. All four proteins can be extracted readily in 154 mM-NaCl from the organ's luminal constituents in which they are present in high concentration. They are referred to as proteins 1, 2, 3 and 4 in order of their elution during DEAE-cellulose column chromatography. Specific primary antibodies were harvested from goats that had been inoculated with the purified vesicular proteins; secondary antibodies were obtained from a donkey inoculated with goat γ-globulins. Double-antibody-immunoprecipitation techniques were developed to precipitate the vesicular proteins. Thus proteins newly synthesized from ^{14}C-labelled amino acids could be precipitated and the incorporated radioactivity assessed. Isolated seminal-vesicle mucosa, incubated in only a buffered salt solution containing glucose, readily synthesized the soluble secreted proteins from added [^{14}C]lysine plus [^{14}C]-glycine, [^{14}C]histidine plus [^{14}C]glutamate, [^{14}C]glutamine alone and [^{14}C]arginine alone. The rates of incorporation (d.p.m./mg of total soluble protein) of labelled lysine and glycine and of labelled arginine were linear with time over 180 min. With the other labelled precursors, rates diminished between 60 and 180 min. Labelled protein could be detected after only 10–15 min of incubation. Only 4–9 % of the newly synthesized protein remained associated with the mucosa; the remainder was found in the cell-free incubation medium. The isolated seminal-vesicle mucosal preparation will provide a unique opportunity to study the synthesis and secretion of abundant cell-specific proteins by this androgen-dependent tissue.

The synthesis of non-specific protein fractions by rat seminal vesicle *in vivo* and *in vitro* and of guinea-pig seminal vesicle *in vitro* has been demonstrated (Manyai, 1963). Other studies have been conducted on the regulation of non-specific protein biosynthesis by slices of rat seminal vesicle (Wilson, 1962) and by homogenates of guinea-pig seminal vesicle (Kochakian, 1964). Also, the induction of specific enzymes of rat seminal vesicle, as indicated by assayable enzyme activity, occurs after the injection of testosterone *in vivo* (Singhal & Valadares, 1968; Singhal & Ling, 1969). However, in the foregoing studies, all tissues of the organ, muscle and fibrous, were used. At present no study of protein biosynthesis has used isolated seminal-vesicle mucosa (for a review see Veneziale *et al.*, 1977). Further, no investigation has studied the biosynthesis of specific soluble secreted proteins, although this is indicated because seminal-vesicle mucosa is uniquely structured for the synthesis and secretion of specific proteins (Veneziale *et al.*, 1974; Flickinger, 1974; Veneziale & Deering, 1976a) and is dependent on androgen for the maintenance of various biosynthetic and secretory functions (Mann, 1964). Indeed, one might have predicted, on the

basis of established information, that the tissue could serve as a model preparation for studies on protein synthesis and on the mechanisms by which androgens support the process. Thus I decided to study the general features of the synthesis of four specific soluble secreted proteins in isolated cellularly homogeneous seminal-vesicle mucosa from intact guinea pigs (Veneziale *et al.*, 1974; Büchi & Veneziale, 1977). This work was helped directly or indirectly by previous research on the metabolism of small molecules (Prendergast & Veneziale, 1975; Veneziale & Deering, 1976b), the intrinsic soluble secreted proteins (Veneziale & Deering, 1976a), the metabolism of multiple androgens (Steer & Veneziale, 1977) and the regulation of the RNA polymerases in isolated nuclei (Büchi & Veneziale, 1977) of seminal-vesicle mucosa. From the foregoing research I and my co-workers have learned that the isolated mucosa *in vitro* retains its metabolic capabilities to an extraordinary degree, as demonstrated by its ability to synthesize fructose and citrate, to maintain an ATP:ADP ratio of at least 6, to recover normal energy metabolism after periods of anoxia and to metabolize steroids. All of the results attest to the

maintenance of viability and integrity of the isolated mucosa preparation. Thus I was able to proceed confidently with the studies of protein synthesis that are presented in this paper.

Materials and Methods

Purification of the four soluble secreted proteins of guinea-pig seminal-vesicle lumen

The luminal proteins were purified by means of DEAE-cellulose column chromatography as previously described (Veneziale & Deering, 1976a). Four peaks were found and were ascribed to four proteins designated proteins 1, 2, 3, and 4 in order of elution. Fractions corresponding to each peak were pooled, freeze-dried and then dialysed against 0.45% NaCl. Each of the four highly purified proteins was subsequently rechromatographed in a freshly prepared DEAE-cellulose column (25 mm × 109 mm). The appropriate fractions were then collected and treated as above. As demonstrated by the presence of single bands on polyacrylamide-gel disc electrophoresis (see below), rechromatography on DEAE-cellulose yielded pure preparations of proteins 1, 2 and 4. The preparation of protein 3 includes a contaminant that probably represents a breakdown product of the primary protein (Veneziale & Deering, 1976a).

Preparation of goat antibodies to the individual soluble secreted proteins of guinea-pig seminal vesicle

Vesicle protein (600 μg) in 1.2 ml of 0.9% NaCl was mixed with 1.2 ml of complete Freund's adjuvant. The emulsion was injected intradermally at 15–20 sites on the dorsum bilaterally along the shaved thoraco-lumbar–sacral regions of a goat. Injections of the four individual guinea-pig proteins were made into each of four goats ranging in age from 12 to 24 months. At 2 weeks later each goat received a booster injection of 100 μg of the appropriate protein in 0.9% NaCl subcutaneously. Subsequently, booster injections of 200, 300 and 400 μg were administered 1 week apart. The booster injections were given distal to the sites of swelling and inflammation elicited by the initial inoculations with Freund's adjuvant. Heparinized blood was obtained after the third and fourth booster injections. Erythrocytes and leucocytes were separated by centrifugation (5000g, 30 min) and concentrated merthiolate (final concn. 0.01%) was added to the antisera. Antibody titre to a specific protein and possible cross-reactivity with each of the other three proteins as well as with the proteins of guinea-pig serum, kidney, liver, heart and testes were evaluated by the Ouchterlony (1968) double-diffusion technique. Antisera specificity was also evaluated by immunoelectrophoresis (Zydeck et al., 1965).

Preparation of donkey anti-(goat γ-globulin) serum

Solid anhydrous Na₂SO₄ (final concn. 16%) was slowly added with stirring over 20–30 min at room temperature (22°C) to non-immune serum obtained from a goat not previously injected. The mixture was stirred for 30 min and the precipitate of γ-globulin was obtained by centrifugation at 5000g for 2h. The precipitate was dissolved in 154 mM-NaCl, dialysed against water for 48h and freeze-dried. The freeze-dried powder was dissolved in enough 0.9% NaCl to give a protein concentration of 2 mg/ml. This solution, which contained the goat serum γ-globulin fraction, was then used as antigen in the preparation of donkey anti-goat serum as follows. An emulsion (1.5 ml) of Freund's adjuvant containing 1.2 mg of the goat γ-globulin was injected subcutaneously into a donkey at five separate sites. At 3 weeks later a series of five booster injections was begun. The first four, increasing stepwise from 1 to 4 mg of protein, were given over a period of 4 weeks. At 6 weeks later a fifth booster injection of 5 mg was given. Then 10 days later the donkey was bled and its serum found to have very high titre of antibody to goat γ-globulin, as measured by the Ouchterlony double-diffusion technique. Donkey γ-globulins were precipitated and prepared as were the non-immune goat γ-globulins (see above). The precipitate of donkey anti-goat globulin was taken up in a volume of 0.9% NaCl one-third that of the original donkey serum. Titre of the donkey anti-(goat γ-globulin) globulin was ascertained by neutralization tests, which showed that 200 μl of the solution gave maximum precipitation of the goat γ-globulins in 50 μl of a 1:50 dilution of non-immune goat serum (Mauer, 1971). This titre was sufficiently high for its use as a second-stage antibody preparation, which was needed for maximum precipitation of the first-stage antigen–antibody complex.

Source of seminal-vesicle mucosa

Adult male guinea pigs from the inbred Mayo colony, which has been in existence for 40 years, were selected with an effort to standardize by weight (837 ± 106 g, mean ± S.D., n = 46). Seminal vesicles were excised at the same time (early morning) approx. 1 to 2h after the lights were turned on (16h of light and 8h of dark). The guinea pigs were used on days 1, 2 and 3 after removal from harems of 6–8 females.

Protein synthesis

Under diethyl ether anaesthesia seminal vesicles were excised close to the vas deferens through a lower midline abdominal incision and immediately placed in ice-cold 0.9% NaCl. The vesicles were slit open longitudinally and the mucosa was isolated as previously described (Prendergast & Veneziale, 1975).

Incubation technique. Erlenmeyer flasks (25 ml) with a side arm and perforated stopper were used. A tube carrying humidified gas (O_2/CO_2, 19:1) was attached to the side arm. The tissue was incubated in 2.0 ml of modified Krebs–Ringer bicarbonate solution containing (mM): Na^+, 143; K^+, 10.8; Ca^{2+}, 2.5; Mg^{2+}, 1.18; Cl^-, 123; P_i, 5.9; SO_4^{2-}, 1.18; HCO_3^-, 24.8. The pH was 7.4. To each flask was added 50 μl of 0.4 M-glucose and various ^{14}C-labelled amino acids as indicated in the appropriate Figure legends. When used the amino acids were added to the Erlenmeyer incubation flasks as follows: 50 μl of [U-14C]glycine (8.5 nmol; 2.1×10^6 d.p.m.); 50 μl of L-[U-14C]lysine hydrochloride (3.1 nmol; 2.0×10^6 d.p.m.); 50 μl of L-[U-14C]histidine (2.7 nmol; 1.9×10^6 d.p.m.); 50 μl of L-[U-14C]glutamate (3.6 nmol; 2.3×10^6 d.p.m.); 100 μl of L-[U-14C]glutamine (31.2 nmol; 3.6×10^6 d.p.m.); 100 μl of L-[U-14C]arginine hydrochloride (5.9 nmol; 4.2×10^6 d.p.m.). All amino acids were from Amersham–Searle, Arlington Heights, IL, U.S.A.

Incubation was at 37°C in a Dubnoff shaker (80 strokes/min) with continuous gas flow via the side arm at approx. 0.5 litre/min. When the total amount of newly synthesized protein was to be measured, both tissue and medium were transferred with 60 μl of 0.1 M-EDTA to a Potter–Elvehjem homogenizer. The tissue was then homogenized at 4°C by using 15 strokes. The homogenate was centrifuged at 27 500 *g* for 50 min and the supernatant transferred to a plastic vial to which had been added 2 μl of 10% (v/v) merthiolate. This supernatant, which was usually 2.0 ml in volume, contained the four specific seminal-vesicle proteins which were newly formed and thus labelled with [14]C. It included those that were secreted as well as those retained inside the cells.

Assay for proteins newly synthesized in vitro by isolated seminal-vesicle mucosa

Only plastic tubes and pipettes were used. A portion (20 μl) of the appropriate supernatant fraction of the seminal-vesicle mucosal preparation was mixed with 880 μl of 154 mM-NaCl, 200 μl of specific antiserum, 100 μl of 5% bovine serum albumin in 0.05 M-sodium phosphate buffer, pH 7.5, and 2 μl of 10% merthiolate [(2-carboxyphenylthio)-ethylmercury (sodium salt)]. This mixture was left for 24 h at 4°C and then 200 μl of donkey anti-(goat γ-globulin) globulin was added. The mixture was left for 24 h at 4°C, after which the precipitate was collected by centrifugation at 5000 *g* for 30 min. The supernatant was discarded and the precipitate, which included the specific protein of interest, was washed with 2×1.0 ml of 154 mM-NaCl with rigorous mechanical vortexing and collected each time by centrifugation. Washings were counted for any radioactivity caused by free labelled amino acids and the results showed that a third wash was unnecessary.

The precipitate, which consisted of a complex of newly formed labelled protein, goat antibodies and donkey anti-goat antibodies, was dissolved in 0.5 ml of 0.01 M-HCl. The solution, together with two washes (each of the 0.5 ml of HCl solution), was transferred to a scintillation vial containing 15 ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.) and counted for radioactivity in a Nuclear–Chicago scintillation counter with an efficiency of 84%. Results are reported as d.p.m. in a specific protein/mg of total protein in the fraction analysed or as d.p.m./ml of fraction analysed. Total protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Cycloheximide, actinomycin D and cordycepin (Fan & Penman, 1970; Penman *et al.*, 1970; Singer & Penman, 1972) were from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Distinction between the fractions of newly formed intracellular and extracellular proteins

After incubation with labelled amino acids as above, tissue was removed from its surrounding medium, washed once in ice-cold 0.9% NaCl and then homogenized in 2.0 ml of 0.9% NaCl containing 3 mM-EDTA. The 27 500 *g* supernatant fraction was obtained exactly as described above. This fraction
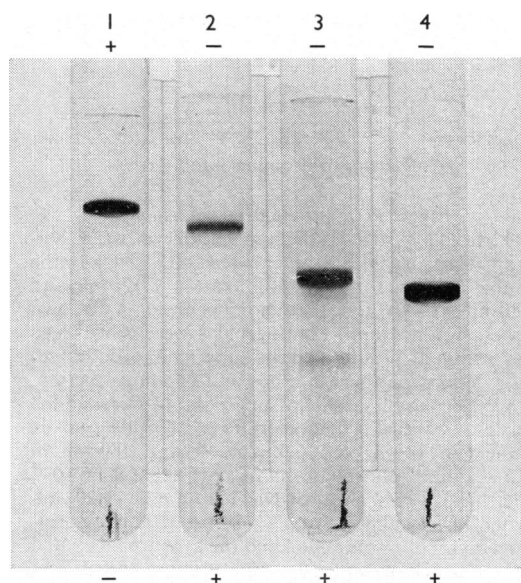

Fig. 1. *Polyacrylamide-gel disc electrophoresis of purified seminal-vesicle proteins*

Protein 1 (2.5–5.0 μg) was evaluated by a cationic system and proteins 2, 3 and 4 by an anionic system as described previously (Veneziale & Deering, 1976*a*).
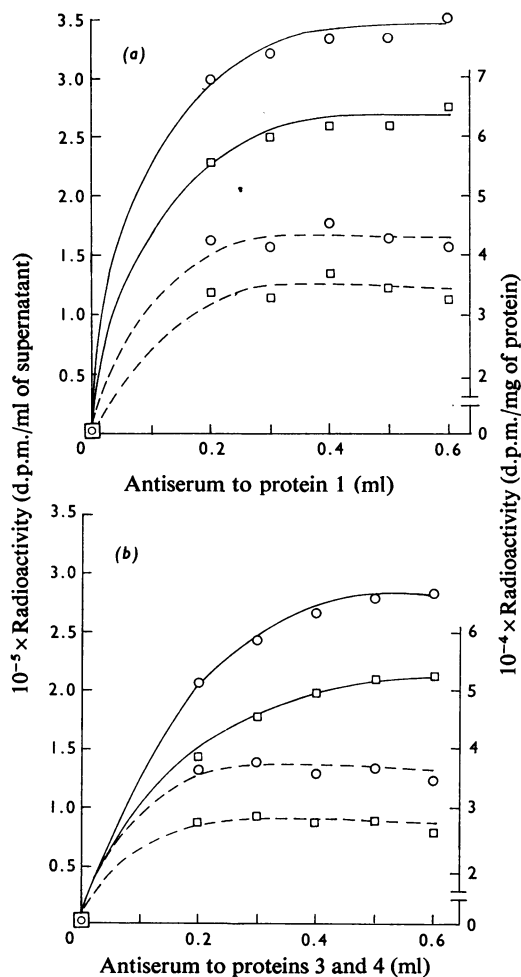
Fig. 2. *First-stage antiserum required*
(*a*) Seminal-vesicle epithelium (mean wet wt. 0.39g) from four organs was incubated with [^{14}C]glycine and [^{14}C]lysine for 1 min, 60min (— — —), 120min and 180min (———) and then homogenized in its own incubation medium. Homogenates were centrifuged for 60min at 27500g and the supernatants collected. For details see the Materials and Methods section. To 50μl samples of the supernatants were added various increasing concentrations of antiserum as indicated, 100μl of 5% bovine serum albumin in 0.5m-sodium phosphate buffer, pH7.5, 2μl of 10% merthiolate and 154mm-NaCl to make 1.202ml. After 24h incubation at 4°C, 200μl of donkey anti-goat γ-globulin was added and the mixture incubated as before. Subsequently, the immunoprecipitates were collected, washed, dissolved and then counted for radioactivity as described in the text. The 120min incubation sample (not shown) was only analysed with 0.4ml of antiserum; the results were 310000 d.p.m./ml of supernatant and 50300d.p.m./mg of supernatant protein. (*b*) As in (*a*) the first-stage anti-

was analysed for specific newly formed intracellular seminal-vesicle proteins. The incubation medium from which mucosa had been removed was centrifuged at 400g for 90s to precipitate any isolated cells that had separated from the intact mucosa. This supernatant fraction was analysed for specific newly formed seminal-vesicle proteins that had been trans-located, in some as yet unknown way, into the medium.

Results

Purification of seminal-vesicle lumenal proteins

Lumenal proteins were purified by DEAE-cellulose column chromatography (Veneziale & Deering, 1976a). Proteins corresponding to peaks 1, 2 and 4 gave only single bands when analysed by anionic, cationic and sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis. Fig. 1 shows results obtained after electrophoresis of protein 1 in 10% (w/v) polyacrylamide gel at pH3.5 containing 5.3m-urea, and of proteins 2, 3 and 4 in 7% poly-acrylamide gel at pH8.3. [For a detailed account of their electrophoretic behaviour on polyacrylamide gels and their partial characterization see Veneziale & Deering (1976a)]. Protein 3 is relatively impure; it contains a band with a mobility similar to protein 4 and a fast-moving band, which may be a low-molecular-weight breakdown fragment of protein 3 (see Veneziale & Deering, 1976a).

Evaluation of antisera

Precipitation lines on Ouchterlony double-diffusion gels indicated immunological recognition and specificity of antiserum against protein 1 for protein 1 and the cross-reaction of antiserum against protein 4 for both proteins 3 and 4, but not proteins 1 and 2. The gels demonstrated that 5μl of antiserum against protein 1 reacted strongly with 625ng of protein 1, but only faintly with 312ng of protein 1. Antiserum against protein 2 reacted strongly with as little as 78ng of protein 2. Antiserum (5μl) against protein 4 reacted individually with 312ng of proteins 3 and 4. Double-diffusion analysis also demonstrated that

serum used was that raised against protein 4. The 120min incubation sample (not shown) was only analysed with 0.4ml of antiserum; the results were 254000d.p.m./ml of supernatant and 41700d.p.m./mg of supernatant protein. In both (*a*) and (*b*) results are expressed as radioactivity/ml of supernatant (○) or radioactivity/mg of protein (□); the 1 min incubation was performed without the addition of antiserum and resulted in no supernatant radioactivity.
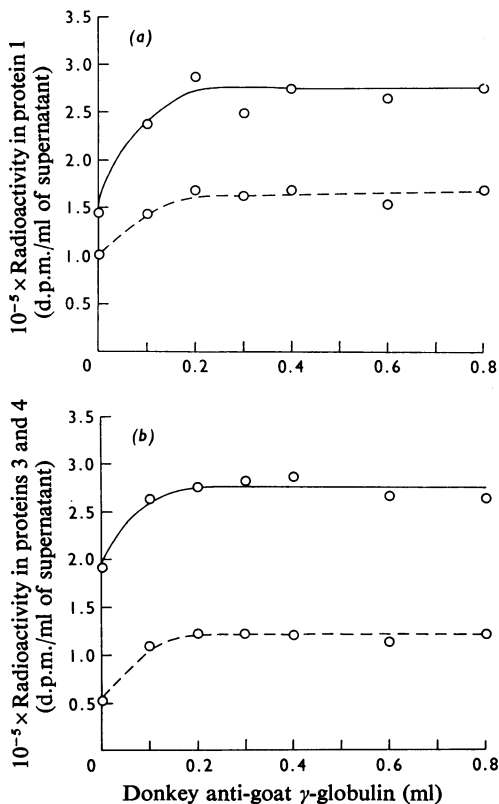
Fig. 3. *Second-stage antibody required*

(a) Seminal-vesicle epithelium (mean wet wt. 0.40 g) from two organs was incubated with [^{14}C]glycine and [^{14}C]lysine for 60 min (——) and 180 min (——) and then homogenized in its own incubation medium. Homogenates were centrifuged for 60 min at 27 500 g and supernatants collected. For details see the Materials and Methods section. To 20 μl samples of the supernatants were added 880 μl of 154 mM-NaCl, 200 μl of antiserum against protein 1, 100 μl of 5% bovine serum albumin in 0.05 M-sodium phosphate buffer, pH 7.5, and 2 μl of 10% merthiolate to make a final volume of 1.202 ml. After 24 h incubation at 4°C increasing volumes of donkey anti-goat γ-globulin were added as indicated with 0.9% NaCl to make the total volume added to each first-stage preparation 800 μl. The mixtures were then incubated as before. Subsequently, the immunoprecipitates were collected, washed, dissolved and then counted for radioactivity as described in the text. (b) The details are as in (a) except that antiserum added was that against proteins 3 and 4.

5 μl of antiserum against protein 3 reacted strongly against protein 3 and moderately against protein 2.

The antisera were also analysed by immunoelectrophoresis, which showed only single arcs between

proteins 1, 2, and 4 and their corresponding antisera. As expected, two arcs were formed in the reaction between my impure protein 3 fraction and antiserum against protein 4. The major one was undoubtedly due to antigen–antibody reaction between protein 3 and antiserum against protein 4, and the second was probably due to reaction between small contaminating amounts of protein 4 or the contaminating breakdown protein product and the antiserum.

By immunoelectrophoresis I also demonstrated weak immunological identification of my impure protein 3 preparation by antiserum against protein 2 and weak identification of protein 2 by antiserum against protein 4.

Multiple additional immunoelectrophoretic analyses were conducted with each of the four antiserum preparations against each of the four proteins. In addition, extensive studies were done by Ouchterlony double-diffusion methods to test possible reactivity of my antisera with extracts of guinea-pig testis, liver, kidney, heart, skeletal muscle and serum. The results were negative.

From the foregoing results the following conclusions were drawn. (1) Antiserum elicited by protein 3 was unacceptable and rejected for this study; (2) antiserum against protein 1 was highly specific and suitable for use in this study; (3) antiserum against protein 2 was specific for protein 2 except for a weak and probably insignificant reaction with protein 3 or some contaminating component of my impure protein 3 preparation; (4) antiserum against protein 4 was specific for both proteins 3 and 4 except possibly for weak and insignificant reaction with protein 2; (5) additional evidence was needed to determine if reactions between antiserum 2 and protein 3 and between antiserum against protein 4 and protein 2 were truly insignificant, compared with the primary antigen–antibody reactions, in the immunoprecipitation methods developed for this study of specific protein synthesis. Such evidence was obtained by means of polyacrylamide-gel disc electrophoresis which disclosed the nature of the immunoprecipitation products of antisera to proteins 2 and 4 (see below).

First-stage antiserum requirements

These were established by immunoprecipitation of the newly formed protein in 50 μl samples of tissue extract by using various increasing volumes of antiserum (see Fig. 2). From the Figure, it should be apparent that 0.5 ml of antiserum (i.e. a volume 10 times that of extract) resulted in maximum precipitation of newly formed proteins 1, 3 and 4. This is the case for protein 2 also, since antiserum against protein 2 was strongest of all the antisera on the basis of the observation that 5 μl formed a precipitin line with only
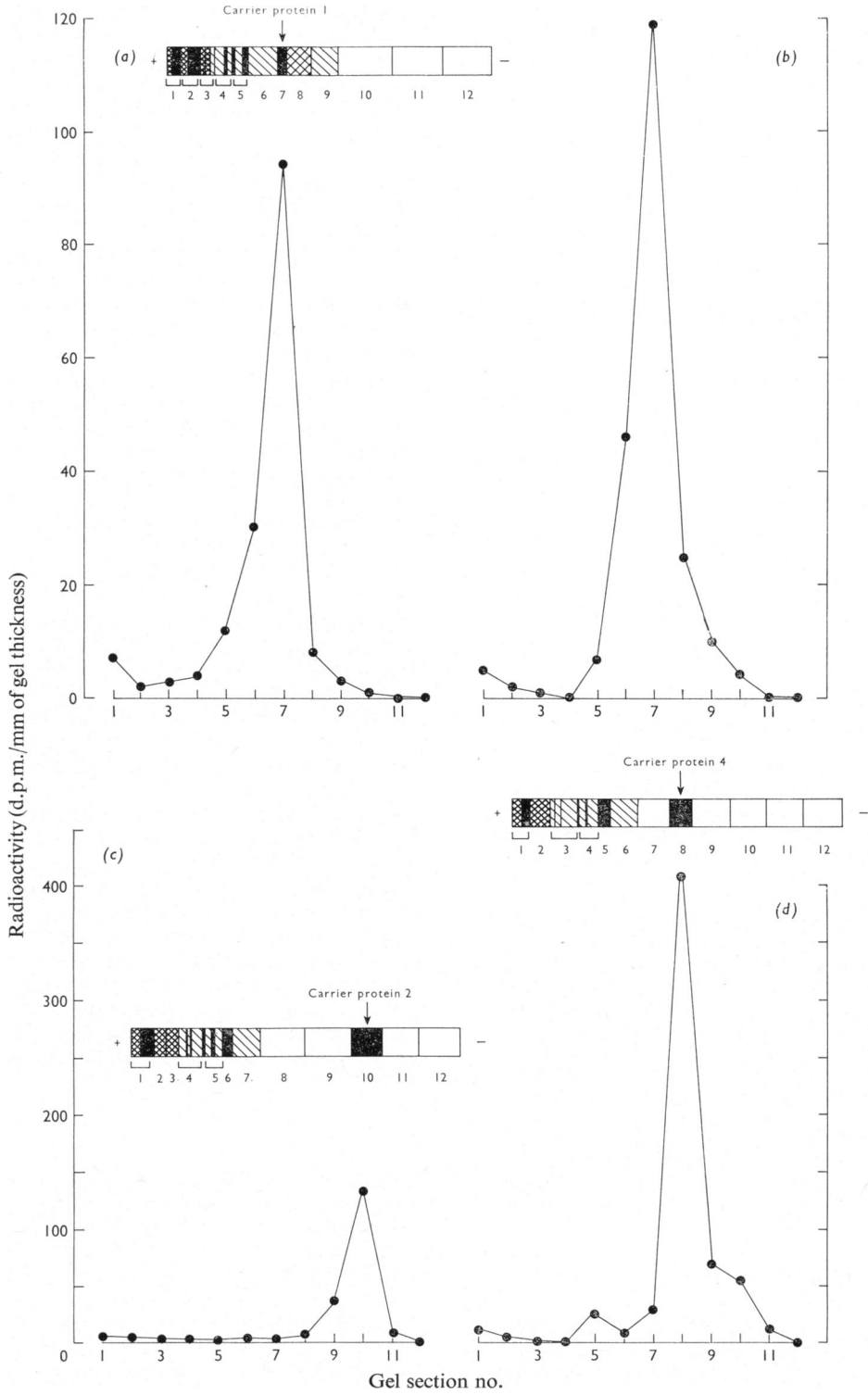
78 ng of protein 2 (see above). Second-stage antibody without prior addition of first-stage antibody did not precipitate newly formed protein. Second-stage donkey anti-goat γ-globulin requirements were established by using increasing amounts of second-stage antibody (Fig. 3). The requirement for maximum precipitation of antigen–antibody was 0.2 ml. The results point out that unless second-stage antibody is used only incomplete precipitation of first-stage complex will occur.

Identification of immunoprecipitable products (Fig. 4)

This was accomplished by means of a cationic polyacrylamide-gel disc electrophoretic system which separates proteins 1 and 2, and both 1 and 2 from 3 and 4. Proteins 3 and 4 co-migrate in this system. In the first electrophoretogram (Fig. 4a) 78% of the recovered radioactivity was localized to the position of protein 1. In the second electrophoretogram (Fig. 4b) 75% of the radioactivity was localized to the position of protein 1. None of the remaining radioactivity was localized to the known positions of the other soluble secreted proteins. Instead, the remaining radioactivity was evenly dispersed throughout the gels and was only slightly above the background value in each gel segment. In the third electrophoretogram (Fig. 4c) 87% of the radioactivity was localized to the position of protein 2 and the remainder distributed evenly throughout the gel. Thus under our assay conditions antisera to proteins 1 and 2 were specific for proteins 1 and 2 respectively. In the fourth electrophoretogram (Fig. 4d) 75% of the radioactivity was localized to the position occupied by proteins 3 and 4. Only 15% was localized to section 10, which would correspond to the position of protein 2 and the 'breakdown product' of protein 3 (Veneziale & Deering, 1976a). The amount localized to section 10, i.e. 15% of the total gel radioactivity, represents a maximum value, because in this experiment I used a combination of four labelled amino acids so that there was a great incorporation of label into all newly synthesized proteins. Thus antiserum

to protein 4 precipitates proteins 3 and 4, and what might be a minority of newly formed protein 2. But, more likely, antiserum to protein 4 precipitates fragments of proteins 3 and 4 that were nascent and incomplete at the time of tissue homogenization and/or fragments that resulted from proteolytic degradation of proteins 3 or 4. Indeed the immunoprecipitation of peptide fragments of a primary protein has already been suggested by studies of the chick oviduct system (Palmiter et al., 1972). In any case the amount of this minor component of radioactivity that was precipitated by antiserum to protein 4 is too small to invalidate use of the antiserum in studies on the biosynthesis of proteins 3 and 4.

Additional evidence was sought for the correct identification of immunoprecipitable product by discriminating choice of labelled amino acids as precursor molecules, on the basis of previous analysis of the proteins for amino acid content (Veneziale & Deering, 1976a). For example, protein 1 is rich in lysine, glycine and glutamine, but poor in histidine and glutamate. In contrast, proteins 2, 3 and 4 are relatively rich in histidine and glutamate. Protein 1 has the least content of arginine, proteins 3 and 4 the greatest and protein 2 an intermediate content (Veneziale & Deering, 1976a). The incorporation of label into proteins that were newly formed from the specific amino acids and that were identified by immunoprecipitation did indeed reflect the foregoing facts about amino acid composition.
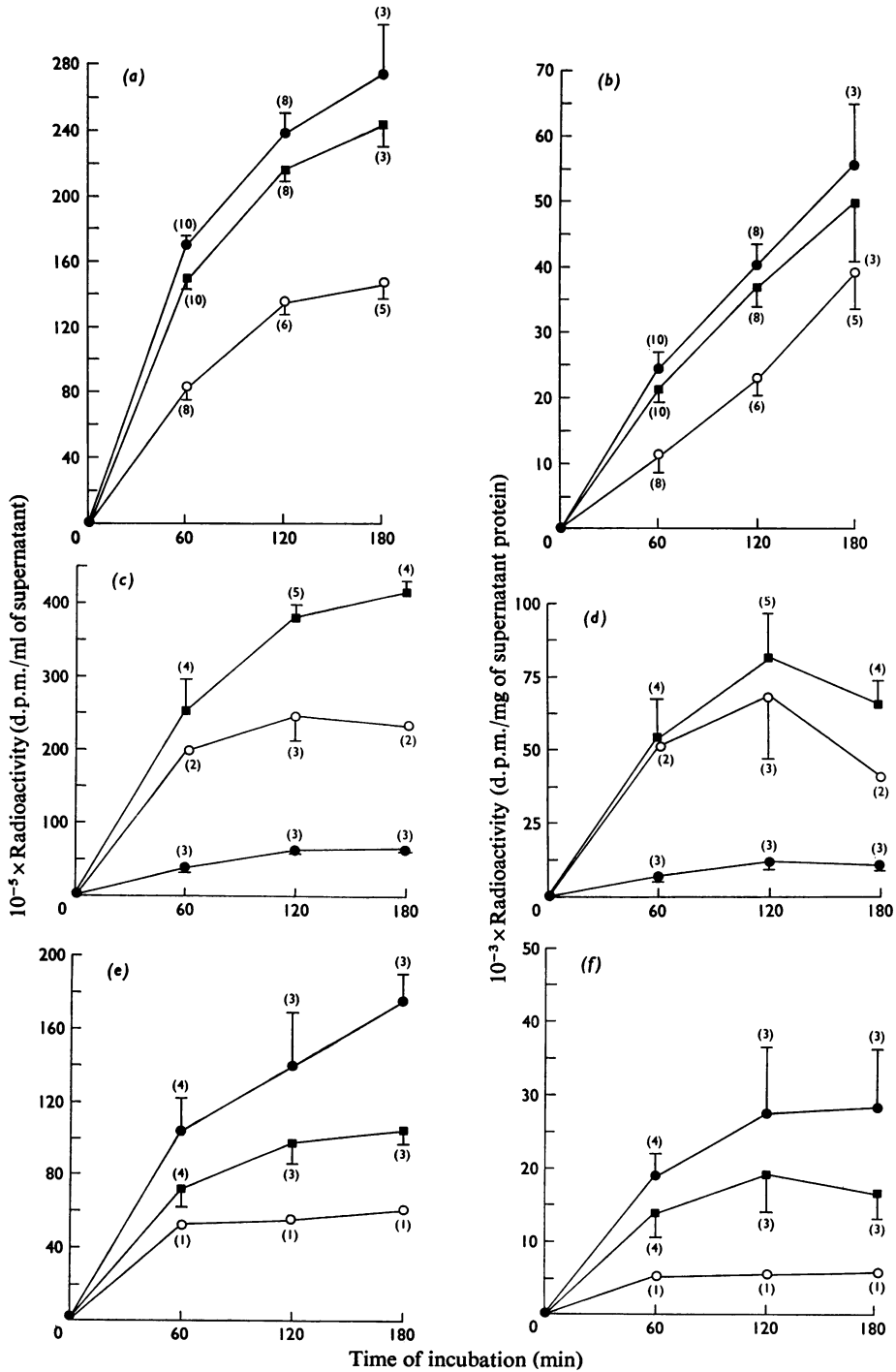
Protein synthesis

Fig. 5 shows that protein synthesis from [^{14}C]-lysine and [^{14}C]glycine occurs essentially linearly with time during 180 min of incubation (Fig. 5b). Evidently, the availability of other amino acids did not limit the process. The highest incorporation, i.e. that into protein 1, reflects not only the relatively higher content of these amino acids in protein 1 (Veneziale & Deering, 1976a), but probably also the capability of the tissue to synthesize more of protein 1 than any of the other three proteins.

Fig. 4. *Electrophoresis of immunoprecipitable protein*

Immunoprecipitable protein newly formed from labelled amino acids was dissolved in 0.4 ml of 8 M-urea to which was added unlabelled carrier protein as indicated. After leaving for 3 h at room temperature, 50 μl was electrophoresed in 10% (w/v) polyacrylamide gel at pH 3.5 containing 5.3 M-urea (see Veneziale & Deering, 1976a) for 6 h. Stained gels were cut into sections that were individually minced, leached out with 1.0 ml of 6 M-guanidine hydrochloride for 12 h at 37°C and then dissolved in Aquasol and counted for ^{14}C radioactivity that was incorporated into protein. The total radioactivity corresponding to the peaks in (a), (b), (c) and (d) were 460, 810, 1220 and 2460 d.p.m. respectively. Relative to total gel radioactivity, the peaks represent recoveries of 78, 83, 87 and 75% respectively. For further details see the text. Mean recovery of radioactivity in the gels relative to that applied at the origin was 81% for four experiments (a)–(d), and 86% for (a), (b) and (d). Actual recovery might be even higher because the thick precipitates of guanidine hydrochloride that formed in Aquasol probably caused some self-absorption of radioactivity for which no correction was made. The immunoprecipitates were derived from experiments with precursors as follows: (a) (protein 1) [^{14}C]-glutamine; (b) (protein 1) [^{14}C]lysine and [^{14}C]glycine; (c) (protein 2) and (d) (proteins 3 and 4) [^{14}C]lysine, [^{14}C]glycine, [^{14}C]-histidine and [^{14}C]glutamate.

With [^{14}C]histidine and [^{14}C]glutamate (Figs. 5c and 5d) the situation was different. Label was incorporated at the slowest rate into protein 1, at the greatest rate into proteins 3 and/or 4 and at an intermediate rate into protein 2. This fits the data which show that histidine and glutamate are present at lowest

Time of incubation (min)

concentration in protein 1 (Veneziale & Deering, 1976a). Interestingly, [14C]-labelled protein synthesis was not linear with time, suggesting that endogenous lysine and/or glycine concentrations were limiting.

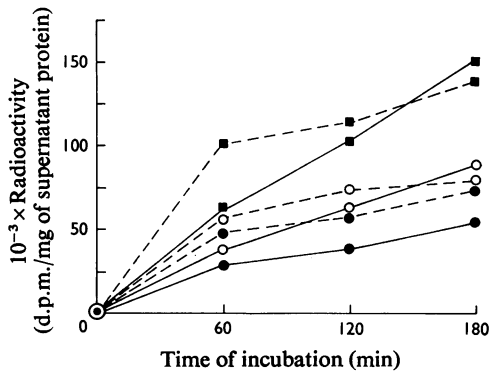Incorporation from [14C]glutamine was predominantly into protein 1, which is not surprising

because this basic protein is especially rich in glutamine (Veneziale & Deering, 1976a; Notides & Williams-Ashman, 1967). After 180 min of incubation with [14C]glutamine the radioactivity of protein 1, 28600 d.p.m./mg of supernatant protein (Fig. 5f), was only one-half that of protein 1, i.e. 55000 d.p.m./mg, after incubation with [14C]lysine and [14C]glycine (Fig. 5b). If one takes into account that the specific radioactivity of [14C]glutamine was only one-quarter that of [14C]lysine and one-half that of [14C]glycine, it appears that incorporation of glutamine carbon into protein 1 was relatively pronounced. [14C]-labelled protein synthesis from [14C]-glutamine was also not linear with time (Figs. 5e and 5f).

Incorporation of [14C]arginine into the proteins was pronounced, being approximately as great as that from [14C]lysine, [14C]glycine, [14C]histidine and [14C]glutamic when used in combination (Fig. 6).

Start of protein synthesis. This is indicated in Table 1. Newly formed protein was detected as early as 10–15 min.

Inhibitors of protein synthesis. These were studied *in vitro* (see Table 2). Cycloheximide was a potent inhibitor. In contrast, neither cordycepin nor actinomycin D inhibited, which would be consistent with the view that isolated seminal-vesicle mucosa contains pre-existent stable mRNA.

Distribution of newly formed protein. The protein distribution between incubated tissue and its surrounding medium was investigated (Table 3). Some 91–96% of the protein was present in the surrounding medium; the remainder was in the tissue. This would be consistent with the great secretory capacity of seminal-vesicle tissue, but it was not clear at this stage of the study if the isolated tissue retained the capacity to secrete proteins entirely in accord with its normal and physiological secretory mechanisms.

Fig. 6. *Protein synthesis from [14C]arginine and from a combination of labelled amino acids*

Four isolated seminal-vesicle mucosa preparations were incubated for 1 min, 60 min, 120 min and 180 min respectively, with [14C]arginine (4.1×10^6 d.p.m.; 5.86 nmol; 720000 d.p.m./nmol) (———). Four other preparations were incubated with a combination of [14C]lysine (1.98×10^6 d.p.m.; 3.1 nmol; 640000 d.p.m./nmol), [14C]glycine (2.10×10^6 d.p.m.; 8.5 nmol; 260000 d.p.m./nmol), [14C]histidine (1.93×10^6 d.p.m.; 2.69 nmol; 720000 d.p.m./nmol) and [14C]glutamate (2.30×10^6 d.p.m.; 3.59 nmol; 640000 d.p.m./nmol) (————). After incubation, tissue was homogenized in its own incubation medium and the homogenate centrifuged at 27 500g for 50 min. The supernatant was then assayed in duplicate for newly formed protein. See the Materials and Methods section for details. After 180 min incubation with [14C]arginine, 557000 d.p.m. was incorporated into protein 1, 744000 into protein 2 and 1260000 into proteins 3 and 4. ●, Protein 1; ○, protein 2; ■, proteins 3 and 4.

Discussion

It is appreciated that in immunoprecipitation analyses multiple lines of evidence for immunological specificity should be obtained whenever

Fig. 5. *Protein synthesis from various amino acids*

Seminal-vesicle mucosa from one organ was incubated in 2.0 ml of medium in the presence of glucose and labelled amino acids. After the appropriate incubation time, tissue was homogenized in its incubation medium and the homogenate centrifuged for 50 min at 27 500g. The supernatant was then assayed in duplicate for newly formed proteins. See the Materials and Methods section for details. Numbers in parentheses are the number of incubations *in vitro* carried out with mucosal tissue. Individual points represent mean values; vertical lines give s.e.m. The data based on volume of supernatant (*a, c* and *e*) were not corrected to a constant mucosa wet weight, which for 51 incubations was 0.42 ± 0.19 (mean ± s.d.). Thus individual 'd.p.m.' values multiplied by 2 give absolute total d.p.m. incorporated into specific proteins by the mucosa from one seminal vesicle during the indicated time intervals. Radioactivity data based on mg of protein present, rather than on less accurate and less meaningful wet-weight data, are given in (*b, d* and *f*). ●, Protein 1; ○, protein 2; ■, proteins 3 and 4. The amino acids used for protein synthesis were as follows: (*a* and *b*), [14C]lysine plus [14C]glycine; (*c* and *d*), [14C]histidine plus [14C]glutamate; (*e* and *f*), [14C]glutamine.

Table 1. *Start of protein synthesis*
See the Materials and Methods section for details.

Radioactivity (d.p.m./mg of supernatant protein)

	Protein 1		Proteins 3 and 4	
Precursors ...	[^{14}C]Glutamine	[^{14}C]Lysine+[^{14}C]glycine	[^{14}C]Glutamine	[^{14}C]Lysine+[^{14}C]glycine
Incubation time (min)				
1	28	63	24	69
10	—	1180	—	1570
15	978	—	1060	—
20	—	4610	—	4840
30	8580	—	5310	—
60	19300	33200	11800	27400

Table 2. *Effect of inhibitors on protein synthesis*

Isolated mucosa was preincubated for 10min with (+) and without (−) added inhibitor. Glucose, [^{14}C]glycine and [^{14}C]lysine were then added and the incubation was continued for 60 and 120min. Tissue was homogenized in its own incubation medium and centrifuged; supernatants were assayed for newly formed protein. See the Materials and Methods section for details. The mucosa tissues for each paired experiment were from the same animal. Thus 11 animals were used.

Radioactivity (d.p.m./mg of supernatant protein)

		Inhibitor ...	Cycloheximide		Cordycepin		Actinomycin D	
	Incubation time (min)	Inhibitor concn. ... Presence or absence of inhibitor	3.6 μM	7.2 μM	100 μM	200 μM	3.2 μM	8 μM
Protein 1	60	+	3430	2590	16100	28500	25500	20000
	60	−	16300	18200	15200	26300	26600	22600
	120	+		8900	31200	42100	43600	38300
	120	−		35300	36800	46700	34300	38600
Protein 2	60	+	1680	1320	6940	19750	11600	10100
	60	−	6360	9180	6780	20400	14800	10900
	120	+		7120	24900	26700	22300	19800
	120	−		21400	25300	31100	18800	21800
Proteins 3 & 4	60	+	2950	2190	12000	28000	20500	17500
	60	−	13200	17800	10900	30900	22300	18900
	120	+		9530	33000	41700	37300	35100
	120	−		32700	37600	45100	27200	33200

possible. In this study double-diffusion and immuno-electrophoresis were used extensively and the results lead us quickly to disqualify one antiserum for use and suggested possible cross-reacting combinations of proteins and antisera in my immunoprecipitation assays. I showed that the rate of incorporation of a specific amino acid precursor was in direct proportion to the content of that amino acid in the protein assayed by the specific immunoprecipitation method. I also demonstrated that the precipitated radioactivity comigrated as single major bands with purified standards of soluble secreted seminal-vesicle proteins after 6h of electrophoresis in a denaturing 10% polyacrylamide gel at pH3.5 and containing 5.3M-urea. The only qualification to this would be the very minor radioactive component precipitated by antiserum to protein 4 and best identified tentatively as possible smaller peptide fragment(s) of proteins 3 and/or 4. Several authors have suggested that sodium dodecyl sulphate/polyacrylamide-gel electrophoresis be used to establish immunospecificity (Cashman & Pitot, 1971; Palmiter *et al.*, 1971). Although I agree, this was not feasible in my study for several reasons, including the fact that proteins 1, 3 and 4 co-migrate in sodium dodecyl sulphate/polyacrylamide gels.

The immunospecificity of antisera to proteins 1 and 2 in our assay procedure was readily demonstrable. I believe that the lack of specificity of antiserum to

Table 3. *Distribution of newly formed* ^{14}*C-labelled protein*

After incubation with [^{14}C]lysine and [^{14}C]glycine tissue was separated from its surrounding medium and the radioactivity in newly formed protein was determined. See the Materials and Methods section for details. Values are means ± S.E.M. for the numbers of samples in parentheses.

Incubation time (min)	Location	Radioactivity (d.p.m./mg of supernatant protein)
Protein 1		
60	Tissue	3530 ± 620 (6)
60	Medium	51 400 ± 4850 (6)
120	Tissue	3580 (2)
120	Medium	88 200 (2)
Protein 2		
60	Tissue	4030 ± 836 (5)
60	Medium	39 200 ± 6150 (5)
120	Tissue	4600 (1)
120	Medium	89 800 (1)
Proteins 3 and 4		
60	Tissue	4330 ± 848 (6)
60	Medium	52 300 ± 4000 (6)
120	Tissue	4170 (2)
120	Medium	96 300 (2)

protein 4, i.e. its immunoreactivity with proteins 3 and 4, was not due to the use of impure protein 4 as antigen. Instead, proteins 3 and 4 probably share similar antigenic sequences, each eliciting cross-reacting antibodies. Perhaps this should have been anticipated on the basis that proteins 3 and 4 are derived from the same cell and are so similar, e.g. with regard to molecular weight, Svedberg constants, isoelectric points and electrophoretic behaviour (Veneziale & Deering, 1976a). The precipitation of protein 3 by antiserum against protein 4 brings to mind the precipitation of conalbumin by antibodies elicited by ovalbumin (Palmiter *et al.*, 1971); however, that situation is not analogous.

Rates of protein synthesis by isolated mucosal preparations were high. For example, after 180 min incubation with [^{14}C]lysine and [^{14}C]glycine containing 4.08×10^6 d.p.m. of radioactivity, 1.38×10^6 d.p.m. or 34% of the starting material was incorporated into the four newly formed proteins. With [^{14}C]histidine and [^{14}C]glutamate 36% was incorporated and with [^{14}C]glutamine 25%. It is possible to calculate the least amount of protein synthesized from the [^{14}C]arginine experiments of Fig. 6 in which 5.86 nmol of [^{14}C]arginine containing 4.2×10^6 d.p.m. was incubated. After 180 min, protein 1 contained 556 000 d.p.m. and protein 2 744 000 d.p.m. From previous work we learned that protein 1 contains 445 nmol of arginine/1000 μg of protein and protein 2 760 nmol/1000 μg. From these data and assuming no dilution of added [^{14}C]arginine by endogenous

arginine one can calculate that 1710 ng of protein 1 and 1330 ng of protein 2 were synthesized. A reasonable estimate is that microgram quantities of protein are formed per 60 min incubation even if the dilution by endogenous arginine is 1:1000.

Protein synthesis is subjected to hormonal regulation in various mammalian target tissues: in liver by growth hormone, cortisol and thyroxin (Korner, 1965; Tata, 1968; Sippel *et al.*, 1975; Kurtz *et al.*, 1976), in mammary tissue by prolactin and oestrogen (Hilf *et al.*, 1967; Topper, 1970; Turkington, 1972), in seminal vesicle and prostate by androgen (Wilson, 1962; Kochakian, 1964; Williams-Ashman *et al.*, 1964) and in uterus by oestrogen (Mueller, 1965). Studies of protein synthesis by sex tissues have usually had the limitation that synthesis of cell-specific protein was not studied. However, androgen-dependent synthesis of basic secretory proteins by minced rat seminal vesicles has been described (Higgins *et al.*, 1976a), and that system has already yielded important information about control of nucleic acid content and cell proliferation by testosterone (Higgins *et al.*, 1976b). The biosynthesis of tissue-specific proteins occurs in oestrogen- and progesterone-dependent chick oviduct, and protein synthesis in that tissue has been studied extensively (Kohler *et al.*, 1969; Palmiter *et al.*, 1970; Palmiter & Wrenn, 1971; Palmiter, 1972). Like the chick oviduct, my isolated seminal-vesicle mucosal preparation will provide a unique opportunity to study the synthesis of abundant soluble secreted cell-specific proteins. Unlike the magnum of the chick oviduct, which is composed of at least three different types of epithelial cell, seminal-vesicle mucosa appears to consist of only one epithelial cell type. My use of this preparation represents the original elaboration of specific protein synthesis in a cellularly homogeneous tissue that is androgen-dependent. Because seminal-vesicle mucosa is both mammalian in origin and androgen-dependent, its capability for protein biosynthesis should be of importance in studies on how various androgens might subserve the functions of synthesis, packaging and secretion of specific proteins. However, I have here concerned myself only with the general features of specific protein synthesis by isolated seminal-vesicle mucosa from intact animals.

References

Büchi, K. A. & Veneziale, C. M. (1977) *Andrologia* in the press

Cashman, D. & Pitot, H. C. (1971) *Anal. Biochem.* **44**, 559–569

Fan, H., & Penman, S. (1970) *J. Mol. Biol.* **50**, 655–670

Flickinger, C. J. (1974) *Anat. Rec.* **180**, 407–426

Higgins, S. J., Burchell, J. M. & Mainwaring, W. I. P. (1976a) *Biochem. J.* **158**, 271–282

Higgins, S. J., Burchell, J. M. & Mainwaring, W. I. P. (1976b) *Biochem. J.* **160**, 43–48

Hilf, R., Michel, I. & Bell, C. (1967) *Recent Prog. Horm. Res.* **23**, 229–295

Kochakian, C. D. (1964) *Acta Endocrinol.* **46**, *Suppl.* 92, 3–16

Kohler, P. O., Grimley, P. M. & O'Malley, B. W. (1969) *J. Cell Biol.* **40**, 8–27

Korner, A. (1965) *Recent Prog. Horm. Res.* **21**, 205–240

Kurtz, D. T., Sippel, A. E. & Feigelson, P. (1976) *Biochemistry* **15**, 1031–1036

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mann, T. (1964) in *The Biochemistry of Semen and of the Male Reproductive Tract*, Methuen, London

Manyai, S. (1963) *Acta Physiol. Acad. Sci. Hung.* **24**, 11–28

Mauer, P. H. (1971) in *Methods in Immunology and Immunochemistry* (Williams, C. A. & Chase, M. W., eds.), vol. 3, pp. 1–58, Ann Arbor–Humphrey Science Publishers, Ann Arbor

Mueller, G. C. (1965) in *Mechanism of Hormone Action* (Karlson, P., ed.), p. 28, Academic Press, New York

Notides, A. C. & Williams-Ashman, H. G. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1991–1995

Ouchterlony, O. (1968) in *Handbook of Immunodiffusion and Immunoelectrophoretic Techniques*, Ann Arbor–Humphrey Science Publishers, Ann Arbor

Palmiter, R. D. (1972) *J. Biol. Chem.* **247**, 6450–6461

Palmiter, R. D. & Wrenn, J. T. (1971) *J. Cell Biol.* **50**, 598–615

Palmiter, R. D., Christensen, A. K. & Schimke, R. T. (1970) *J. Biol. Chem.* **245**, 833–845

Palmiter, R. D., Oka, T. & Schimke, R. T. (1971) *J. Biol. Chem.* **246**, 724–737

Palmiter, R. D., Palacios, R. & Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 3296–3304

Penman, S., Rosbash, M. & Penman, M. (1970) *Proc. Natl. Acad. Sci.* **67**, 1878–1885

Prendergast, F. G. & Veneziale, C. M. (1975) *J. Biol. Chem.* **250**, 1282–1289

Singer, R. H. & Penman, S. (1972) *Nature (London)* **240**, 100–102

Singhal, R. L. & Ling, G. M. (1969) *Can. J. Physiol. Pharmacol.* **47**, 233–239

Singhal, R. L. & Valadares, J. R. E. (1968) *Biochem. J.* **110**, 703–711

Sippel, A. E., Feigelson, P. & Roy, A. K. (1975) *Biochemistry* **14**, 825–829

Steer, R. C. & Veneziale, C. M. (1977) *Andrologia* in the press

Tata, J. R. (1968) *Nature (London)* **219**, 331–337

Topper, Y. J. (1970) *Recent Prog. Horm. Res.* **26**, 287–308

Turkington, R. W. (1972) in *Biochemical Actions of Hormones* (Litwack, G., ed.), vol. 2, pp. 55–80, Academic Press, New York

Veneziale, C. M. & Deering, N. (1976a) *Andrologia* **8**, 73–82

Veneziale, C. M. & Deering, N. (1976b) *Andrologia* **8**, 271–276

Veneziale, C. M., Brown, A. L. & Pendergast, F. G. (1974) *Mayo Clin. Proc.* **49**, 309–313

Veneziale, C. M., Steer, R. C. & Buchi, K. (1977) in *Advances in Sex Hormone Research* (Thomas, J. A. & Singhal, R. L., eds.), vol. 3, pp. 1–50, University Park Press, Baltimore

Williams-Ashman, H. G., Liao, S., Hancock, R. I., Jurkowitz, L. & Silverman, D. A. (1964) *Recent Prog. Horm. Res.* **20**, 247–301

Wilson, J. D. (1962) *J. Clin. Invest.* **41**, 153–161

Zydeck, F. A., Muirhead, E. E. & Schneider, H. (1965) *Nature (London)* **205**, 189–190