Some Factors Affecting the Production, by Cultured Baby-Hamster Kidney Cells, of BHK Glycoprotein I which Cross-React Immunologically with Tamm–Horsfall Glycoprotein

By FREDERICK J. BLOOMFIELD,* DIANA R. DUNSTAN,*† CHARLES L. FOSTER,‡
FRANCA SERAFINI-CESSI*§ and R. DEREK MARSHALL*||

*Department of Chemical Pathology and ‡Department of Cellular Biology and Histology,
St. Mary's Hospital Medical School, London W2 1PG, U.K.

(Received 29 July 1976)

Cultured baby-hamster kidney cells (BHK-21/C13), which are adapted to grow in suspension (strain 2P), produce a glycoprotein, termed BHK glycoprotein I, which cross-reacts immunologically with hamster urinary Tamm–Horsfall glycoprotein. BHK glycoprotein I was isolated in an electrophoretically (sodium dodecyl sulphate/polyacrylamide gel) homogeneous form by application of affinity chromatography to the medium in which cells had been cultured. Insolubilized anti-(Tamm–Horsfall glycoprotein immunoglobulin G) was used as the adsorbent. The amount of BHK glycoprotein I associated with the cultured cells was found by both radioimmunoassay and immunofluorescence to be related to the amount of Ca^{2+} in the medium and to the particular stage of the cell cycle. 5'-Nucleotidase was also shed by the cells into the culture medium in amounts related to the stage of the cell cycle. The turnover of hamster Tamm–Horsfall glycoprotein *in vivo* appeared to be considerably more rapid than can be accounted for by cell turnover. Hamster Tamm–Horsfall glycoprotein was shown to be ineffective in inhibiting agglutination of chicken erythrocytes caused by influenza virus.

There is in human urine a glycoprotein which acts *in vitro* as a potent inhibitor of haemagglutination induced by myxoviruses (Tamm & Horsfall, 1950, 1952), and which is probably produced in the kidney tubules (Keutel, 1965; Cornelius *et al.*, 1965; Friedman, 1966; McKenzie & McQueen, 1968; Schenk *et al.*, 1971; Hoyer *et al.*, 1974). The function of the glycoprotein is unknown, although its pathology is of considerable interest for several reasons. Firstly, urinary casts, produced in the nephrotic syndrome, are composed to a large extent of aggregated Tamm–Horsfall glycoprotein (McQueen, 1962; Fletcher *et al.*, 1970), and it was suggested that the oligoanuric phase of acute renal failure could result from intratubular precipitation of the glycoprotein (Patel *et al.*, 1964). Cell-mediated immunity against the glycoprotein was reported in a very large proportion of patients with either active chronic hepatitis or primary biliary cirrhosis associated in either case with renal tubular acidosis (Tsantoulas *et al.*, 1974). Finally, circulating anti-

bodies, of the IgG γ class, against Tamm–Horsfall glycoprotein were reported in the sera of patients with acute pyelonephritis (Hanson *et al.*, 1976).

The urine of the hamster contains a closely related glycoprotein (Dunstan *et al.*, 1974), and cultured baby-hamster kidney cells (BHK-21/C13; MacPherson & Stoker, 1962) adapted to grow in suspension (Capstick *et al.*, 1966) produce a glycoprotein (or glycoproteins) which was found to cross-react immunologically with hamster urinary glycoprotein.

A procedure for isolating the glycoprotein, which will be called BHK glycoprotein I, in an apparently homogeneous state from the medium in which cells have been cultured is now described. Studies are also described of the accumulation of the glycoprotein in the cultured cells as a result of altering various parameters.

Some of the results were presented at the Third International Symposium on 'Glycoconjugates: Functions in Animals', which was held at the University of Sussex, Sussex, U.K. (6–12 July 1975).

Materials

Culture medium (Glasgow modification of minimum essential medium; MacPherson & Stoker,

¶ Abbreviations: IgG, IgM, immunoglobulins G and M.

† Present address: Medical Research Council Headquarters, Park Crescent, London W1, U.K.

§ Present address: Istituto di Patologia Generale, Università di Bologna, Bologna, Italy

|| To whom reprint requests should be addressed.

1962), foetal calf serum, antibiotic (penicillin-streptomycin) solution and tryptose phosphate broth were purchased from Gibco-Biocult, Paisley, Renfrewshire, Scotland, U.K. CNBr-Sephadex and Sephadex G-200 were bought from Pharmacia (G.B.) Ltd., London W.5, U.K. The Radiochemical Centre, Amersham, Bucks., U.K. supplied D-[^{14}C]glucosamine hydrochloride (55 Ci/mol), KB^3H_4 (308 Ci/mol), [^3H]thymidine (2.5 Ci/mol), [$^2\text{-}^3\text{H}$]AMP (ammonium salt; 5000 Ci/mol) and Na^{125}I . DEAE-cellulose (DE-23) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., trypsin from Sigma (London) Chemical Co., London S.W.6, U.K., and fluorescein-labelled sheep anti-(rabbit immunoglobulin) from Wellcome Research Laboratories, Beckenham, Kent, U.K. Samples of cultured baby-hamster kidney cells (BHK-21/C13, substrain 2P) were supplied by the Wellcome Laboratory, Pirbright, Surrey, U.K. Syrian hamsters were obtained from Coombehurst Breeding Establishment, Baughurst, Basingstoke, Hants., U.K.

Methods

Isolation and determination of Tamm-Horsfall glycoprotein

Isolation of hamster urinary Tamm-Horsfall glycoprotein was done by procedures that involved chromatography on Sepharose 4B (Dunstan *et al.*, 1974). The glycoprotein was homogeneous, as assessed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Plate 4a).

Antiserum to Tamm-Horsfall glycoprotein was raised in rabbits by intramuscular injection every 2 weeks of 0.5 ml of an emulsion containing equal volumes of the glycoprotein (1 mg/ml of water) and complete Freund's adjuvant (Calbiochem, Hereford, U.K.). Blood, taken from the ear veins of the immunized rabbits, was allowed to clot and the serum was either used directly or various partially purified fractions were prepared, as described below.

Determination of Tamm-Horsfall glycoprotein and of BHK glycoprotein I was done by radioimmunoassay (Dunstan *et al.*, 1974).

The Tamm-Horsfall glycoprotein content of Syrian-hamster kidney was measured by a method closely similar to that described by Grant & Neuberger (1973).

Polyacrylamide-gel disc electrophoresis in the presence of sodium dodecyl sulphate

This was carried out at 4°C by the procedure of Marshall & Zamecnik (1969) with 9.6% (w/v) polyacrylamide gels. The ice-cold buffer used during electrophoresis completely surrounded the tubes containing the gels, so that cooling was as effective as possible. The samples were not pretreated with dithiothreitol.

Electrophoresis on cellulose acetate

Cellulose acetate strips (2.5 cm × 16 cm) were used. The electrophoresis was carried out in sodium barbitone buffer (0.2 M in barbitone), pH 8.6, for 3 h at 1.5 mA per strip. Scanning of the strips for radioactivity was done with a Panax gas-flow strip scanner, and staining was effected with Amido Schwartz [1% in 7% (v/v) acetic acid].

Double-diffusion studies

Ouchterlony plates were prepared with 1% agar in glycine/NaOH buffer (0.1 M in glycine, pH 8.0). Samples containing either Tamm-Horsfall glycoprotein or mixtures of bovine serum albumin and Tamm-Horsfall glycoprotein were incubated before being added to the plate with 0.1% sodium dodecyl sulphate for 1 h at 37°C. The albumin and Tamm-Horsfall glycoprotein were each dissolved separately in the detergent solution 1 h before the resultant solutions were mixed, and kept at room temperature (22°C) for this period.

Tests for inhibition by Tamm-Horsfall glycoprotein of haemagglutination induced by influenza virus

These tests were done at 21°C by a modification of established procedures (Salk, 1944) with the use of Japanese influenza virus [A₂/Japan/305/57(H₂N₂)] in phosphate-buffered saline (Dulbecco A; see below) (10 HA units/0.25 ml; 1 HA unit is the minimum amount of virus needed to cause agglutination under the test conditions), and chicken erythrocytes in suspension in 0.9% NaCl (0.5%, v/v). In wells were placed 0.25 ml of 0.9% NaCl or 0.25 ml of solutions of Tamm-Horsfall glycoproteins, which were isolated from human, rabbit and hamster urine (1 mg/ml), in 0.9% NaCl followed in each case by 0.25 ml of the influenza-virus preparation. After 40 min, 0.25 ml of the erythrocyte suspension was added, and the samples were examined for haemagglutination 20 min later.

Growth of BHK-21/C13/2P cells

The substrain of cells used may be grown either in suspension or attached to a glass surface (Capstick *et al.*, 1966). In those experiments in which BHK glycoprotein I was isolated from the culture medium, the first of these methods was used. D-[^{14}C]glucosamine was incorporated into the culture medium to facilitate recognition of the isolated labelled glycoprotein.

Cells (5×10^5 /ml) were seeded in suspension in stirred Bellco spinner flasks in 40 ml of Glasgow modification of minimum essential medium (MacPherson & Stoker, 1962) containing D-[^{14}C]glucosamine (7.6 μCi ; 1.34×10^7 c.p.m.; 0.138 μmol), in addition to 10% (v/v) calf serum and 10% (v/v)

tryptose phosphate broth. The cells increased in number to 1×10^6 per ml within 48 h at 37°C and were almost wholly viable when tested with Trypan Blue (Phillips, 1973). Counting was done in a Neubauer haemocytometer. The suspension was centrifuged (500g, 5 min, 22°C) and the cells were set aside. The supernatant was dialysed against water (2 litres; three changes, containing 0.02% NaN_3 , for 3 days), and the non-diffusible material was freeze-dried (product M; 240 mg). It contained 1080 c.p.m./mg, which is equivalent to 1.93% of the D-glucosamine originally supplied to the medium.

Cells used in a number of experiments were grown in the quiescent state (Bürk, 1970) on glass. If the quiescent cells were to be used directly for radioimmunoassay they were seeded at 3×10^6 cells per 15 ml of culture medium, composed of Glasgow medium containing 0.5% calf serum, in 125 ml medical 'flats'. Streptomycin (50 µg/ml) and penicillin (50 i.u./ml) were incorporated into the cultures. The cells were cultured at 37°C for 60 h and then released from the glass surface, after washing with the culture medium, by one of three methods: (a) by incubation for 15 min in 0.02% neutralized EDTA in Dulbecco A phosphate-buffered saline (8.10 mM- Na_2HPO_4 , 1.47 mM- KH_2PO_4 , 137 mM- NaCl , 2.68 mM- KCl ; pH 7.3) (Dulbecco & Vogt, 1954; 10 ml per 100 ml medical flat); (b) by incubating with 10 ml of 0.1% crystalline trypsin at 22°C for 10 min; (c) by gentle mechanical agitation in the presence of 10 ml of culture medium. In each case the sample was centrifuged (500g, 5 min, 22°C) and the pellet of cells washed several times with phosphate-buffered saline.

For those experiments involving study of the cell cycle, which was induced by culturing quiescent cells in the presence of 10% (v/v) calf serum (Bürk, 1970), larger numbers of quiescent cells were grown and harvested with EDTA. Portions of the washed pellet (1.2×10^6 – 1.3×10^6 cells) were seeded in 100 ml medical flats, and the cells were cultured at 37°C in 10 ml of the medium used to grow the cells in suspension, except that no labelled glucosamine was added. At appropriate time-intervals a culture bottle was opened and the cells were harvested with EDTA. The cells were counted, and the total protein and BHK glycoprotein I contents were measured, as was also thymidine incorporation.

Total protein and BHK glycoprotein I contents of the cells

A sample of cells (about 0.5×10^6 – 1×10^6 cells) in 2 ml of ice-cold buffer (1.7 mM- Na_2HPO_4 , 0.52 mM- K_2HPO_4 , 44 mM- NaCl ; pH 7.0), containing 0.0011% sodium dodecyl sulphate, was sonicated for 1 min in an MSE 50W ultrasonic disintegrator at 1 A. The resultant solution was assayed colorimetrically

for total protein (Lowry *et al.*, 1951), with bovine serum albumin as standard, and for BHK glycoprotein I by radioimmunoassay (Dunstan *et al.*, 1974).

Thymidine incorporation

A sample of the cells (10% of the total in each bottle) was incubated at 37°C for 20 min in culture medium (10 ml) to which was added 100 µl of [^3H]-thymidine (50 µCi *in toto*, final concentration 0.2 µM). The cells were recovered after centrifugation (500g, 5 min, 22°C) and washed thoroughly with Dulbecco A phosphate-buffered saline. The pellet was taken up in the same buffer and filtered through a Millipore filter. The cells were washed with 5% (w/v) trichloroacetic acid, and after the disc has been dried it was counted for radioactivity in the scintillator described below for ^{14}C .

5'-Nucleotidase activity

This was measured, in samples of medium in which cells had been cultured, by a procedure similar to that of Peters *et al.* (1972). Substrate solution was prepared by adding 125 µl of [^3H]AMP [250 µCi in 10 ml of aq. 50% (v/v) ethanol] to 50 ml of piperazine/HCl buffer (60 mM in piperazine, pH 9.0) containing 24 mM- MgCl_2 , 12 mM-glycerol 2-phosphate, 0.12 mM-AMP and 0.1% Triton X-100. For the assays, 0.5 ml volumes of substrate solution were pipetted into conical centrifuge tubes and 0.1 ml volumes of the media from the cell cultures were added. Enzyme blanks were also done. Incubations were at 37°C for times of up to 30 min. Reaction was stopped by adding and mixing 0.25 ml of 0.25 M- ZnSO_4 , followed by 0.25 ml of 0.25 M- $\text{Ba}(\text{OH})_2$ to precipitate unchanged AMP. The samples were stored at 4°C for 30 min before centrifugation (1000g; 5 min; 4°C). A portion (0.25 ml) of the supernatant was counted for radioactivity in 5 ml of scintillator [4 parts of toluene, containing 0.5% of 2,5-diphenyloxazole and 0.02% of 1,4-bis-(5-phenyloxazolyl-2-yl)benzene, and 1 part of Triton X-100 (Turner, 1973)]. The results obtained were calculated from standard curves in terms of nmol of substrate hydrolysed/min (munits), and expressed per ml of culture medium.

Preparation of ^3H -labelled modified human Tamm-Horsfall glycoprotein

The sialic acid moieties were converted into 5-acetamido-3,5-dideoxy-L-arabino-2-[^3H]heptulosonic acid residues by a procedure similar to that described by Van Lenten & Ashwell (1971). Human Tamm-Horsfall glycoprotein (35 mg) was stirred with 50 ml of sodium acetate buffer (0.1 M in acetate,

pH 5.6) containing 0.15M-NaCl and the mixture was cooled to 2°C. NaIO₄ (0.12M, 8.4ml) was added and the mixture kept at 2°C for 10min. Excess of ethylene glycol was added and the preparation was dialysed in the cold-room for 16h against 0.05M-sodium phosphate, pH 7.4, containing 0.15M-NaCl.

KB³H₄ (3.8mg) in 0.1M-NaCl (1ml) was added to the cold non-diffusible material and the mixture was allowed to warm to room temperature, and then left for 30min with stirring. Non-radioactive KBH₄ (10mg) was added and stirring was continued for a further 30min. The mixture was dialysed against 0.05M-sodium phosphate, pH 7.4, containing 0.15M-NaCl and then against Dulbecco A buffer (see above). The material in the dialysis bag was centrifuged and the supernatant was freeze-dried. The product had a specific radioactivity of 8.0×10^6 c.p.m./mg. Its mobility on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) was identical with that of the unmodified glycoprotein.

Counting of radioactivity

Counting of ¹⁴C- or ³H-labelled samples was usually effected in 10ml of scintillator composed of 2 parts of toluene, containing 0.5% of 2,5-diphenyl-oxazole and 0.02% of 1,4-bis-(5-phenyloxazolyl-2-yl)-benzene, and 1 part of Triton X-100 (Turner, 1973). A Nuclear-Chicago scintillation counter was used, and corrections were applied where necessary for quenching by protein.

Radioactivity counting of ¹²⁵I-labelled samples was done with a model 578 Packard Auto-gamma counter.

Isolation from antiserum of protein fractions which were used for affinity chromatography

Affinity-chromatography columns were made by linking various protein fractions isolated from immune rabbit serum to CNBr-treated Sepharose 4B. The protein fractions used were (i) rabbit whole antiserum, (ii) partially purified rabbit serum albumin, (iii) partially purified total antibody which includes IgG and IgM, (iv) immunoglobulin precipitated by (NH₄)₂SO₄ and (v) purified IgG. In all cases antiserum raised in rabbits was used for the isolation of the fractions.

The albumin (ii) and IgG plus IgM (iii) fractions were obtained by chromatography on a Sephadex G-200 column (Flodin & Killander, 1962). Serum (4ml) was applied to a column (38cm × 2cm) of Sephadex G-200 which was eluted with 0.1M-Tris/HCl buffer, pH 8.0, containing 0.2M-NaCl. The fractions containing the albumin (61–102, B of Fig. 1) were combined, as were the fractions containing the antibody (33–55, A). The two pooled volumes were dialysed separately against 0.9% NaCl con-

Fig. 1. Gel filtration at 4°C on a column (38cm × 2cm) of Sephadex G-200 of antiserum (4ml) raised in rabbits against hamster urinary Tamm-Horsfall glycoprotein

The eluting buffer was 0.1M-Tris/HCl buffer (0.1M in Tris), pH 8.0, containing 0.2M-NaCl. Fraction size was 25 drops. The first peak contains IgM, the second IgG and the third albumin (Flodin & Killander, 1962). Combined fractions A and B were used as the source of partially purified antibody and partially purified albumin respectively for the production of affinity columns, as described in the text.

taining 0.02% NaN₃, and the solutions were freeze-dried.

Antibody, which is relatively free from IgM, was also obtained by precipitating it from serum (4ml) by the addition of 0.5vol. of saturated (NH₄)₂SO₄ at pH 6.5 (Stelos, 1967). The precipitated antibody was dialysed against water and freeze-dried. Pure 7S immunoglobulin was obtained by chromatography dialysed (against starting buffer containing 0.02% NaN₃) rabbit antiserum (4ml) on a column (1.6cm × 15cm) of DEAE-cellulose at pH 8.6 in Tris/phosphate buffer (0.04M-Tris, 0.005M-phosphate; Sober & Peterson, 1958). The fractions containing IgG were combined, and the solution was dialysed against water and freeze-dried.

The various products were linked to Sepharose 4B by a modification of the procedure of Cuatrecasas (1970). Five amounts of 4g of CNBr-activated Sepharose 4B were swollen in 1mm-HCl and washed on sintered-glass funnels with 800ml volumes of the same acid.

To the filtered portions of swollen gel were added severally the serum or fractions prepared therefrom as described above: (i) a mixture of 4ml of rabbit antiserum and 21ml of 0.1M-NaHCO₃, containing 0.5M-NaCl, (ii) the freeze-dried albumin dissolved in

25 ml of 0.1 M-NaHCO₃/0.5 M-NaCl (iii) the freeze-dried partly purified IgG plus IgM fraction from the Sephadex G-200 column in 25 ml of 0.1 M-NaHCO₃/0.5 M-NaCl, (iv) the freeze-dried (NH₄)₂SO₄-precipitated antibody in 25 ml or 0.1 M-NaHCO₃/0.5 M-NaCl and (v) freeze-dried IgG from DEAE-cellulose chromatography in 25 ml of 0.1 M-NaHCO₃/0.5 M-NaCl. Each preparation was stirred very gently with a magnetic stirrer for 2 h at room temperature, and the modified gel was collected by filtration, and finally washed, with 4 × 25 ml of 0.1 M-NaHCO₃/0.5 M-NaCl, on sintered-glass funnels. The gels were stirred with 1 M-ethanolamine/HCl (20 ml, pH 8.0) for about 1.5 h and then allowed to settle. The supernatant in each case was decanted and the gels were washed successively, with three washing cycles in all, with 0.1 M-sodium acetate buffer, pH 4, containing 1 M-NaCl, and with 0.1 M-sodium borate buffer, pH 8.5, containing 1 M-NaCl. The gels were washed overnight by gentle stirring with chloride/phosphate buffer, pH 7.0 (1.7 mM-Na₂HPO₄, 0.52 mM-KH₂PO₄, 44 mM-NaCl), which also contained 0.02% NaN₃. The gels were separated by gentle centrifugation and the supernatants were decanted. The precise way in which the modified gels were used is described in the Results section.

Immunofluorescence studies

Samples of quiescent cells [2×10^5 in 1 ml volumes of medium containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth] were cultured on cover-slides at 37°C for 18 h or 42 h. Another sample of quiescent cells in the appropriate medium (see above) was allowed to attach to the cover-slide. The preparations were washed with phosphate-buffered saline and fixed for 1 h with neutral 10% (v/v) formalin, which contained 10% (w/v) CaCl₂. The fixed cells were rinsed thoroughly with several changes of phosphate-buffered saline and then covered with a few drops of rabbit anti-(hamster Tamm-Horsfall glycoprotein pure IgG) (a 1:8 dilution, v/v, in Dulbecco A buffer of the IgG isolated from 4 ml of serum and redissolved in that volume of Dulbecco A buffer) for 10 min. The cells were again thoroughly washed with phosphate-buffered saline and then covered with a few drops of fluorescein-labelled sheep anti-(rabbit IgG) [concentration as for anti-(Tamm-Horsfall glycoprotein IgG)]. They were again washed and finally mounted for examination by fluorescence microscopy.

The treated cells were examined with a Leitz microscope fitted with a Leitz dark-ground condenser and a quartz/iodine light-source provided with a Turner filter (Gillett and Sibert FITC 3). A barrier filter (Wratten B15) was incorporated into the microscope. The illumination, time exposure and photographic processing were standardized throughout.

Results

The presence of a product (or products) immunologically cross-reacting with hamster urinary Tamm-Horsfall glycoprotein was demonstrated to be present in the medium in which baby-hamster kidney cells BHK-21/C13/2P had been cultured (Dunstan *et al.*, 1974). In the present series of experiments a procedure was devised for isolating the material (BHK glycoprotein I) from the medium in a chromatographically homogeneous form. Affinity chromatography was used in the isolation procedure, and the results of the experiments show the necessity for the use of purified antibody as the adsorbing entity for BHK glycoprotein I on the chromatographic columns.

Amounts of 15 mg (16200 c.p.m.) of product M, which had been obtained by freeze-drying the medium in which cells had been cultured in the presence of D-[¹⁴C]glucosamine (see above), were dissolved with gentle stirring in 20 ml of chloride/phosphate buffer (buffer B). These solutions were added to the several modified gels prepared as described above.

Incubation was, in each case, for 2 h at room temperature with occasional shaking, and a small column (0.8 cm × 15 cm) was made from each gel, together with its supernatant. The gels were allowed to settle in the columns, and elution was carried out first with the excess buffer used in the incubation, followed by buffer B until no further radioactivity or 280 nm-absorbing material was eluted from the column. The total volume of eluate at this stage was about 25–30 ml in each case, but elution was continued until about 60 ml was collected. The level of buffer B in each glass column was allowed to fall to the top of the gel, when 3.5 ml of 1% sodium dodecyl sulphate solution in buffer B was added. After the levels of the detergent solutions had been allowed to fall to the top of the gels, flow from the columns was stopped and the gels were incubated for 0.5 h at 23°C. Elution was then effected with the 1% detergent solution in buffer in each case, and fractions of volume approx. 1 ml were collected. Portions (50 μl) of the fractions were counted for radioactivity and in each case the radioactive fractions were combined. These preparations were dialysed at 4°C against water (2 litres, two changes containing 0.02% sodium azide and one further change without azide), and the non-diffusible materials were freeze-dried. The residues were dissolved in 600 μl of Tris/glycine buffer, pH 8.4 (0.025 M in Tris) containing 0.1% sodium dodecyl sulphate.

Gel electrophoresis on measured portions of these solutions was carried out in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969; Khalkhali & Marshall, 1976). The results obtained (Plate 1) show that a homogeneous

Table 1. *Total protein and BHK glycoprotein I contents of BHK-21/C13/2P cells cultured on glass in a quiescent state and under non-synchronous conditions, when released from the glass by (a) treatment with trypsin, (b) treatment with EDTA or (c) mechanical agitation*

The conditions of the experiments are described in the text. Replicate analyses agreed within 8%, although differences of up to 15% were observed from one subculture of cells to another.

State of cells	Method of removal from glass	Total protein (pg/cell)	BHK glycoprotein I (pg/cell)
Quiescent	(a) Trypsin	1217	2.1
	(b) EDTA	1220	65
	(c) Mechanical agitation	1127	53
Non-synchronous growth	(a) Trypsin	200	3.6
	(b) EDTA	400	5.7
	(c) Mechanical agitation	347	2.6

preparation of BHK glycoprotein I was obtained only from the affinity column in which antibody purified on DEAE-cellulose was used as the adsorbent [Plate 1(v)]. Its mobility was very similar to that exhibited by hamster urinary glycoprotein [Plate 1(vi)]. Other protein bands were apparent in these gels on which electrophoresis of the product from the medium had been performed with fractions from immunized rabbit serum isolated on other types of columns. It is likely that there is non-specific binding of proteins from the culture medium to various fractions, other than IgG, on the affinity columns.

One of the main questions arising from the finding of the glycoprotein in the culture medium concerns the reason and mechanism for its release. It was decided therefore to examine the amount of BHK glycoprotein I in cells that had been treated in different ways.

BHK glycoprotein I contents of cells released from a surface by different techniques

Cells were cultured on glass, either under conditions leading to a quiescent state (Bürk, 1970) or in non-synchronous growth conditions, to a limiting density. Removal of the cells from the glass was effected in both cases in each of the three ways described above: with EDTA, with trypsin or by gentle mechanical agitation. The total protein and the BHK glycoprotein I contents of the six samples of cells were measured.

The results obtained (Table 1) are of considerable interest. The use of crystalline trypsin results in the loss of over 96% of the BHK glycoprotein I from the quiescent cells, when compared with the cells removed from the surface by other methods. The results of experiments reported by Snow & Allen (1970) suggest that trypsin releases macromolecules predominantly from the cell surface. If this is presently the case, it is probable that a large proportion of the BHK glycoprotein I content of the quiescent cells is associated with the cell membrane. The

cells released from the surface with EDTA are associated with the largest amount of BHK glycoprotein I, in spite of the probability that some intracellular material had been released by this treatment (Snow & Allen, 1970).

There is much less BHK glycoprotein I associated with non-synchronous cells grown to maximum cell density; the largest amount is again associated with those cells released from the surface with EDTA.

It is likely that much of the BHK glycoprotein I of the cells in the quiescent state is associated with the cell surface. In view of this, and because Tamm-Horsfall glycoprotein appears to bind Ca^{2+} ions strongly *in vitro* (Cleave *et al.*, 1972), it was decided to examine the effects of different concentrations of Ca^{2+} ions on the BHK glycoprotein I contents of cultured cells.

BHK glycoprotein contents of BHK-21/C13/2P cells cultured in medium containing different Ca^{2+} ion concentrations

In these experiments the cells were grown on a glass surface in the presence of Ca^{2+} ion concentrations ranging from 0.56 to 3.56 mM, under conditions leading to quiescent cells. They were removed from the surface with EDTA. The results (Table 2) show that the amount of glycoprotein associated with the cells decreased markedly as the Ca^{2+} ion concentration in the medium was increased. This decrease in glycoprotein content might be due to an increased rate of its release into the medium, and if this is the case the experiment might be a model for examining the way in which Tamm-Horsfall glycoprotein is released from renal tubular cells into the tubular urine. Further examination of this aspect must await the development of methods for assaying the relatively low concentrations of BHK glycoprotein I compared with the total concentration of protein in the culture medium. Measurements of the rate of production and release of the specific glycoprotein into the medium might help in explaining

why the kidney tubules release such relatively large amounts of Tamm–Horsfall glycoprotein into the tubular urine. The experiments described below were aimed at assessing the relative amounts in the urine and in the whole kidney of the Syrian golden hamster.

Tamm–Horsfall glycoprotein concentrations of Syrian-golden-hamster urine and kidney

The Tamm–Horsfall glycoprotein contents of 24h collections of hamster urine were measured by radio-immunoassay. The average daily output is of the order of 0.8–1.0mg/24h (Table 3). The kidneys of the hamster 1 and hamster 2 contained 1.7 and 1.8mg of Tamm–Horsfall glycoprotein/g of kidney respectively. The average weight of one kidney was 0.44g, so that the half-life for the excretion of Tamm–Horsfall glycoprotein from the hamster is of the order of 19h, a value of a similar order to that found for the rabbit (9h) and man (16h; Grant & Neuberger, 1973). This rapid turnover *in vivo* is unlikely to be due to cell turnover, but, nevertheless, it seemed worth while to examine the BHK glycoprotein I content of cells at different stages of the cell cycle.

Total protein and BHK glycoprotein I contents of cells at various stages of the cell cycle

This series of experiments was done by initially culturing the cells in the quiescent state, attached to glass surfaces. In all cases the cells were released from the glass surface with EDTA. The viable cells were subsequently cultured in the presence of 10%

(v/v) calf serum at 37°C, and there was a doubling in cell number beginning after about 19h and being largely complete within the following hour (Fig. 2). The S phase of the cells occurred over the time-period 11–16h after stimulation of growth with 10% calf serum. This stage, assessed by thymidine incorporation, appeared to be bimodal (Fig. 2), and is of a type exhibited by a number of cell lines (Remington & Klevecz, 1973; Howard *et al.*, 1974).

Fig. 2. Some changes occurring in cultures of BHK-21/C13/2P cells grown into the quiescent state (Bürk, 1970) and subsequently grown in the presence of 10% (v/v) calf serum (Howard *et al.*, 1974) on glass surfaces
○, Cell count; ●, [3H]thymidine incorporation; □, total protein; ■, BHK glycoprotein I; ▲, 5'-nucleotidase activity in the culture medium. Details of the experiment are described in the text.

Table 2. BHK glycoprotein I contents of BHK-21/C13/2P cells cultured in the quiescent state on glass surfaces in the presence of different concentrations of Ca²⁺ ions

The cells were released from the glass with EDTA.	
Ca ²⁺ concentration in the medium (mM)	BHK glycoprotein I content (pg/cell)
0.56	70
1.56	66
2.56	37
3.56	25

Table 3. Tamm–Horsfall glycoprotein contents of 24h collections of hamster urine

Day no.	Hamster 1		Hamster 2	
	Volume of urine (ml)	Tamm–Horsfall glycoprotein (mg)	Volume of urine (ml)	Tamm–Horsfall glycoprotein (mg)
1	2.5	0.58	2.5	0.98
2	3.0	1.0	3.2	1.08
3	3.8	0.76	—	—
4	3.8	0.80	—	—
	Average 0.78		Average 1.03	

The BHK glycoprotein I content of the cells showed noteworthy features. As the change occurred from the quiescent state to the beginning of the G1 phase there was a rapid loss of the glycoprotein from about 78 pg/cell to about 6 pg/cell. Throughout the G1 and S phases the amount remained constant, but then increased at the beginning of the G2 phase. The precise point at which the M phase begins is unknown, and the increase might also be occurring during the early part of the M phase, reaching a peak value (40 pg/cell) at least 1 h before physical division of the cells occurred. By the time the cells divided (between 19 and 20 h after stimulus to growth), the amount of BHK glycoprotein I per cell had fallen markedly, and at the end of division was down to about 6 pg/cell.

The results of these experiments can be interpreted only with reservations, because, for reasons mentioned above, measurements have not been made of the amounts of BHK glycoprotein I released into the medium by the cells. But with this reservation, it may be suggested that there is a specific burst of synthesis of BHK glycoprotein I immediately after the S phase. The alternative explanation is that there is increased retention of the glycoprotein by the cells at this time. Whichever explanation is correct, the results may be used to propose that there is increased accumulation of the specific glycoprotein by the cells during the G2 phase of the cell cycle. The accumulation at that time might be of considerable significance if, as has been suggested (Shodell, 1975), one function of this phase involves reorganization of the cell surface in preparation for the start of mitosis.

Immunofluorescence methods were also used to demonstrate the presence of BHK glycoprotein I in the cultured cells.

Demonstration of BHK glycoprotein I in cultured cells by immunofluorescence

Cells that had been cultured in the quiescent state, or that had subsequently been grown for either 18 or 42 h in the presence of 10% (v/v) calf serum, were harvested with EDTA. The cells were identified by the indirect immunofluorescence procedure, and the results, which were recorded under standardized conditions of illumination, time exposure and photographic processing, are shown in Plate 2. The greatest intensity of fluorescence is seen in the quiescent cells, with smaller amounts after 18 h of growth in the presence of 10% serum, the time at which the BHK glycoprotein I content is highest (Fig. 2), and also after 42 h of growth, when the cells are in non-synchronous growth conditions. There is evidence of small foci of brighter fluorescence in the last-mentioned cells especially, and these may indicate the presence of the glycoprotein in the cytoplasm of

the stained cells. Similar foci in the stained quiescent cells are largely masked by the overall greater intensity of fluorescence.

Great care is required in interpreting fluorescence intensity in different preparations, partly because fluorescein undergoes rapid photodecomposition and even the small margin of error implicit in any standardized photographic process could produce significant variations. Moreover, fixing of the cells could induce changes. Nevertheless, the fact that the results tally well with radioimmunoassays of BHK glycoprotein I contents of cells at different stages in the cell cycle encourages the belief that they represent the visual expression of the immunogen.

It seemed reasonable, in the light of the possibility that much of the glycoprotein of the cells in the quiescent state at least is associated with the plasma membrane, to question whether the decrease in the amount of BHK glycoprotein I associated with the cells, which begins 17.5 h after culturing, is due to shedding of the plasma membrane. To consider this aspect, measurements were made of the activity of 5'-nucleotidase in the culture medium throughout the cycle. This enzyme is known to be associated with the plasma membrane of BHK-21/C13 cells (Gahmberg & Simons, 1970).

5'-Nucleotidase activities in culture medium throughout the cell cycle

5'-Nucleotidase was measured by procedures (Peters *et al.*, 1972) that are specific for the enzyme. In any case, alkaline phosphatase activity, assayed with 4-methylumbelliferyl phosphate as substrate (Peters *et al.*, 1972), in both sonicated cells and culture medium, apart from that in added serum, at all stages of the cell cycle was negligible. The results (Fig. 2) show that there is increased 5'-nucleotidase activity in the culture medium during the transition of the cells from the quiescent state to the G1 phase, and this falls to almost zero towards the end of the G1 phase (that is after about 8 h of growth of the cells). It then begins to rise during the S phase of the cycle and reaches a maximum at about the beginning of the G2 phase, falling again to zero after a further 8–10 h. This latter decrease might occur towards the end of a greatly shortened G1 phase of the second cycle (Bürk, 1970).

It seems likely that 5'-nucleotidase loses its activity in the culture medium rapidly, with a half-life of less than 1 h. This is not due to production by the cells of an inhibitor of the enzyme, because assays were done with culture medium from the 2 h-growth state in the presence of medium from the 9 h-growth state, and the latter fraction did not inhibit the enzyme activity of the former.

The results suggest that release of 5'-nucleotidase from the cells begins before the beginning of the

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) of fractions obtained by affinity chromatography on modified Sepharose 4B of the proteins from the medium in which BHK-21/C13/2P cells had been cultured in suspension

The sample of Bromophenol Blue used as a marker dye did not destain under the conditions used. It is apparent at the lower end of the gels. The Sepharose 4B had been covalently substituted (Cuatrecasas, 1970) by (i) antiserum raised in rabbits against hamster Tamm-Horsfall urinary glycoprotein, (ii) partially purified rabbit serum albumin, (iii) partially purified total antibody, (iv) immunoglobulin precipitated by $(\text{NH}_4)_2\text{SO}_4$ and (v) pure IgG. A sample of hamster urinary Tamm-Horsfall glycoprotein was also subjected to electrophoresis and the stained gel is shown in (vi). Details of the experiments are given in the text.

EXPLANATION OF PLATE 2

Immunofluorescence, by the indirect method, exhibited by fixed BHK-21/C13/2P cells grown on a glass surface (a) in the quiescent state (Bürk, 1970), (b) after subsequent culture in the presence of 10% (v/v) calf serum for 18 h and (c) after subsequent culture for 42 h
Magnification $\times 360$.

EXPLANATION OF PLATE 3

Double-diffusion studies of rabbit anti-(human Tamm-Horsfall glycoprotein) serum (centre well)
All outer wells contained 10 μ g of human Tamm-Horsfall glycoprotein in the presence of 0.1% sodium dodecyl sulphate.
Well I contained, in addition, bovine serum albumin at one-fifth the molar quantity of Tamm-Horsfall glycoprotein,
well II albumin in equal molar quantity and well III at five times the molar amount.

EXPLANATION OF PLATE 4

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) of (a) hamster Tamm-Horsfall glycoprotein, (b) equimolar amounts of hamster Tamm-Horsfall glycoprotein and bovine serum albumin and (c) monomeric bovine serum albumin

The mixing of the proteins in the experiment, the results of which are shown in (b), was done after incubating the proteins separately in the detergent for 1 h at room temperature. In the other experiments, (a) and (c), the proteins were incubated under identical conditions before electrophoresis.

G2 phase and thus before the cell-associated accumulation of BHK glycoprotein I. If it is assumed that the 5'-nucleotidase that appears in the culture medium is derived from the plasma membrane of the cells, the results suggest that the accumulation of BHK glycoprotein I is an event that is distinct from turnover of the plasma membrane.

There is a further notable point with regard to 5'-nucleotidase. If BHK-21/C13 cells are derived from those of the renal tubule, it might be deduced that the latter would also shed 5'-nucleotidase into the tubular urine. Unpublished results (F. J. Bloomfield & R. D. Marshall) suggest that the origin of urinary 5'-nucleotidase may be, at least in part, kidney tubular cells.

None of the experiments so far described yield results that can be used with certainty to explain the mechanism of release of BHK glycoprotein I from cells either *in vivo* or *in vitro*. The albumin in the culture medium might play a role here, in view of the immunological similarity of the glycoprotein to urinary Tamm-Horsfall glycoprotein, and the ability of the latter to bind to albumin (McQueen, 1962; McQueen & Engel, 1966). This binding is strong and is only partly reversed in the presence of sodium dodecyl sulphate. It is not unreasonable to believe that BHK glycoprotein I would behave in a similar manner.

Binding of urinary Tamm-Horsfall glycoprotein to serum albumin

The electrophoretic pattern of human ^3H -labelled Tamm-Horsfall glycoprotein is altered when albumin is added to the material before electrophoresis (Fig. 3).

There is also an apparently lower rate of diffusion of human Tamm-Horsfall glycoprotein in Ouchterlony plates in the presence of serum albumin (Plate 3). Sodium dodecyl sulphate (0.1%) was originally present with these proteins and it is unlikely that the proteins would have shed all of the detergent during diffusion (Pitt-Rivers & Impiombato, 1968). It seems likely that interaction of the Tamm-Horsfall glycoprotein with albumin leads to the production of a complex of higher molecular weight which diffuses more slowly in the agar gel.

Finally hamster Tamm-Horsfall glycoprotein exhibits a single band on polyacrylamide-gel disc electrophoresis in the presence of 0.1% sodium dodecyl sulphate (Marshall & Zamecnik, 1969), with an apparent mol.wt. of about 82000 (Dunstan *et al.*, 1974), and when mixed with bovine serum albumin in the presence of the detergent a number of bands in addition to those due to monomeric forms of the two standard proteins are apparent (Plate 4).

The results of these experiments raise the question as to whether Tamm-Horsfall glycoprotein, or BHK glycoprotein I, can be estimated accurately by

Fig. 3. Electrophoresis on cellulose acetate strips in sodium barbitone buffer (0.2M in barbitone) pH8.6 of (a) ^3H -labelled modified human Tamm-Horsfall glycoprotein (5 μg ; 40000 c.p.m. applied in 5 μl of buffer) and (b) a mixture of 5 μg of ^3H -labelled modified Tamm-Horsfall protein and 150 μg of bovine serum albumin in 5 μl of buffer. In (b) the mixture was incubated at room temperature for 30 min before electrophoresis. The strips were cut laterally into pieces 5 mm wide, and these were counted for radioactivity [32000 c.p.m. was found in the single peak (a) and in (b) there was 9400 c.p.m. in the slower fraction and 16900 c.p.m. in the faster]. The position of albumin (stained with Amido Schwartz) is shown hatched. Tamm-Horsfall glycoprotein stains only very weakly with this dye. Other details are given in the text.

radioimmunoassay in the presence of albumin, which may well be bound to cultured cells, even though there is sodium dodecyl sulphate in the assay medium. Control experiments done by assaying human Tamm-Horsfall glycoprotein in the presence of added bovine serum albumin (amounts from 0 to 2 mg/ml) indicated that there was relatively little interference over the range tested. At a concentration of 2 mg/ml there was a decrease of 10% in the value obtained, compared with that found in the absence of added albumin.

Inhibitory action of Tamm-Horsfall glycoprotein on haemagglutination induced by influenza virus

Hamster urinary Tamm-Horsfall glycoprotein is devoid of sialic acid residues (Dunstan *et al.*, 1974; Serafini-Cessi & Marshall, 1975), and the glycoprotein from the rabbit has *O*-acetylated sialic acid

residues (Neuberger & Ratcliffe, 1970). A comparison was therefore made of the ability of these two glycoproteins and of human Tamm-Horsfall glycoprotein to inhibit agglutination of chicken erythrocytes induced by Japanese influenza virus. The human glycoprotein, not unexpectedly (Tamm & Horsfall, 1950, 1952), was found to be an inhibitor, but neither the rabbit nor the hamster material acted in this way as assessed by the technique described in the Methods section. The results indicate, for rabbit and hamster at least, that the glycoprotein is unlikely to be involved in a function that is dependent on binding influenza virus and probably other myxoviruses also.

Discussion

An aneuploid line of cultured baby-hamster kidney cells BHK-21/C13/2P (Capstick *et al.*, 1966) was found to synthesize and release a glycoprotein, called BHK glycoprotein I into the culture medium. This glycoprotein is closely similar to, if not identical with, urinary Tamm-Horsfall glycoprotein, which is produced by kidney tubules and excreted in the urine of the hamster, with a turnover half-life of about 19h. The finding may suggest that the line of cells is derived from the particular tubular cells. This latter question requires resolution, because the cultured cells could also have arisen from other types of precursor cells and might have acquired the facility to synthesize BHK glycoprotein I either through dedifferentiation or other change.

BHK glycoprotein I can be isolated from the medium in which cells have been cultured in a form which is homogeneous, as is apparent from the results obtained when the product is subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969). The isolation may be achieved by affinity chromatography on pure immune IgG which is linked to Sepharose 4B and which may be isolated from rabbit antiserum raised against hamster urinary Tamm-Horsfall glycoprotein.

Cells in a quiescent state (Bürk, 1970) are relatively rich in BHK glycoprotein I, although the amounts present are inversely related to the Ca^{2+} concentration and possibly to other constituents of the medium. The action of trypsin leads to the release of over 95% of the glycoprotein from the cells. On the other hand there is much less BHK glycoprotein I associated with cells grown to maximum cell density and less than half of that present is released by the action of trypsin under the same conditions. These results might suggest that the quantitative subcellular distribution of BHK glycoprotein I is different in the cells in the two states. A not unreasonable suggestion is that serum albumin or Ca^{2+} ions might play a role in binding and

removing the glycoprotein, possibly from the surface, of cells grown to maximum cell density. For this latter purpose the culture medium contains about 4g of albumin/litre, but in that required for quiescent cells there is only about 0.2g/litre. But other components of the serum may also play a part.

The implication of the results obtained when the cells were grown through the cell cycle are not immediately apparent as regards the situation *in vivo*. There is an increase in BHK glycoprotein I probably during the G2 phase with a subsequent decrease before physical division of the cells. The highest concentration of the glycoprotein associated with the cells occurs about 0.8h before physical division, and it might be suggested that this is the time when nuclear division begins. Presumably the glycoprotein is released into the culture medium at this time, but its intracellular origin is not clearly defined. It is unlikely that renal tubular cells undergo sufficiently frequent cell divisions *in vivo* to release the similar urinary glycoprotein into the tubular urine during the G2 plus M phase, and there must be another mechanism of release to account for so much urinary glycoprotein.

The bimodal incorporation of thymidine during the S phase of the cell cycle might support the suggestion that replication of DNA occurs at different periods of time (Howard *et al.*, 1974), but there is also the possibility that there is more than one cell type with slightly varying lengths for different periods of the cycle. Cultures from a variety of organs, including kidney, of mouse are believed to yield two predominant cell types (Franks & Wilson, 1970; Franks, 1972), and BHK-21/C13/2P cells can be examined ultrastructurally and cytochemically in the light of this consideration. Preliminary results suggest that the line of cells being used may consist predominantly of two kinds.

We thank Dr. John Skehel, National Institute for Medical Research, London N.W.7, U.K. for the influenza virus and for helpful advice. The Wellcome Foundation, Pirbright, Surrey, U.K. kindly provided samples of the cultured cells. F. S.-C. was a fellow of the Accademia Nazionale dei Lincei (Roma). The work was kindly supported by the Medical Research Council and the National Kidney Fund.

References

- Bürk, R. R. (1970) *Exp. Cell Res.* **63**, 309-316
- Capstick, P. B., Garland, A. J., Masters, R. C. & Chapman, W. C. (1966) *Exp. Cell Res.* **44**, 119-128
- Cleave, A. J., Kent, P. W. & Peacocke, A. R. (1972) *Biochim. Biophys. Acta* **285**, 208-223
- Cornelius, C. E., Mia, S. A. & Rosenfeld, S. (1965) *Invest. Urol.* **2**, 453-457
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059-3065
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167-182

- Dunstan, D. R., Grant, A. M. S., Marshall, R. D. & Neuberger, A. (1974) *Proc. R. Soc. London Ser. B* **186**, 297–316
- Fletcher, A. P., McLaughlin, J. E., Ratcliffe, W. A. & Woods, D. A. (1970) *Biochim. Biophys. Acta* **214**, 299–308
- Flodin, P. & Killander, J. (1962) *Biochim. Biophys. Acta* **63**, 403–410
- Franks, L. M. (1972) in *Ultrastructural Features of Cells and Tissues in Culture* (Töro, I. & Rappay, G., eds.), pp. 31–35, Akademiai Kiado, Budapest
- Franks, L. M. & Wilson, P. D. (1970) *Eur. J. Cancer* **6**, 517–523
- Friedman, T. (1966) *Experientia* **22**, 624–625
- Gahmberg, C. G. & Simons, K. (1970) *Acta Pathol. Microbiol. Scand.* **78**, 176–182
- Grant, A. M. S. & Neuberger, A. (1973) *Biochem. J.* **136**, 659–668
- Hanson, L. Å., Fasth, A. & Jodal, U. (1976) *Lancet* **i**, 226–228
- Howard, D. K., Hay, J., Melvin, W. T. & Durham, J. P. (1974) *Exp. Cell Res.* **86**, 31–42
- Hoyer, J. R., Resnick, J. S., Michael, A. F. & Vernier, R. L. (1974) *Lab. Invest.* **30**, 757–761
- Keutel, M. J. (1965) *J. Histochem. Cytochem.* **13**, 155–160
- Khalkhali, Z. & Marshall, R. D. (1976) *Carbohydr. Res.* **49**, 455–473
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacPherson, I. A. & Stoker, M. G. P. (1962) *Virology* **16**, 147–151
- Marshall, R. D. & Zamecnik, P. C. (1969) *Biochim. Biophys. Acta* **181**, 454–464
- McKenzie, J. K. & McQueen, E. G. (1968) *J. Clin. Pathol.* **22**, 334–339
- McQueen, E. G. (1962) *J. Clin. Pathol.* **15**, 367–373
- McQueen, E. G. & Engel, G. B. (1966) *J. Clin. Pathol.* **19**, 392–396
- Neuberger, A. & Ratcliffe, W. A. (1970) *Biochem. J.* **129**, 683–693
- Patel, R., McKenzie, J. K. & McQueen, E. G. (1964) *Lancet* **i**, 457–461
- Peters, T. J., Müller, M. & de Duve, C. (1972) *J. Exp. Med.* **136**, 1117–1139
- Phillips, H. J. (1973) in *Tissue Culture* (Druse, P. F. & Patterson, M. K., eds.), pp. 606–408, Academic Press, New York, San Francisco and London
- Pitt-Rivers, R. & Impiombato, F. S. A. (1968) *Biochem. J.* **109**, 825–830
- Remington, J. A. & Klevecz, R. R. (1973) *Exp. Cell Res.* **76**, 410–418
- Salk, J. E. (1944) *J. Immunol.* **49**, 87–98
- Schenk, E. A., Schwartz, R. H. & Lewis, R. A. (1971) *Lab. Invest.* **25**, 92–95
- Serafini-Cessi, F. & Marshall, R. D. (1975) *Biochem. Soc. Trans.* **3**, 1110–1112
- Shodell, M. (1975) *Nature (London)* **256**, 578–580
- Snow, C. & Allen, A. (1970) *Biochem. J.* **119**, 707–714
- Sober, H. A. & Peterson, E. A. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 1116–1126
- Stelos, P. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 3–9, Blackwell Scientific Publications, Oxford
- Tamm, I. & Horsfall, F. L. (1950) *Proc. Soc. Exp. Biol. Med.* **74**, 108–114
- Tamm, I. & Horsfall, F. L. (1952) *J. Exp. Med.* **95**, 71–97
- Tsantoulas, D. C., McFarlane, I. G., Portmann, B., Eddlestone, A. L. W. F. & Williams, R. (1974) *Br. Med. J.* **iv**, 491–494
- Turner, J. C. (1973) *Tech. Protein Biosynth.* **3**, 67–124
- Van Lenten, L. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1889–1894