A Study of the Cell Surface of Tumour, Foetal and Lymph-Node Cells by Cell Electrophoresis after Antibody and Enzymic Treatment

By JEAN WOO and DONALD B. CATER

Department of Pathology, University of Cambridge, Cambridge CB2 1QP, U.K.

(Received 24 February 1972)

1. Rabbit anti-(rat foetal liver) serum, absorbed with adult rat liver cells, decreased the electrophoretic mobility of foetal liver cells by 51% and rat hepatoma cells by 45%, indicating the presence of a foetal-type antigen on the hepatoma cell membrane. 2. The chemical nature of the surface antigen was investigated. Incubation with neuraminidase had no effect on adult liver cells but decreased the electrophoretic mobility of foetal liver cells by 51% and of hepatoma cells by 34%; the effect of antiserum was decreased to one-fifth. 3. Sialic acid, or the supernatant from neuraminidase-treated cells, partially blocked the decrease in electrophoretic mobility induced by antiserum. 4. The pH-electrophoretic mobility curves of hepatoma cells treated with antisera were consistent with a sialic acid-containing antigen on the surface of the tumour cells. 5. Treatment with ribonuclease did not decrease the electrophoretic mobility of adult-liver cells, but decreased that of the foetal liver cells by 17% and hepatoma cells by 29%. 6. In parallel studies made with mouse BP8 ascites-tumour cells ribonuclease decreased the electrophoretic mobility by 39%, that of normal mouse lymph-node cells by 4.8% and allergized mouse lymph-node cells by 13.3%. 7. Trypsin treatment also decreased the electrophoretic mobility of hepatoma cells by 22%.

Tumour cells often have a high surface-charge density as measured by cell electrophoretic mobility, and when antibody adheres to the cell surface the electrophoretic mobility is decreased (Hartveit *et al.*, 1968). This seemed a convenient technique for the study of adherence of anti-(foetal liver) serum to hepatoma and foetal liver cells, with the advantage that the chemical composition of the antigen could be investigated by the treatment of the cells with neuraminidase, RNAase* and trypsin before the application of the antiserum and by the change of shape produced in the pH-electrophoretic mobility curve by the antiserum.

It has been suggested that tumour-specific transplantation antigens might be foetal antigens or closely allied to foetal antigens. Abelev *et al.* (1963) reported that hepatoma cells and the sera of newborn mice have an identical antigen. Abelev (1968) showed that a foetoprotein (α fG) is synthesized in embryos, in the liver tissue of 14-day-old rats and in mouse hepatoma cells and some primary liver carcinomas in man. Foetoproteins were also found in primary hepatocellular cancer by Purves *et al.* (1970*a,b*). Krupey *et al.* (1967) and Gold *et al.* (1968) have isolated and characterized a tumour-specific antigen from human gut cancers similar to antigens isolated from embry-

onic gut, pancreas and liver in the first two trimesters of gestation. They call these the carcinoembryonic antigens of the human digestive system. It is a surface antigen, and the sera of most patients with non-metastatic carcinoma have IgM antibody to carcinoembryonic antigen. Hollinshead *et al.* (1970) found that 17 out of 19 patients with carcinoma of the colon or rectum gave delayed-hypersensitivity skin reactions with a soluble fraction from the membranes of autologous tumour cells and that carcinoembryonic antigen was detected in many of these extracts. Stonehill & Bendich (1970) reported that anti-embryonic serum would precipitate with extracts from many tumours and tissues with a high cell turnover rate. A common antigen on normal embryonic hamster cells and on polyoma-virus-transformed cells was reported by Pearson & Freeman (1968), and an antigen on unfertilized mouse eggs has also been found on virus-SV-40-transformed mouse cells (Baranska *et al.*, 1970).

We were stimulated to undertake the work described in this paper by Dr. N. G. Anderson, who found that injecting foetal liver cells into adult rats gave some protection against challenge with hepatoma cells, indicating a common antigen between hepatoma and foetal cells (personal communication). Some of the work of this group on production of anti-tumour immunity by foetal cells has since been published (Ambrose *et al.*, 1971*a,b*).

* Abbreviations: RNAase, ribonuclease; IgM, immunoglobulin M.

Materials and Methods

Cell electrophoresis

The cell-electrophoresis apparatus used was that described by Seaman & Heard (1960) as modified by Mehrishi (1962). The chamber had a circular cross-section and all measurements of electrophoretic mobility were made at 25°C.

Preparation of antisera

Preparation of two rat foetal antigens by the method of Stonehill & Bendich (1970). The livers of rat embryos (white Wistar rats, 14 days gestation) were washed in phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 9.6 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.2) and homogenized in a glass homogenizer. The embryos, minus their livers, were treated likewise. Suspensions were centrifuged at 100g_{av.} for 10 min and the supernatants, after dialysis against three × 500 vol. of phosphate-buffered saline, were freeze-dried in 1 ml portions. All this was done at 4°C.

Preparation in rabbits of anti-(foetal rat) sera. Each of the freeze-dried homogenates, resuspended in water, was emulsified with an equal volume of Freund's complete adjuvant. Antigen (1 ml; 20 mg/ml) plus 1 ml of Freund's complete adjuvant was injected each week subcutaneously into a rabbit for 3 weeks. At 1 month after the first injection a booster dose of 1 ml of the antigen in 1 ml of Freund's incomplete adjuvant was injected intramuscularly. Then 10 days after the booster dose blood was collected weekly for 4 weeks. The sera were inactivated at 56°C for 30 min. The anti-(foetal liver) serum was designated anti-FL serum, and the anti-embryo serum anti-E serum. Anti-FL serum was absorbed with adult liver cells (from a pregnant rat) or foetal liver cells, or hepatoma cells. These absorbed sera were designated anti-FL serum (A), (F) and (H) respectively. Absorption was done by shaking 1 vol. of cells and 2 vol. of serum at 37°C for 30 min and then centrifuging for 10 min at 2800g_{av.}. The sera were stored at -20°C until used.

Preparation of the cell suspensions

Foetal liver cells. These were obtained from white Wistar rats of 14 or more days gestation. The foetal livers were dispersed in 0.05 M-EDTA in phosphate-buffered saline by two strokes of a loosely fitting glass homogenizer, then they were washed three times, and at each wash the layer of contaminating erythrocytes was sucked off and discarded.

Adult liver cells. These were obtained from the same pregnant rat by perfusing the liver with 0.05 M-EDTA in phosphate-buffered saline through a branch of the portal vein. A relatively bloodless liver lobe was cut

into small pieces and homogenized in 145 mM-NaCl, and the cell suspension was washed three times.

Hepatoma 223 cells. This is a hepatoma transplantable in August-strain rats. The cells were taken from rats injected intraperitoneally 7 days previously. The ascitic fluid was centrifuged at 80g_{av.} for 1 min. The fluid and erythrocyte layer were removed, and the hepatoma cells were resuspended in phosphate-buffered saline and washed three times to remove contaminating erythrocytes. The cells were then resuspended at a known number of cells/ml in 145 mM-NaCl.

BP8 ascites-tumour cells. This is a mouse tumour; 5 × 10⁴ cells were injected intraperitoneally into isogenic C3H mice. At 7 days the cells were harvested and treated as described above for the hepatoma cells.

Lymph-node cells. These were prepared from normal or allergized C57 Bl mice. C57 Bl mice were allergized by injecting BP8 cells (4 × 10⁶/mouse) emulsified with an equal volume (0.1 ml) of Freund's complete adjuvant (Difco Laboratories, Detroit 1, Mich., U.S.A.) divided into four subcutaneous sites. At weekly intervals fresh BP8 cells (4 × 10⁶/mouse) were injected subcutaneously for 3 or 4 weeks. Lymph nodes were taken 6 days after the last injection and the cells were released by squeezing the nodes with a silicone-rubber bung. The cells were then washed as described above for the hepatoma cells.

Treatment of cells

Treatment of cells with antisera. The cells were incubated with serum at a known quantity/10⁶ cells for 1 h at 0°C. The complement activity in the serum had been destroyed by heating at 56°C for 30 min. This would also destroy most of the endogenous neuraminidase activity of these sera. They were washed and resuspended in saline buffer (145 mM-NaCl, 0.1 mM-NaHCO₃, pH 7.2). They were warmed to 25°C before insertion into the electrophoresis chamber so that readings could be taken at once.

All readings were completed within 3 h of removal of the cells from the animals. At the end of all readings cell death was estimated by phase-contrast microscopy or by Trypan Blue (2%) dye-exclusion test. Any agglutination was noted and the pH was measured.

Treatment of cells with neuraminidase. Cell suspensions were incubated with 1 unit/10⁶ cells for 15 min at 36°C, then they were washed three times and resuspended in saline buffer, pH 7.2.

Treatment of cells with RNAase. Cell suspensions were incubated with 10 µg of RNAase/10⁶ cells for 30 min at 37°C. The cells were then washed and resuspended in 145 mM-NaCl-0.1 mM-NaHCO₃ buffer, pH 7.2.

Treatment of cells with trypsin. The cells were

incubated for 60min at 37°C with trypsin (1mg/10⁶ cells in 0.5ml) in Hanks solution at pH7.2, and washed with 145mM-NaCl-0.1mM-NaHCO₃ buffer, pH7.2, before electrophoretic mobility measurements were made.

Chemicals and enzymes

N-Acetylneuraminic acid (synthetic, crystalline) was from Sigma, St. Louis, Mo., U.S.A. (batch no. 106B-0704). Bromoacetate and potassium ferricyanide (A.R. grade) were from Hopkin and Williams, Chadwell Heath, Essex, U.K. Neuraminidase (EC 3.2.1.18), from the filtrate of *Vibrio cholerae* cultures, made by Behringwerke A.-G. and stated by them to give negative tests for proteases, aldolase and lecithinase C, was obtained from Hoechst Pharmaceuticals Ltd., Hounslow, Middx., U.K. RNAase (type 1A; 5×crystallized extract from bovine pancreas; stated by the makers to be protease-free and essentially salt-free) was from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Trypsin was from Hopkin and Williams.

Results and Discussion

Effects of rabbit antisera to foetal rat liver (anti-FL serum) and to embryo tissue (anti-E serum) on the electrophoretic mobility of adult liver cells, foetal liver cells and hepatoma cells of the rat

The results in Table 1 show that both antisera decreased the electrophoretic mobility of foetal-liver and hepatoma cells by comparable amounts, which were much greater than those for adult-liver cells. The foetal and tumour cells have a higher negative surface-charge density compared with that of the

normal adult liver cells; the significance of this is discussed below.

The anti-(foetal liver) serum, which we have called anti-FL serum, was then absorbed with adult liver to give anti-FL serum (A), and likewise with foetal liver cells and hepatoma cells to give anti-FL serum (F) and anti-FL serum (H) respectively. The effects of these sera on the electrophoretic mobility of the three cell types are shown in Table 2. Anti-FL serum, after absorption with adult liver cells, still decreased the electrophoretic mobility of foetal liver cells by 51% and hepatoma cells by 45%. Anti-FL serum, after absorption with foetal liver cells, decreased the electrophoretic mobility of hepatoma cells by only 20%, and anti-FL serum, after absorption with hepatoma cells, decreased the electrophoretic mobility of foetal liver cells by only 9.3%, but there was not complete removal of antibodies against adult or foetal antigens, since the electrophoretic mobility was decreased by 4.1 and 9.3% for adult and foetal liver cells respectively. The results suggest that the hepatoma cell surface has some antigens in common with foetal liver cells, but perhaps in lower concentrations, and that it also has some adult antigens, not present on foetal cells but present in much higher concentrations on adult liver cells.

The anti-embryo serum, which we have called anti-E serum, was almost as effective as anti-FL serum in decreasing the electrophoretic mobility of foetal liver and hepatoma cells. This could have been due to incomplete removal of the livers from the embryos during the preparation of the antigen, or, more likely, to the presence in the foetal gut of the carcino-embryonic antigens described by Krupey *et al.* (1967) and Gold *et al.* (1968).

Table 1. *Electrophoretic mobility of adult liver cells, foetal liver cells and hepatoma cells before and after treatment with rabbit antisera to rat foetal liver and rat foetus*

The cells were isolated (for experimental details see the text), washed in phosphate-buffered saline and suspended in 145mM-NaCl-0.1mM-NaHCO₃ buffer, pH7.2. The cells were incubated with antiserum (0.5ml/10⁶ cells) at 0°C for 1h. The cells were washed three times by centrifugation, then suspended in 145mM-NaCl-0.1mM-NaHCO₃ buffer, pH7.2, and brought to 25°C before measurement of the electrophoretic mobility of 15-32 randomly selected cells (numbers in parentheses). The change compared with the control is given as a percentage, and the significance, by Student's *t* test (*P* < 0.001), is indicated by *** (see Table 2).

Electrophoretic mobility ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$)

Cell suspension	No antiserum	Anti-(foetal liver) serum	Anti-embryo serum
Adult liver cells	-0.761 ± 0.010 (20)	-0.65 ± 0.036 (15) -14.3% ***	-0.614 ± 0.005 (15) -19.3% ***
Foetal liver cells	-0.942 ± 0.015 (23)	-0.438 ± 0.006 (20) -53.5% ***	-0.474 ± 0.009 (15) -49.5% ***
Hepatoma cells	-0.956 ± 0.010 (32)	-0.490 ± 0.011 (20) -49.0% ***	-0.539 ± 0.008 (20) -43.6% ***

Table 2. *Electrophoretic mobility of adult liver cells, foetal-liver cells and hepatoma cells before and after treatment with rabbit antiserum to rat foetal liver absorbed with rat adult liver (A), foetal liver (F) and hepatoma cells (H)*

For experimental details see Table 1. The change compared with the control is given as a percentage. Significance by Student's *t* test: $P > 0.05$, (N.S.); $0.05 > P > 0.01$, *; $P < 0.001$, ***.

Cell suspension	Electrophoretic mobility ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$)			
	No antiserum	Anti-(foetal liver) serum (A) (absorbed with liver cells)	Anti-(foetal liver) serum (F) (absorbed with foetal liver)	Anti-(foetal liver) serum (H) (absorbed with hepatoma cells)
Adult liver cells	-0.733 ± 0.009 (18)	-0.720 ± 0.008 (20) -1.8% (N.S.)	-0.644 ± 0.009 (20) -12.2% ***	-0.703 ± 0.008 (25) -4.1% *
Foetal liver cells	-0.881 ± 0.006 (20)	-0.431 ± 0.006 (20) -51.0% ***	-0.860 ± 0.006 (20) -2.4% (N.S.)	-0.799 ± 0.012 (26) -9.3% ***
Hepatoma cells	-0.954 ± 0.010 (27)	-0.524 ± 0.011 (25) -45.0% ***	-0.761 ± 0.009 (22) -20.2% ***	-0.978 ± 0.010 (20) +2.5% (N.S.)

Fig. 1. *Electrophoretic mobility of hepatoma cells after treatment with doubling dilutions of anti-(foetal liver) serum (absorbed with adult liver cells)*

Hepatoma 223 cells were freshly removed from a rat, washed in phosphate-buffered saline and suspended in 145 mM-NaCl-0.1 mM-NaHCO₃ buffer, pH 7.2. Samples containing approx. 2×10^7 cells were treated with various dilutions of anti-(foetal liver) serum (A) (0.5 ml/ 10^6 tumour cells) at 0°C for 1 h. The cells were washed three times by centrifugation, suspended in 145 mM-NaCl-0.1 mM-NaHCO₃ buffer, pH 7.2, and warmed to 25°C before measurement of electrophoretic mobility as described in the text. Measurements were made of 20-23 single cells, randomly selected, from each sample treated with serum, and the means and s.e.m. so obtained are indicated in the figure. Complement activity in the serum was destroyed by heating at 56°C for 30 min before use. Cell viability was measured at the end of each experiment and was usually greater than 90%.

Titration of antiserum anti-FL serum (A) against hepatoma cells

The electrophoretic mobility of hepatoma cells was measured after incubation for 1 h at 0°C with doubling dilutions from 1/10 to 1/1280 per 10^6 cells

of anti-FL serum. The titration curve is shown in Fig. 1. The maximum decrease in electrophoretic mobility remained constant up to a dilution of 1/60, a dilution of 1/240 gave a 50% effect and no effect was observed with dilutions of 1/640 and 1/1280. The high titre of

the end point of the anti-FL serum (A) titration curve indicates a high affinity of the antiserum for hepatoma surface antigen.

Attempts to characterize the chemical composition of the foetal antigen

Effect of neuraminidase treatment. The electrophoretic mobilities of foetal liver and hepatoma cells were statistically higher than that of adult liver cells. Neuraminidase had no effect on adult liver cells, whereas it produced a large decrease in the electrophoretic mobility of foetal liver and hepatoma cells (51 and 34% respectively). The results are shown in Table 3 and suggest that sialic acid residues are present in the foetal liver and tumour cells linked to components by glycosidic bonds that are susceptible to neuraminidase. These sialic acid residues are near to the electrokinetic slip-plane and are therefore on or in the cell plasma membrane, and are also sufficiently exposed to react with the enzyme. It is possible that the adult liver cells had sialic acid residues that were non-susceptible or not exposed to the neuraminidase. Unfortunately, time and facilities did not permit the chemical estimation of the quantity of sialic acid released from the cells. The possibility also exists that some components were removed from the surface of the cells, either foetal or adult, by treatment with EDTA. Codington *et al.* (1970) found that TA₃ ascites cells lost 16% of their sialic acid and 11% of cell weight after being shaken for 80 min with EDTA at 3°C. In our experiments the adult liver cells had contact with EDTA during perfusion, and the foetal cells during homogenization, but in both cases for the shortest possible time (less than 5 min). It is, however, possible that the so-called foetal antigen was present on the adult liver cell surface but was removed more easily by the chelating agent.

Effect of antiserum on the neuraminidase-treated

cells. Another batch of neuraminidase-treated cells was subsequently treated with antisera, either anti-FL serum (A) or unabsorbed anti-embryo serum, before measurement of their electrophoretic mobilities. The results in Table 2 showed that anti-FL serum (A) caused a large decrease in the electrophoretic mobilities of foetal liver and hepatoma cells. However, after these cells had been treated with neuraminidase (Table 4) only a further small decrease in electrophoretic mobility resulted from addition of anti-FL serum (A). This suggests that most of the binding sites for anti-FL serum (A) had been removed by the neuraminidase treatment or were no longer available to antibody.

Blocking effect by sialic acid on the decrease in electrophoretic mobility of hepatoma cells produced by anti-FL serum (A)

Sialic acid [1.8 mg in 0.5 ml of 1/100 dilution of anti-FL serum (A)] was added to 2×10^6 cells in 5 ml of 145 mM-NaCl and adjusted to pH 7.2 with 100 mM-NaOH. The cells were incubated at 0°C for 1 h and washed before the electrophoretic mobilities were measured. The results in Table 5 show that sialic acid alone did not alter the electrophoretic mobility of hepatoma cells but it significantly decreased the fall in electrophoretic mobility caused by anti-FL serum (A). The treatment with sialic acid or sialic acid plus anti-FL serum (A) did not significantly increase cell death or clumping of the hepatoma cells. Here sialic acid appears to be acting as a hapten that blocks the specific foetal-antigen-binding site of the anti-FL serum (A). Sialic acid alone caused no significant change in the electrophoretic mobility of the cells; therefore the effect is unlikely to be due to the sialic acid remaining adsorbed on the cell membrane after washing.

Table 3. *Effect of neuraminidase and RNAase on electrophoretic mobility of adult liver cells, foetal liver cells and hepatoma cells*

Treatments were with either 1 unit of neuraminidase/ 10^6 cells for 15 min at 36°C, followed by three washings, or $10 \mu\text{g}$ of RNAase/ 10^6 cells for 30 min at 37°C, followed by three washings. Results are expressed as means $\pm$ s.e.m. of the numbers of cells measured in parentheses. For other details see Table 2.

Cell suspension	Electrophoretic mobilities ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$)		
	No treatment	+ Neuraminidase	+ RNAase
Adult liver cells	-0.803 ± 0.008 (23)	-0.802 ± 0.010 (18) 0%	-0.808 ± 0.014 (16) +0.6% (N.S.)
Foetal liver cells	-0.984 ± 0.013 (25)	-0.480 ± 0.008 (20) -51.2% ***	-0.818 ± 0.013 (30) -17.0% ***
Hepatoma cells	-1.071 ± 0.020 (25)	-0.704 ± 0.012 (22) -34.3% ***	-0.760 ± 0.010 (24) -29.0% ***

Table 4. *Effect of rabbit anti-(rat foetal liver) serum (absorbed with adult rat liver) and anti-embryo serum on the electrophoretic mobility of neuraminidase-treated adult liver cells, foetal liver cells and hepatoma cells of the rat*

Experimental details of neuraminidase treatment were the same as in Table 3: incubation with antiserum, washings and measurement of cell electrophoretic mobility were as in Table 1.

Cell suspension	Electrophoretic mobility ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$)		
	Neuraminidase + 145 mM-NaCl	Neuraminidase + anti-(foetal liver) serum (A)	Neuraminidase + anti-embryo serum
Adult liver cells	-0.686 ± 0.007 (24)	-0.698 ± 0.006 (27)	-0.704 ± 0.009 (22)
Foetal liver cells	-0.420 ± 0.006 (20)	-0.384 ± 0.010 (17) -8.6%	-0.374 ± 0.004 (18) -11.0%
Hepatoma cells	-0.562 ± 0.006 (20)	-0.514 ± 0.010 (20) -8.5%	-0.448 ± 0.007 (20) -20%

Table 5. *Blocking effect by sialic acid (or supernatants from neuraminidase-treated cells containing sialic acid) of the decrease in the electrophoretic mobility of hepatoma cells caused by 1/100 anti-(foetal liver) serum (A)*

For experimental details see the text. Electrophoretic mobilities are given as means $\pm$ S.E.M. of the numbers of cells measured in parentheses. Sialic acid alone caused no significant change in the electrophoretic mobility. Student's *t* test showed all other percentage changes are significant ($P < 0.001$). The percentage changes in the last column indicate the extent to which sialic acid (or the sialic acid-containing supernatants) have blocked the change induced by the antiserum alone.

Treatment of hepatoma cells	Electrophoretic mobility ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$)	Change in mobility (%)	Blocking of antiserum effect (%)
None (145 mM-NaCl)	-0.935 ± 0.009 (23)		
Sialic acid (0.9 mg/10 ⁶ cells)	-0.929 ± 0.007 (24)	-0.6	
Anti-serum only, 1/100 anti-FL serum (A)	-0.660 ± 0.010 (20)	-29.5	
Sialic acid + 1/100 antiserum	-0.831 ± 0.014 (25)	-11.2	62
Supernatant X from neuraminidase-treated hepatoma cells [residual neuraminidase inhibited with K ₃ Fe(CN) ₆]	-0.797 ± 0.014 (22)	-14.7	
Supernatant X + 1/100 antiserum	-0.770 ± 0.010 (22)	-17.7	40
Supernatant Y from neuraminidase-treated hepatoma cells (residual neuraminidase inhibited by 56°C for 30 min)	-0.864 ± 0.009 (24)	-7.6	
Supernatant Y + 1/100 antiserum	-0.761 ± 0.017 (23)	-18.2	38

Blocking effect by the supernatant from neuraminidase-treated hepatoma cells on the decrease in electrophoretic mobility of hepatoma cells produced by anti-FL serum (A)

The supernatant from neuraminidase-treated hepatoma cells should contain sialic acid, but it also contains neuraminidase, which must be inactivated. Table 5 also includes the results of an experiment using 1 ml of 0.2% (w/v) potassium ferricyanide to inactivate the residual neuraminidase activity. The ferricyanide-inactivated 'supernatant X' blocked at least 40% of the ability of anti-FL serum (A) to lower the electrophoretic mobility of hepatoma cells. The super-

natant and the antiserum caused a slight amount of agglutination in the hepatoma cells.

The use of ferricyanide to block the residual neuraminidase activity in this experiment is open to criticism, because it is a metabolic poison and might damage the cells via the sodium pump. However, cell death was not significantly increased. It is also possible that ferricyanide could combine with the antibody molecule, thus preventing its combination with the antigen. This possibility cannot be discounted, since the ferricyanide probably combines with a peptide group when it inactivates neuraminidase. Therefore heat-inactivation was used as an alternative

method of destroying the neuraminidase in the supernatant.

In this experiment the 'supernatant Y' from neuraminidase-treated hepatoma cells was heated at 56°C for 30 min to inactivate or minimize residual neuraminidase activity (Buzzell & Hanig, 1958). The results shown in Table 5 perhaps indicate that the 'supernatant Y' had some residual neuraminidase activity, but it clearly did block at least 38% of the antiserum effect.

pH-electrophoretic mobility relationship of hepatoma cells in the presence of anti-FL serum (A) and anti-FL serum (H)

The electrophoretic mobilities of hepatoma cells at different pH values between 3 and 10 are shown in Fig. 2. The shapes of the control and anti-FL serum (H) curves are similar, with a slightly lower electrophoretic mobility of the latter at pH 7 and a shift of the curve to the left at low pH and to the right at high

Fig. 2. *pH-electrophoretic mobility curves of hepatoma cells after treatment with 145mm-NaCl (●), anti-(foetal liver) serum (absorbed with hepatoma cells) (○), and anti-(foetal liver) serum (absorbed with adult liver cells) (△)*

Hepatoma 223 cells were washed as described in Fig. 1 and incubated either in saline or with antisera (0.5 ml/10⁶ cells) for 1 h at 0°C. The cells after incubation were washed three times with 145mm-NaCl-0.1 mm-NaHCO₃ buffer, pH 7.2, and finally suspended in 145mm-NaCl and adjusted to different pH values by addition of 145mm-HCl or -NaOH immediately before the electrophoretic mobilities of 16-24 randomly selected cells were measured at each of the pH values. The mean obtained in each case is indicated in the figure.

pH values, but the slopes are parallel with those of the control curve. The anti-FL serum (A) curve, however, shows a 33% decrease in the electrophoretic mobility at pH 7.2 and an absence of the slope between pH 3 and 4. The slope between pH 8 and 9 is parallel with that of the control curves. This indicates that only cell-surface groups with pK 3-4 are affected by the anti-FL serum (A). The flattening of this slope suggests that these cell-surface groups (sialic acid would be an obvious candidate) are covered by antibody molecules.

Unfortunately, readings of electrophoretic mobility cannot be extended to lower pH values because of cell lysis, so that an accurate pK of the group being titrated in this experiment cannot be determined. However, if the curves for hepatoma cells in 145mm-NaCl and in anti-FL serum (H) are extrapolated to cut the abscissae the end point of the curve would be about pH 3, which is near the pK of sialic acid (pK_a 2.6). The slight decrease in electrophoretic mobility and shift to the left of the anti-FL serum (H) curve are probably due to a non-specific electrostatic effect of the serum. The slopes are parallel with those of the curve for the hepatoma in 145mm-NaCl, showing that none of the ionogenic groups are covered by anti-FL serum (H).

Note that the anti-FL serum (A) curve is also parallel with the control curves in the high-pH region. This would indicate that the groups responsible for this slope (probably basic amino groups) are not covered by the antiserum. The absence of a slope at pH 3-4 shows that anti-FL serum (A) has reacted specifically with the groups of pK 3-4 and covered them, markedly decreasing the net negative charge and therefore decreasing the electrophoretic mobility.

Effect of RNAase on the electrophoretic mobility of adult liver cells, foetal liver cells and hepatoma cells of rat

The cells were incubated with RNAase (10 μg/10⁶ cells) at 37°C for 30 min and then washed three times and resuspended in 145mm-NaCl (Table 3). There was no significant decrease in the electrophoretic mobility of adult liver cells after treatment with RNAase, but the foetal liver cells showed a 17% decrease and hepatoma cells a 29% decrease. The results also show that foetal liver cells and hepatoma cells have a higher electrophoretic mobility than adult liver cells, by 17 and 29% respectively. It is tempting to equate this with the comparable decrease in electrophoretic mobility produced by RNAase treatment. However, neuraminidase decreased the electrophoretic mobility of foetal liver cells by 51% and of hepatoma cells by 34%, the enzyme-treated cells having electrophoretic mobilities well below the values for normal liver.

Fig. 3. Histograms showing the electrophoretic mobility of BP8 ascites-tumour cells before and after incubation with RNAase

Mouse ascites-tumour BP8 cells were incubated for 30 min at 37°C with gentle shaking in the presence of ribonuclease (100 $\mu\text{g}/10^7$ cells in 1 ml of Hanks balanced salt solution). The treated and control cells not exposed to the enzyme were washed three times in 145 mM-NaCl-0.1 mM- NaHCO_3 buffer, pH 7.2 and the electrophoretic mobilities of 20 randomly selected cells in each group were measured as described in the text. ▨, Untreated; ■, treated with RNAase. The mean mobilities were -2.471 ± 0.090 and $-1.497 \pm 0.044 \mu\text{m} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$ before and after incubation respectively.

It would be dangerous to postulate from this evidence alone that RNA is present in the cell membranes of foetal liver and hepatoma cells. Ribonuclease carries a net positive charge at pH 7, and could theoretically decrease the electrophoretic mobility by simple adsorption on the cell membrane. If this were so, however, the electrophoretic mobility of normal liver cells should also be affected. To study further the question of RNA in cell membranes the parallel experiments on BP8 tumour cells and normal and allergized lymph-node cells were undertaken.

Effect of RNAase treatment on mouse BP8 ascites-tumour cells and lymph-node cells from allergized and normal C57 Bl mice

BP8 cells were incubated with RNAase (10 $\mu\text{g}/10^6$ cells) for 30 min at 37°C, and after washing they were resuspended in 145 mM-NaCl and the electrophoretic mobility was determined. The results are drawn as a histogram in Fig. 3, and show that RNAase caused a significant decrease of 39% in the electrophoretic mobility of BP8 cells, and suggest the presence of groups susceptible to RNAase on the cell membrane. It is important to determine whether the decrease in electrophoretic mobility is caused by adsorption of the enzyme on the cell membrane or by enzymic removal of RNA from the cell surface. The isoelectric point of RNAase is 9.6 (Glick *et al.*, 1967) and the pH of the BP8 cell suspension is 6.5. Therefore the enzyme molecule will have an overall positive charge,

which might decrease the electrophoretic mobility by adsorption on the cell membrane. As it is not easy to inactivate RNAase without altering its ionogenic groups this problem was approached in four ways.

(a) The electrophoretic mobility of the BP8 cells was measured after 1, 10, 25 and 30 min of incubation with RNAase, and the results (Fig. 4) showed an approximately exponential curve. (A similar experiment with hepatoma cells showed the same type of curve.)

(b) The RNAase activity was inhibited by incubation at 4°C. RNAase was added to BP8 cells at 4°C and a portion of the suspension was immediately taken and warmed to 25°C, and the electrophoretic mobility measurements were made as quickly as possible. This sample had an electrophoretic mobility of -2.069 ± 0.112 (20) and corresponded to the action of RNAase for 1 min in Fig. 4. The rest of the cells were kept at 4°C for 30 min and then the electrophoretic mobility measurements were made as quickly as possible at 25°C, giving a value of -2.018 ± 0.049 (20), which is not significantly different from the previous sample. Both these experiments argue against the decrease in electrophoretic mobility being due to an electrostatic effect from an adsorption of the enzyme protein on the cell membrane.

(c) A third and rather less satisfactory method was undertaken to disprove the adsorption hypothesis. After incubation with RNAase the BP8 cells were washed three, six and ten times and the electrophoretic mobility was determined. If the enzyme were

adsorbed, then increasing the number of washes should remove some of it and cause an increase in the electrophoretic mobility. In fact the electrophoretic mobility decreased slightly but not significantly and was -1.168 ± 0.062 (22), -1.143 ± 0.057 (26) and -1.123 ± 0.033 (23) after three, six and ten washes respectively.

(d) The electrophoretic mobility of washed BP8 cells decreases by 26% after the addition of 5mM- Ca^{2+} . Inactivated RNAase was prepared by the method of Barnard & Stein (1959), i.e. 0.31 M-bromoacetic acid freshly dissolved and neutralized to pH 7.2 was added to RNAase (60 $\mu\text{g}/\text{ml}$). The inactivated RNAase did not prevent the binding of Ca^{2+} to the cell membrane and the electrophoretic mobility decreased by 24%. Conversely, RNAase alone decreased the electrophoretic mobility by 37.4% and in the presence of Ca^{2+} by only a further 4%.

Effect of RNAase on the electrophoretic mobility of lymph-node cells from normal and allergized C57 Bl mice

The distribution of the electrophoretic mobility of lymph-node cells from untreated and from allergized

Fig. 4. Effect of the time of incubation with RNAase on the electrophoretic mobility of BP8 ascites-tumour cells

The cells were treated with the enzyme (100 $\mu\text{g}/10^7$ cells in 1 ml of Hanks balanced salt solution). At various times cells were harvested from the incubation mixture by centrifugation, washed three times in and resuspended in 145 mM-NaCl-0.1 mM- NaHCO_3 , pH 7.2, for measurement of the electrophoretic mobilities of 20-22 randomly selected cells as indicated in the text. The means and S.E.M. are plotted.

C57 Bl mice is shown in Fig. 5. The wide scatter in electrophoretic mobility of lymph-node cells from the allergized mouse should be noted. This is because there are really two populations in this case: (1) a normal population of lymph-node cells corresponding to that in the control mouse and (2) a new population of fast-moving lymph-node cells in the allergized mouse. In some mice, especially when highly allergized, these populations can be seen to be separate (Hartveit *et al.*, 1968). Another feature often noticed was the presence of a few lymph-node cells in the allergized mice that were slower than the normal population, and these could be plasma cells or their precursors.

The effect of RNAase on the lymph-node cells of a normal mouse was small: there was a decrease of 4.8% (not significant) compared with a much larger effect, a 13.3% decrease in the electrophoretic mobility of the lymph-node cells in the allergized mouse ($P < 0.01$). The effect appears to be predominantly on the faster lymph-node cells in each case.

Neuraminidase treatment (1 unit/ 10^6 cells at 36°C for 15 min) caused a 49% decrease in the electrophoretic mobility of allergized lymph-node cells compared with a 23% decrease in that of normal lymph-node cells. After neuraminidase treatment, Mitchell & Cater (1971) found decreases in the electrophoretic mobility of allergized lymph-node cells of 36, 32 and 27% in three different experiments. The incubation conditions were slightly different from ours, and they did not examine lymph-node cells from normal mice.

The high electrophoretic mobility shown by BP8 ascites-tumour cells and allergized lymph-node cells could be due to a high content of RNA and sialic acid on the cell surface, and the effect of RNAase and neuraminidase on the electrophoretic mobility of these cells is consistent with this hypothesis.

It is difficult to determine whether the RNAase-susceptible RNA is an integral structural component of the cell membrane or an artifact produced by RNA liberated from damaged cells becoming adsorbed on the cell membrane. Weiss (1968) argued that if the RNA were adsorbed then repeated washings would tend to remove it and minimize the effect of RNAase. He found that repeated washing did not reverse the RNAase-induced decrease in electrophoretic mobility, and we also found that the decrease in electrophoretic mobility tended to increase rather than diminish with multiple washings. Weiss & Mayhew (1967) deliberately contaminated cells with cell sap after RNAase treatment and found that no change in electrophoretic mobility occurred. We have not done this, but our finding that RNAase has no effect on adult liver cells and little effect on foetal liver cells, hepatoma cells and allergized lymph-node cells argues in favour of the RNA being an integral part of

Fig. 5. Electrophoretic mobility of lymph-node cells from normal and allergized C57 Bl mice: histograms showing the effect of incubation with RNAase ($10\mu\text{g}/10^6$ cells) for 30 min at 37°C

(a) Lymph-node cells from normal C57 Bl mice; electrophoretic mobility = 1.285 ± 0.027 (22). (b) Lymph-node cells from the same batch as (a) after treatment with RNAase ($10\mu\text{g}/10^6$ cells) for 30 min at 37°C ; electrophoretic mobility = 1.223 ± 0.025 (21), a change of -4.8% , which is not significant ($P = 0.1$). (c) Lymph-node cells from allergized C57 Bl mice; electrophoretic mobility = 1.386 ± 0.054 (20). (d) Lymph-node cells from the same batch as (c) after treatment with RNAase (as above); electrophoretic mobility = 1.201 ± 0.043 (20), a change of -13.3% ($P < 0.02$). ■, Untreated; □, treated with RNAase.

the growing dividing cells. After all, the adult liver cells and normal lymph-node cells are as likely to be contaminated by cell sap during preparation as are the other cell types under investigation.

Further evidence is provided by the effect of RNAase on the binding of Ca^{2+} to BP8 cells. The additional decrease of mobility due to Ca^{2+} after RNAase treatment was only one-quarter of the decrease in electrophoretic mobility due to Ca^{2+} alone. This indicates that the RNAase had destroyed three-quarters of the binding sites for Ca^{2+} . Weiss & Mayhew (1967), after extensive experiments on the effects of RNAase and neuraminidase on the binding of Ca^{2+} to Ehrlich ascites-tumour cells and a human osteogenic sarcoma cell line, concluded that RNAase was more effective than neuraminidase in decreasing the binding of Ca^{2+} .

The electrophoretic mobility of lymph-node cells from allergized mice is higher than that of lymph-node cells from normal mice by about the same amount as the decrease produced by RNAase treatment. However, any idea that the increased electrophoretic mobility of allergized lymph-node cells is due to an increase of their surface RNA should be regarded with some caution, because of the increased

effect of neuraminidase on these cells. Mayhew & Weiss (1968) reported that RNAase decreased the electrophoretic mobility of normal human lymphocytes by 12.6% . This is almost identical with our present findings with lymph-node cells from allergized mice, and may be significant, because human blood lymphocytes are known to be a population of immunologically competent cells.

It is interesting to speculate in biological terms on the function(s) of RNA on the surface cells.

One possibility is that cell-surface RNA is associated with mitotically active cell types. In favour of this idea is the presence of the RNAase effect in tumours, foetal tissues and actively dividing cells, but not in cells that have ceased to divide, such as erythrocytes and platelets, or that divide very infrequently, such as adult liver cells. Mayhew & O'Grady (1965) found that human osteosarcoma cells had a higher electrophoretic mobility in mitosis than in the DNA-synthetic stage. This might or might not be due to RNA, but Mayhew & Weiss (1968) found this same cell line when grown in 5% calf serum showed a 23.6% decrease in electrophoretic mobility after RNAase treatment, compared with a 13% decrease when it had been grown in 0.5% calf serum. They

concluded that the surface density of sialic acid remained fairly constant but when the cells grow poorly the RNA content fell.

Another possibility is that the RNA serves an immunological function. The presence of surface RNA might play a part in cell recognition. It could explain why a mixture of cell types sorts itself out into homotypic aggregates in tissue culture. There would seem to be a greater need for cell recognition during foetal life. On this basis the presence of RNA on tumours would be a reversion towards a foetal cell type. Ever since Fishman (1961) reported that macrophages passed an antigen-RNA complex to lymphocytes during the chain of events leading to antibody formation, there have been an increasing number of publications on the transference of specific immunity by RNA, or more correctly by RNA contaminated with protein. There are reports on transference of anti-tumour immunity *in vivo* by 'RNA' from lymphocytes (Alexander *et al.*, 1967; Kennedy *et al.*, 1969) and also *in vitro* (Mannick & Egdahl, 1962; Pilch & Ramming, 1970; Bell & Dray, 1970). Thus there is strong evidence for RNA being passed from one cell to another (see also Kolodny, 1971), or from culture medium to cell, and during such passage the RNA must be on the surface of the cell at least temporarily. Thus the RNA on the surface of lymph-node cells from allergized mice could have been placed there by stimulated macrophages. If this hypothesis is correct then there is evidence in the present paper that would suggest that the RNA stays there as a 'recognition coat'. There is no reason to suppose that the possibilities outlined above are mutually incompatible.

Effect of trypsin on the electrophoretic mobility of hepatoma cells and the action of the anti-FL serum (A)

The hepatoma cells were incubated with trypsin (1 mg/10⁶ cells) for 60 min at 37°C and then thoroughly washed three times before addition of the antiserum, to avoid the latter being degraded. The trypsin decreased the electrophoretic mobility of hepatoma cells by 22.3%, and after trypsin treatment the anti-FL serum (A) did not produce a significantly greater decrease in electrophoretic mobility (24.5%). Trypsin did not appear to cause the decrease in electrophoretic mobility by damaging the cells, as cell death after trypsin was the same (10%) as in the controls.

There is much evidence that trypsin treatment of cells removes sialoglycopeptide. From trypsin-treated Ehrlich ascites tumour Langley & Ambrose (1964, 1967) obtained and partially characterized a sialoglycopeptide. Codington *et al.* (1970) released sialic acid by trypsin or by Pronase from mouse mammary tumour cells, and Walborg *et al.* (1969) obtained sialoglycopeptide from Novikoff rat hepatoma (ascites form) by digestion with papain.

Moscona (1971) has shown that binding sites for certain lectins are exposed at the surface of chick embryonic cells but not on normal adult cells unless these cells are first treated with trypsin. These results were published after we had completed our experiments, but in retrospect we should have studied the effect of antiserum on trypsin-treated adult liver cells, as it is possible that a foetal antigen might be present in the normal adult cell but is only made available to antibody after removal of a trypsin-sensitive layer.

Demonstration of foetal antigen by immunological techniques

Mixed agglutination. If a common surface exists on hepatoma and foetal liver cells, then the anti-(foetal liver) serum purified by absorption with adult liver cells should produce mixed clumps of cells containing both cell types, as well as separate clumps of hepatoma and foetal liver cells (unless the antibody is incomplete). From the titration of antiserum activity (Fig. 1), the most dilute anti-FL serum (A) to have agglutinating activity would be in the 1/160–1/320 range.

Adult liver or foetal liver cell suspensions (10⁶ cells/ml) were incubated with an equal volume of 1/100 antiserum for 30 min at room temperature and the cells collected by centrifugation (80g_{av.}), washed twice and made up to the original cell concentration. An equal number of hepatoma cells were added and the cells brought together by centrifugation (80g_{av.}) and then examined under phase-contrast microscopy on silicone-treated slides. Since foetal and adult liver cells are larger than hepatoma cells, mixed agglutination was shown by a cluster of hepatoma cells round the liver cells. The percentage of liver cells showing adherent hepatoma cells was recorded (percentage of rosettes). We found that 1/100 anti-FL serum (A) gave a positive 20% and 1/1000 gave a 2% mixed agglutination between foetal liver cells and hepatoma cells; other values were: 1/100 anti-FL serum (F), 2%, 1/1000, 0%; anti-FL serum (H) 1/100, 0%; 145 mM-NaCl, 0%. It is noteworthy that Furusawa *et al.* (1965) reported mixed agglutination between Ehrlich ascites-tumour cells and embryonic mouse erythrocytes.

In an experiment with hepatoma cells alone, 1/100 anti-FL serum (A) gave a statistically higher (77.9±2.3%) agglutination than did anti-FL serum (F) (54.9±3.4%), anti-FL serum (H) (40.25±2.5%) or 145 mM-NaCl (31.75±2.7%).

Complement lysis. Hepatoma cells (10⁵ cells/ml) were mixed with an equal volume of 1/40 antiserum and left for 15 min at room temperature. Three dilutions of fresh guinea-pig serum were added and the mixtures incubated at 37°C for 30 min. Cytolysis was read by phase-contrast microscopy. With 1/40 anti-FL serum (A) and 1/10 guinea-pig serum there was

88% cell lysis, a significant increase over the control lysis of 20% with 145 mM-NaCl or anti-FL serum (H).

Anti-globulin test. This was performed by the technique of Coombs & Gell (1968), by using adult liver, foetal liver and hepatoma cells against normal rabbit serum, unabsorbed anti-(foetal liver) serum and the absorbed sera anti-FL sera (A), (F) and (H). The normal rabbit serum gave a negative reaction; the anti-FL serum gave a low-titre reaction, strongest with foetal cells; anti-FL serum (A) gave a weak reaction with hepatoma cells and a rather stronger reaction with foetal cells. Anti-FL serum (F) and anti-FL serum (H) gave no reaction with hepatoma cells but still some reaction with foetal liver cells. This rather equivocal answer obtained by using goat anti-(rabbit globulin) serum (absorbed with sheep erythrocytes) may be consistent with the report of Gold *et al.* (1968) that the antibodies against carcino-embryonic antigens are macroglobulins. No anti-(rabbit IgM) serum was available.

Thus we have obtained evidence for a common antigen(s) between hepatoma cells and rat foetal liver cells both from our main investigations by the technique of cell electrophoresis and from subsidiary experiments with more conventional immunological techniques. A common antigen between foetal and tumour cells might explain why the host often appears immunologically tolerant to specific antigens of its own tumour and suggests the possibility of using foetal cells to produce anti-tumour immunity as a therapeutic measure in cancer patients.

We are grateful to Dr. J. N. Mehrishi for help with the electrophoresis apparatus, Dr. Constance Grove for help with recording experiments and to Professor R. R. A. Coombs and Dr. L. B. Fraser for helpful discussions. D. B. C. is a Senior Gibb Fellow of the Cancer Research Campaign.

References

- Abelev, G. I. (1968) *Cancer Res.* **28**, 1344
- Abelev, G. I., Perova, S. D., Khramkova, N. I., Postnikova, Z. A. & Irlin, I. S. (1963) *Transplantation* **1**, 174
- Alexander, P., Delorme, E. F., Hamilton, L. D. G. & Hall, F. G. (1967) *Nature (London)* **213**, 569
- Ambrose, K. R., Anderson, N. G. & Coggin, J. H., Jr. (1971a) *Nature (London)* **233**, 194
- Ambrose, K. R., Anderson, N. G. & Coggin, J. H., Jr. (1971b) *Nature (London)* **233**, 321
- Baranska, W., Koldovsky, P. & Koprowski, H. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 193
- Barnard, E. A. & Stein, W. D. (1959) *J. Mol. Biol.* **1**, 339
- Bell, C. & Dray, S. (1970) *J. Immunol.* **105**, 541
- Buzzell, A. & Hanig, M. (1958) *Advan. Virus Res.* **5**, 289
- Codington, J. F., Sanford, B. H. & Jeanloz, R. W. (1970) *J. Nat. Cancer Inst.* **45**, 637
- Coombs, R. R. A. & Gell, P. G. H. (1968) in *Clinical Aspects of Immunology* (Gell, P. G. H. & Coombs, R. R. A., eds.), 2nd edn., p. 31, Blackwell, Oxford
- Fishman, M. (1961) *J. Exp. Med.* **114**, 837
- Furusawa, M., Adachi, H. & Asayama, S. (1965) *Exp. Cell Res.* **40**, 151
- Glick, G. M., Gorney, H. J. & Barnard, E. A. (1967) *Biochem. J.* **102**, 7c
- Gold, P., Gold, M. & Freedman, S. O. (1968) *Cancer Res.* **28**, 1331
- Hartveit, F., Cater, D. B. & Mehrishi, J. N. (1968) *Brit. J. Exp. Pathol.* **49**, 634
- Hollinshead, A., Glew, D., Bunnag, B., Gold, P. & Herberman, R. (1970) *Lancet* **i**, 1191
- Kennedy, C. T. C., Cater, D. B. & Hartveit, F. (1969) *Acta Pathol. Microbiol. Scand.* **77**, 196
- Kolodny, G. M. (1971) *Exp. Cell Res.* **65**, 313
- Krupey, J., Gold, P. & Freedman, S. O. (1967) *Nature (London)* **215**, 67
- Langley, O. K. & Ambrose, E. J. (1964) *Nature (London)* **204**, 53
- Langley, O. K. & Ambrose, E. J. (1967) *Biochem. J.* **102**, 367
- Mannick, J. A. & Egdahl, R. H. (1962) *Science* **137**, 976
- Mayhew, E. & O'Grady, E. A. (1965) *Nature (London)* **207**, 86
- Mayhew, E. & Weiss, L. (1968) *Exp. Cell Res.* **50**, 441
- Mehrishi, J. (1962) *Annu. Rep. Brit. Emp. Cancer Campgn.* **40**, 380
- Mitchell, D. M. & Cater, D. B. (1971) *Brit. J. Exp. Pathol.* **52**, 152
- Moscona, A. A. (1971) *Science* **171**, 905
- Pearson, G. & Freeman, G. (1968) *Cancer Res.* **28**, 1665
- Pilch, Y. H. & Ramming, K. P. (1970) *Cancer (Philadelphia)* **26**, 630
- Purves, L. R., Bersohn, I. & Geddes, E. W. (1970a) *Cancer (Philadelphia)* **25**, 1261
- Purves, L. R., Van de Merwe, E. & Bersohn, I. (1970b) *Lancet* **ii**, 464
- Seaman, G. V. F. & Heard, D. H. (1960) *J. Gen. Physiol.* **44**, 251
- Stonehill, E. H. & Bendich, A. (1970) *Nature (London)* **228**, 370
- Walborg, E. F., Jr., Lantz, R. S. & Wray, V. P. (1969) *Cancer Res.* **29**, 2034
- Weiss, L. (1968) *J. Theor. Biol.* **18**, 9
- Weiss, L. & Mayhew, E. (1967) *J. Cell. Physiol.* **69**, 281