2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate and methyl [17β-acetoxy-3-methoxyoestra-1,3,5(10)-trien-16α-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate, have been synthesized from oestrone.

2. Oestriol glucuronide was extracted from human-pregnancy urine with butanol, and purified by chromatography on an alumina column and a Celite partition column.

3. This glucuronide was methylated and acetylated and resolved into two components by fractional crystallization.

4. Comparison of the two components with the two synthetic isomers indicated that both ring D glucuronides of oestriol monoglucuronide exist in human-pregnancy urine.

The authors thank Professor Sir Charles Dodds, M.V.O., F.R.S., for the interest he has shown in this work. Generous gifts of chemicals were received: oestrone from Dr T. F. Gallagher, Sloan-Kettering Institute, New York, oestrone and isopropenyl acetate from Dr V. Petrow, British Drug Houses Ltd., and glucuronolactone from Corn Products Co.

REFERENCES

Brown, J. B. (1955). *Biochem. J.* **60**, 185.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Carpenter, J. G. D. & Kellie, A. E. (1961). *Biochem. J.* **78**, 1P.
Cohen, S. (1950). *J. biol. Chem.* **184**, 417.
Cohen, S. & Marrian, G. F. (1936). *Biochem. J.* **30**, 57.
Cohen, S., Marrian, G. F. & Odell, A. D. (1936). *Biochem. J.* **30**, 2250.
Crépy, O., Jayle, M. F. & Meslin, F. (1957*a*). *Acta endocr., Copenhagen*, **24**, 233.
Crépy, O., Jayle, M. F. & Meslin, F. (1957*b*). *C.R. Soc. biol., Paris*, **151**, 322.
de Boer, T. J. & Backer, H. J. (1954). *Rec. Trav. chim.* **73**, 229.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
Edwards, R. W. H. (1960). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., p. 418. Ed. by Smith, I. London: William Heinemann Ltd.
Felger, C. B. & Katzman, P. A. (1961). *Fed. Proc.* **20** 199.
Grant, J. K. & Marrian, G. F. (1948). *Biochem. J.* **43**, v.
Grant, J. K. & Marrian, G. F. (1950). *Biochem. J.* **47**, 1.
Ittrich, G. (1958). *Hoppe-Seyl. Z.* **312**, 1.
Johnson, W. S. & Johns, W. F. (1957). *J. Amer. chem. Soc.* **79**, 2005.
Leeds, N. S., Fukushima, D. K. & Gallagher, T. F. (1954). *J. Amer. chem. Soc.* **76**, 2943.
Lewbart, M. L. & Schneider, J. J. (1955). *Nature, Lond.*, **176**, 1175.
Mueller, G. P. & Johns, W. F. (1961). *J. org. Chem.* **26**, 2403.
Neeman, M. & Hashimoto, Y. (1961). *Tetrahedron Letters*, **5**, 183.
Pelzer, H. (1959). *Hoppe-Seyl. Z.* **314**, 234.

Biochem. J. (1962) **84**, 307

The Isolation of Mouse Antigens Carrying H2-Histocompatibility Specificity: Some Preliminary Studies

BY D. A. L. DAVIES
Microbiological Research Establishment, Porton, Salisbury, Wilts.

(*Received* 12 *January* 1962)

The rejection of tissue transplants, whether grafts of normal or malignant tissue, is the result of an immunological reaction on the part of the recipient against antigens present in the donor cells (Medawar, 1958*a*). Although transplantation studies are now being carried out with a variety of mammalian and avian species as inbred lines become available, the genetic system underlying the distribution of transplantation (T) antigens is best understood in the mouse (Snell, 1957).

In mice, antigens governing histocompatibility are determined by several genetic loci, H1, H2, H3 etc. Because H2 antigens are generally 'stronger' than others and some of these are present on the surfaces of erythrocytes as haemagglutinogens, they have been the most extensively studied and many alleles or pseudo-alleles are known at this locus (Amos, Gorer & Mikulska, 1955; Gorer, 1956; Snell, 1958; Gorer & Mikulska, 1959).

On appropriate immunization of mice of one inbred strain with the products of another, the subsequent graft of a tumour specific for the first strain can lead to enhancement of the growth of the tumour in a recipient which would otherwise reject such a tumour (Kaliss, 1958; Snell, Winn, Stimpfling & Parker, 1960). The distribution of histocompatibility (H) haemagglutinogens and of enhancing (E) antigens is the same as that of the T antigens, which give, under appropriate circumstances, hastened rejection of, for example, skin grafts. This leads to the view, now gaining acceptance, that T, E and H antigens are either the same substance or at least are closely associated in some way. Certain differences between the properties of

materials isolated, shown by using different tests for measurement of activity, have to be accounted for, but a preparation which hastened the rejection of allogeneic skin grafts also absorbed out haemagglutinins (Brent, Medawar & Ruszkiewicz, 1961). The T, E and H antigens are associated with the insoluble fraction of cells and this has hindered attempts to determine the chemical nature of the active substance.

Shortage of starting material and the relative insensitivity of the tests have also been troublesome, but an active cell-free T antigen, associated with the nuclear fraction, was obtained by Billingham, Brent & Medawar (1956). It was subsequently shown that nucleic acid was not the active component (Hašková & Hrubešová, 1958; Medawar 1958*b*; Castermans, 1961*a*), but it seemed possible that a mucoid substance was involved. Recent preparations contain protein, carbohydrate and lipid (Billingham, Brent & Medawar, 1958; Brent *et al.* 1961; Castermans, 1961*b*). It has been shown (Kandutsch & Reinert-Wenck, 1957; Kandutsch, 1960) that E antigen contains protein, carbohydrate and lipid, and is essentially insoluble, though some activity in soluble form was found after treatment with a non-ionic detergent. The H antigen is associated with the membranes of cells (Herzenberg & Herzenberg, 1961) and the material appears to be largely protein and lipid. The chemical work has been reviewed by Kandutsch (1961) and it is apparent that the true nature of the active component is not yet known.

The H2 antibody in mouse anti-mouse sera is 'incomplete' and does not agglutinate erythrocytes directly, but haemagglutination can be induced, for example, by including dextran and normal human serum in the system (Gorer & Mikulska, 1954). Inhibition of this reaction to give a measure of histocompatibility activity was described by Davies & Hutchison (1961), and this test is more sensitive than the graft-rejection or tumour-enhancement tests. With this method ascites-tumour cells grown in inbred mice were a convenient source of H2 antigen. Also, Davies (1961, 1962*a*) showed that cell-free ascitic fluid carried specific activity and that the active substance was a lipoprotein. Ascitic fluid seemed to be a more promising starting material than cell homogenates and it was felt that characterization of the antigen from this source would ease the task of obtaining specific material from cells. Some preliminary results are given in this paper.

MATERIALS AND METHODS

Mice. Breeding colonies of the inbred lines C3H (Heston sub-line), C57BL, Balb/c and A were provided by the Laboratory Animals Centre, Medical Research Council, Carshalton, Surrey. Animals of each strain were mated at random within their own colonies for up to three generations, since strict inbreeding would not provide the required number of mice. At the time of third-generation breeding, new inbred colonies were established for replacement.

Tumours. The following ascites tumours were used: BP8 (a benzpyrene-induced sarcoma) for C3H; EL4 (a leukaemia) for C57BL; CL2 (a leukaemia) for Balb/c; Sa-1 (a sarcoma) for A; and the Landschütz (LAN) sub-line of the Ehrlich-ascites carcinoma which is relatively non-specific and grows readily in all the strains of mice used.

Growth and recovery of tumours. Tumours were transplanted at intervals of about 10 days to batches of ten mice by intraperitoneal inoculation of 0·1 ml. of a 1:100 dilution of whole ascitic-tumour-cell suspension, with 'buffered saline' (0·02 M-KH_2PO_4, 0·06 M-Na_2HPO_4, 0·07 M-NaCl, pH 7·2) as diluent; sodium citrate was used as anticoagulant. When not in current use tumours were maintained at −70° in calf serum containing 20% of glycerol. For large-scale production, batches of 1000 mice of a single strain (wt. 30–40 g. each) were inoculated and peritoneal fluids recovered after 10 days (EL4/C57BL), 12 days (CL2/Balb/c), 13 days (BP8/C3H) or 14 days (Sa-1/A and LAN). Recovery was by suction into aspirators, suitable amounts of anticoagulant being added periodically; with tumours EL4 and CL2 little free fluid was produced and cells were washed out of the peritoneal cavity with 'buffered saline'. The fluid volumes recovered were usually 5–10 l. Tumour cells were separated from suspension by slow centrifuging, the speed depending on the tumour used. The cells were washed several times with 'buffered saline' to remove erythrocytes and used for the preparation of non-H2 antigens either directly or after freeze-drying and storage at −20°. The original supernatant ascitic fluid, after removal of tumour cells, was passed through an Alfa-Laval continuous-flow centrifuge to remove erythrocytes and cell debris, and used directly for H2-antigen isolation or stored freeze-dried at −20° for other purposes.

Chemical methods. Total N was determined by the Kjeldahl method with the distillation apparatus of Markham (1942) and the mixed bromocresol green–methyl red indicator of Ma & Zuazaga (1942). Phosphorus was determined by a modification of the method of Martland & Robison (1926) on samples containing 5–30 μg. of P. Sulphur was determined by the Carius method. Carbohydrate was estimated (in terms of glucose) with orcinol, according to Synge & Wood (1958); total lipid was extracted by refluxing with chloroform–methanol (2:1, v/v) until no more material could be removed; protein was estimated with ninhydrin (Moore & Stein, 1948) after hydrolysis at 100° for 16 hr. with 6 N-HCl, with leucine as standard. Other methods used were: for hexosamine (Rondle & Morgan, 1955); for sialic acid (Warren, 1959); and for uronic acid (Dische & Borenfrend, 1951). Amino acids were separated by paper chromatography by standard methods and sugar chromatography was as described by Davies (1957).

Antisera. Rabbit antisera were prepared against normal mouse serum, mouse-tumour cells, or their products, by several intramuscular injections in Freund's adjuvant at intervals of 1–2 weeks. Booster injections of material without adjuvant and given intravenously were followed 10 days later by sample bleedings. About 1 mg. (dry wt.)

of material was given at each injection. Animals were bled out when an adequate antibody response, judged by agar-diffusion patterns, had been achieved. Sera were stored at 0° freeze-dried and made up at 70 mg./ml. as required, this being the concentration of fresh mouse serum.

The H2 antisera for haemagglutination tests were obtained by immunizing batches of mice with several subcutaneous injections, at weekly intervals, of spleen cells or tumour cells from different mouse strains. Mice were bled from the heart and sera pooled, or, more usually, antibody was recovered as immune ascitic fluid by injecting a non-specific, or preferably a syngeneic, ascites tumour about 12–14 days before antibody was due to be recovered, i.e. a few days before the last immunizing injection. A much larger amount of antibody could be obtained in this way than by recovering immune serum. The antisera or immune ascitic fluids were preserved freeze-dried at 0°, distributed in small amounts in sealed containers because antibody titres declined on freezing and thawing. Immune ascitic fluids were dissolved at 40 mg./ml. for use, this being the concentration of fresh mouse ascitic fluid.

Since it is not established whether the several H2 specificities carried by each mouse strain are different structural features of one molecular complex or are different chemical entities, a characteristic H2 specificity of each strain was chosen and a relatively specific antibody used for this factor, to avoid misleading results should different molecular antigenic entities be involved which might separate on fractionation. Thus, for example, for following the isolation of C3H-specific material, in terms of which most of the results in this paper are given, antibody was induced in (C57BL × Balb/c) F_1 hybrids by immunizing with spleen cells from A mice. By using C3H erythrocytes with this antibody, the reaction was restricted to that of one H2 'antigen' (H2-K), although several specificities can be recognized in cells from C3H mice which are controlled from the same H2 locus (e.g. H2-C, H2-D^k, H2-E and H2-K). Other combinations to give various specificities are described elsewhere (Davies & Hutchison, 1961; Davies, 1962*b*).

Agar diffusion. This was carried out as described by Crumpton & Davies (1956), or by similar methods on a reduced scale to be accommodated on microscope slides when rapid results were more important than maximum resolution.

Haemagglutination and haemagglutination inhibition. The method has been described by Davies & Hutchison (1961). Briefly, the incomplete mouse H2 antibody was detected by inducing haemagglutination with dextran and normal human serum (Gorer & Mikulska, 1954). For comparison of the specificities of whole cells or of insoluble but dispersible preparations, an equal volume of suspension was added to each tube of a series of antiserum dilutions [prepared in dextran, 1% (w/v) in 'buffered saline'] and incubated. After centrifuging, samples of the supernatant were re-incubated with erythrocytes (in 1:2 normal human serum, diluted in 'buffered saline', which had been inactivated at 56° and absorbed with similar erythrocytes), and haemagglutination was read microscopically. The difference between the haemagglutination titre thus obtained and the titre of the unabsorbed antiserum was taken as the measure of activity. For soluble preparations and those giving stable suspensions or emulsions, antiserum was used at a small number of haemagglutinating doses (in 1% dextran) and the potential inhibitor diluted out. Inhibition titres were obtained after incubation with appropriate kinds of erythrocytes (in 1:2 normal human serum). For simplicity, only the specificities H2-C to K are considered below.

EXPERIMENTS AND RESULTS

The BP8-tumour cells from C3H mice gave good inhibition of the haemagglutination of C3H erythrocytes in Balb/c anti-C3H serum (detection of H2-D^kEK); syngeneic 6C3HED-lymphosarcoma cells grown in C3H mice or 'L' cells grown in tissue culture but originally derived from C3H mice (Earle, 1943) gave less powerful inhibition on a dry wt. basis. The allogeneic Sa-1-tumour cells cross-reacted (for H2-E) but cells not carrying H2-E or H2-K specificities did not inhibit in this system. EL4 and CL2 cells gave good inhibition in their own homologous systems.

Preparation of H2 *haemagglutinogen from* C3H *mice*

Batches of 1000 mice growing BP8-tumour cells yielded 7–9 l. of peritoneal fluid. After separation of tumour cells and removal of erythrocytes and debris, this fluid carried specific haemagglutination-inhibiting activity corresponding to that of the tumour cells. In this apparently soluble form it was possible to measure activity by the dilution–inhibition method, and inhibitory activity could be obtained at 5–10 mg./ml. Sodium chloride (0·9%) washings of cells carried negligible activity and were discarded. Incubation of cell-free ascitic fluid at 37° overnight did not decrease the activity, and destructive enzymes were taken to be absent. Activity was labile to heat (60° for 20 min.), to acid (below pH 5·5) and to alkali (over pH 9). Much activity was lost on freeze-drying or freezing and thawing; samples were therefore taken of all fractions over 10 successive extractions and these were freeze-dried to give estimates of concentration of solutions and to obtain analytical figures. The bulk of the material was not dried at any stage.

Precipitation with ammonium sulphate gave little suggestion of fractionation: activity was present in fractions, recovered after dialysis, precipitated at 20, 20–30, 30–50 and over 50% saturation. Some activity in the 30–50% saturation fraction appeared to be non-specific since the corresponding fraction of a non-specific tumour fluid (LAN) behaved similarly. Attempts to use diethylaminoethyl- and carboxymethyl-substituted celluloses and starch-block electrophoresis were unsuccessful because activity could not be eluted. Non-specific adsorption seemed to be a property of the active component.

Dialysis of ascitic fluid against water gave a precipitate which carried all the activity (active at

100 μg./ml.). Precipitation of material by the addition of increasing amounts of water to the ascitic fluid showed that some further resolution was possible by this method. Material precipitated by 1 vol. of water was relatively inactive (1 mg.– 500 μg./ml.); the precipitates with 1–2 and 2–3 vol. of water were very active (25–50 μg./ml.), and dialysis of the final supernatant against water gave a small inactive precipitate. As it was not practicable to recover material in this way because of the large volumes involved the following procedure was adopted. Fresh ascitic fluid (about 8 l.) was diluted by the slow addition of 0·75 vol. of cold water and left overnight at 2–3°. The precipitate, which had no activity, was removed and the supernatant (about 14 l.) was dialysed for 40 hr. against slowly running water in a rocking dialyser in the cold. The resulting precipitate (DP) represented 1–2 % of the original indiffusible material and was 50–100 times as active an inhibitor (active at about 60 μg./ml.) as the original ascitic fluid. Activity could not be detected in the dialysis-supernatant fraction (DS) containing the bulk of serum proteins. The weights (ranges from 10 extractions) of these and subsequent fractions are summarized in Fig. 1.

The fraction DP was partly soluble in salt solutions. On resuspension in 'buffered saline' at 0·5 % (w/v) and centrifuging at 80 000*g* for 2 hr. all activity appeared in the sediment while most of the material remained in solution. Advantage was taken of this to remove most of the remaining serum components precipitated in the dialysis step. The fraction DP was therefore centrifuged four times from 'buffered saline', three times from 0·9 % sodium chloride and once from water in the Spinco (model L) at 80 000*g*; after removal of salt in the last spin the sedimented fraction (SP) gave a rather stable emulsion in water which could be sedimented at higher *g* values, leaving no significant amount of material in the supernatant. The pooled Spinco supernatants (SS) were inactive, and the activity of the SP fraction was 3–8 μg./ml. The fraction SP could now be centrifuged as a water emulsion at 2000*g* for 30 min. to remove dirt. Examination in the analytical ultracentrifuge showed at least two components and agar diffusion against rabbit antisera showed one cell-bound antigen and weak reactions for three or four serum components.

The marked increase in the amount of lipid in the Spinco-precipitated fraction (SP) suggested that this might belong to the active constituent, which would be consistent with the properties so far revealed. The SP fraction was therefore suspended at 1·5–2·0 % (w/v) and centrifuged to equilibrium in a density gradient (Spinco SW-39 rotor,

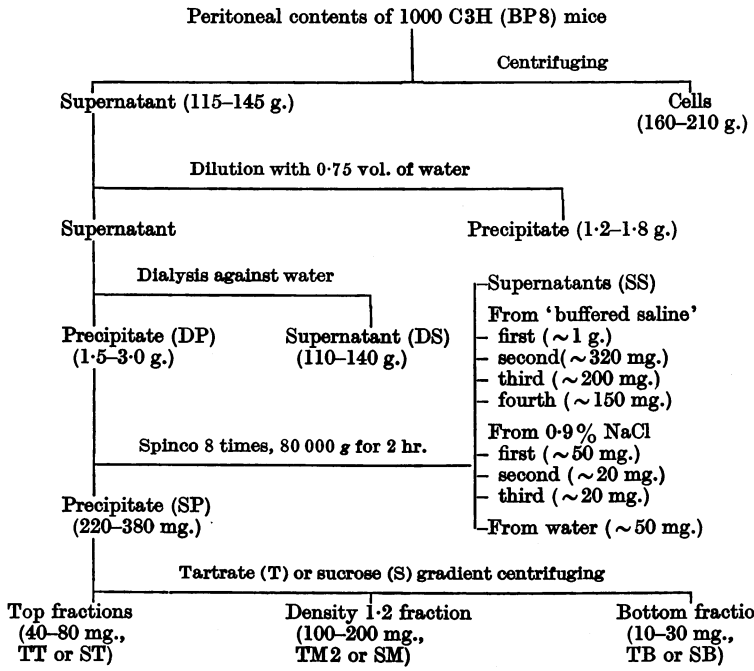

Fig. 1. Isolation procedure for H2 antigen from ascitic fluid. Weights are ranges obtained in a series of 10 batches of 1000 C3H mice, with BP8 tumour, in which samples were taken for analysis at each stage. Weights of products are corrected for samples removed for analysis. All weights are from dialysed freeze-dried products.

100 000*g* for 3 hr., or SW-25 rotor, 50 000*g* for 6 hr.). Potassium tartrate gradients (5–50 % of salt, density 1·03–1·28) were used initially and gave some of the products described below. Of the several bands which separated, two gave specific activity (TM1 and TM2 at density 1·14 and 1·2 approx.) after dialysis to remove salt. The pooled top bands (TT) and bottom bands (TB) were inactive. Only one of the active bands (TM2) induced haemagglutinins when injected into appropriate strains of mice, but, when TM2 material was re-run on a similar gradient, more than one band again appeared suggesting that some degradation occurred in the strong salt. Sucrose gradients covering the same density range have subsequently been used, where most of the material appears at the 1·2 density level and there is less indication of degradation. After dialysis to remove salt or sucrose the suspensions were concentrated by fanning the dialysis sacs before sterilization and removal of samples to be freeze-dried for analysis and determination of concentration. The TM2 and SM (sucrose middle fraction) materials give the same analysis and make opalescent emulsions in water which settle out to some extent on storage; they are somewhat less active than the Spinco-precipitated (SP) fractions, and this is thought to be due to differences in the states of dispersion.

Analytical figures and activities are shown in Table 1. Protein estimates have been reduced by 10 % for the addition of water on hydrolysis.

It has not been possible to recover material on the necessary scale under sterile conditions and the preparation is therefore carried out at 2–3°. Dirt tends to accumulate in the active fraction at each stage up to the gradient centrifuging, where it sediments through to the bottom fraction. However, the final product is sterilized by u.v. irradiation (20 min. for a layer of solution not exceeding 0·5 cm. in depth) since other means of sterilization have proved harmful to the active material.

Isolation of analogous products

The LAN tumour is 'non-specific' and grows progressively, either as an ascites tumour on intraperitoneal injection or as a solid tumour when injected subcutaneously, in all the mouse strains which have been used. Material has been prepared in the manner described from LAN-ascitic fluids from batches of A, C3H and outbred mice; the SM (sucrose middle fraction) products gave the same analytical figures as the analogous specific product of BP8/C3H but lacked specificity in that they did not inhibit haemagglutination in the H2–K or H2–D^{k}EK systems. Certain other specificities have been found in the products, however, which were not detected with the intact LAN-tumour cells. The material is obtained in similar yields to that of the BP8/C3H product when related to tumour-cell weight, but gives a greater overall yield in corresponding batches of 1000 mice because of the greater weight of cells produced per mouse. Results for this and the other tumours used are shown in Table 2.

Analogous specific products have been prepared from C57BL and Balb/c mice with EL4- and CL2-ascites leukaemias respectively. The SM fractions carried the H2 specificities of the mouse strains. In these instances much smaller yields of cells were obtained and little fluid was produced. The cells were washed out with 4–6 l. of 'buffered saline'/1000 mice. The same isolation procedure was successful but although the amount of starting material was less the yields of product were relatively high; for Balb/c antigen the weight of product was often as high as that from BP8/C3H, and the EL4/C57BL product was frequently up to half this amount. This probably reflects the greater fragility of the leukaemic cells revealed in other experiments (Haughton & Davies, 1962). The general composition of these substances is the same as that of the BP8/C3H and LAN antigens.

Table 1. *Analysis of* BP8/C3H *ascitic fluid and its products*

Experimental details are given in the text.

Material	N (%)	P (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Activity* (μg./ml.)
Ascitic fluid	13·3	0·3	97	6·0	2·5	5 000–10 000
Precipitate with 0·75 vol. of water	13·0	0·7	96	12·5	5·8	> 500
Dialysis precipitate (DP)	13·4	0·7	79	10	2·5	60
Dialysis supernatant (DS)	14·1	0·2	99	5·6	2·5	0
Spinco precipitate (SP)	10·6	1·2	50	30	4·0	3–8
Spinco supernatant (SS)	14·1	0·4	97	4·0	2·1	0
TT/ST†	9·8	1·2	68	26	3·0	0
TM1	8·4	1·1	42	40	3·5	50
TM2/SM	9·5	1·0	56	35	3·7	10–20
TB/SB	9·0	1·2	50	25	9·5	0

* Minimum wt./ml. of solution giving haemagglutination inhibition.
† 'T' fractions from tartrate gradients; 'S' fractions from sucrose gradients.

Table 2. *Products from different kinds of tumour cells*

Experimental details are given in the text.

Tumour	Mouse strain	Products of 1000 mice: Vol. of ascitic fluid (l.)	Wt. of cells (g.)	Wt. of soluble fraction (g.)
BP8	C3H	7–9	160–200	115–150
LAN	A	12	200	200
LAN	Outbred	14	200–300	200–300
EL4	C57BL	4	8–12	16–25
CL2	Balb/c	4	50–60	80–110

Similar treatment of normal mouse serum

No H2-specific activity can be detected in normal mouse serum, and to find whether any insoluble fraction from such serum would persist through the purification procedure to contaminate the product (ascitic fluid having the composition of serum diluted about 1:2, plus certain cell products), the following experiment was performed. 'A' mice (500) were bled from the heart and the serum pooled; a sample which was freeze-dried showed the concentration to be 70 mg./ml., and the starting vol., 180 ml. of serum, had 12·6 g. of material. This was diluted with an equal volume of 0·9 % sodium chloride to reproduce the approximate concentration of ascitic fluid and then diluted with 0·75 vol. of water and left in the cold overnight. Analogous fractions to those of BP8-ascitic fluid were recovered at each stage. An insoluble fraction (35 mg.) was recovered after dilution with water and the supernatant dialysed; the new precipitate (DP) was resuspended and centrifuged in the Spinco eight times as described above. The pooled supernatants (SS fractions) were dialysed and freeze-dried to give 120 mg. of material. The sedimented fraction (SP) was suspended in 3 ml. of water and centrifuged through a sucrose gradient to give, after dialysis and freeze-drying, ST (0·42 mg.), SM (0·2 mg.) and SB (2·7 mg.) fractions. The SS fraction (120 mg.) is therefore essentially all of the DP fraction and corresponds to 1 % of the starting material. The dry wt. of serum was about 10 % of the wt. of ascitic fluid normally used, and, although the SM fraction did not have the appearance of lipoprotein but was probably just the accumulated dirt, 10 times the SM fraction would be only 2 mg. to contaminate a specific H2 antigen, i.e. to the extent of 1–2 %.

PROPERTIES OF THE ANTIGENS

Physical properties

The antigens (SM gradient products) are essentially insoluble in water but give emulsions which settle out to some extent on storage; traces of salt (0·1 M-sodium chloride or less) flocculate the material. The material is not soluble in dimethylsulphoxide, formamide, dimethylformamide, dioxan, sodium thioglycollate solutions or chloroform–methanol–water. Stable 'solutions' can be obtained at 1 % (w/v) in 1 % (w/v) lysolecithin, 0·2 % sodium dodecyl sulphate, 0·2 % deoxycholate, aq. 50 % (v/v) 2-chloroethanol, 0·5 M-sucrose, or at pH over 10·5. Partial solution occurs in 66 % (v/v) acetic acid or 5 M-urea. The solution (probably emulsion) in sucrose is very stable, but in aq. 2-chloroethanol there appears to be a separation of protein and lipid. When stored at 2–3° in aqueous suspension after sterilization there is no loss of activity over several months. Lability is discussed below with the biological properties. Preliminary examination in the electron microscope did not reveal any structure.

Examination for physical homogeneity by the usual methods is unsatisfactory because of solubility troubles. Precipitation by traces of salt precludes free electrophoresis, but on cellulose acetate paper no movement of protein-staining material occurs at pH 7·2 although at pH 9 a faint area of protein-staining material migrates, leaving most of the substance at the starting point.

In the analytical ultracentrifuge a 1 % suspension in water shows strong light-absorption which clears rapidly between 30 000 and 60 000 g (Spinco model E) to give a gel on the bottom of the cell. No other components can be detected on subsequent centrifuging at higher g values. Thus a soluble but rapidly sedimenting second component mentioned above, detected on ultracentrifuging the SP fraction, is removed in the density-gradient fractionation. At pH 9 (tris buffer) an aqueous suspension (1 %, w/v) sediments very rapidly but with a suggestion of some material failing to separate from the meniscus, which would be consistent with the existence of a degradation product at this pH as suggested by paper electrophoresis. Ultracentrifuge runs in other solvents cannot yet be interpreted. These results give little information about the material itself but show inability, thus far, to detect impurities.

When an aqueous suspension was centrifuged at 3000*g* for 20 min., the supernatant removed and centrifuged at 5000*g* for 30 min., and the final supernatant and two precipitates were freeze-dried, the following figures were obtained: precipitate 1, 13·0 mg., N 9·5 %, P 1·0 %; precipitate 2, 15·8 mg., N 9·35 %, P 1·18 %; supernatant, 18·3 mg., N 8·6 %, P 1·26 %. This fall in the N:P ratio reflects some heterogeneity with respect to the proportions of protein and lipid.

Chemical properties

Analysis of the TM 2 and SM fractions did not differ and gave the following percentage figures: N 9·5; S 0·5; P 1·0; protein 65; carbohydrate 3·5; lipid 35; hexosamine (not included in the carbohydrate figure obtained by the orcinol method) 1·4; sialic acid 0·3; no uronic acid; no nucleic acid, checked by absorption at 260 mμ in solution at pH 11.

Lipid was extracted from freeze-dried material by refluxing until nothing more was removed. Ether–ethanol (1:1, v/v) extracted above half of the total extractable lipid containing 0·8 % of N and 1·6 % of P. Chloroform–methanol (2:1, v/v) would extract this fraction and as much again; the fraction extracted only by chloroform–methanol contained 2·5 % of N and 2·9 % of P. This fraction, but not that soluble in ethanol–ether, contained sugar residues; 10 mg. amounts of this lipid were hydrolysed with N-sulphuric acid for 6 hr. at 100°, neutralized with barium hydroxide, centrifuged and the supernatant was passed through a Zeo-Karb 225 resin column. The volume of the aqueous eluate of the column was reduced to 10 μl. and examined on chromatograms: galactose and glucose were recognized. With shorter hydrolysis times (for example 20 min.) a third sugar, not yet identified, was found. Total neutral sugar, estimated by orcinol on the total extractable lipid, was 0·6–2·6 %, depending on the strain of mouse used; sulphuric acid–cysteine tests (Dische & Shettles, 1948) suggested that a 6-deoxy hexose might be present in small amount, and 0·3–0·4 % of hexosamine was present in the lipids of each of LAN, C3H, Balb/c and C57BL.

The residues remaining after lipid extraction were very insoluble and contained (%): N 14·5; P 0·6; protein 90; carbohydrate 2; hexosamine 1·7. Amino acids detected chromatographically after strong acid hydrolysis were glycine, alanine, valine, leucine or isoleucine or both, proline, phenylalanine, cysteine, arginine, lysine, aspartate, glutamate, serine, threonine, tyrosine and at least one unidentified compound. Some closely bound lipid, not removed by solvent extraction, is revealed by the release of some fatty acids, recoverable in ether, after strong acid hydrolysis; the amount, calculated as phospholipid, would be about 10 % of the residue fraction.

Biological properties

Immunological homogeneity. Precipitation on agar diffusion has not been obtained with mouse anti-mouse sera, presumably because the antibody is incomplete. Examination for homogeneity has been carried out with rabbit anti-mouse sera. Fig. 2 (*a*) shows an agar diffusion pattern of fractions tested by rabbit antiserum against normal mouse serum. Fractions of increasing H 2 activity down the left side of the photograph show a progressive loss of normal mouse serum components and the final product (SM) gives only a trace reaction. This amount, estimated by dilution on agar plates in parallel with normal mouse serum, is less than 1 % contamination. The corresponding inactive fractions down the right side of the photograph show enrichment for the various serum components removed on fractionation.

Fig. 2 (*b*) shows a similar arrangement but with a rabbit antiserum against mouse cells, after absorption with normal mouse serum, and only reveals antigens confined to the cells or their products (G. Haughton, W. Boyle & D. A. L. Davies, unpublished work). Of these antigens (four of seven known components can be seen on the photograph), only one is present in the final product (SM) and this reacts strongly. This is believed to represent the specificity of the protein component (which occurs in the free state in ascitic fluid) of the complex which is present in normal mouse cells. This substance and the nature of its species specificity is described by Haughton (1962). The H 2 antigen therefore shows only one significant component when tested by agar diffusion, but the poor dispersion of the substance limits one to the conclusion that no significant amount of impurity can be detected. Further light on this is revealed by the response of rabbits to injection of the purified material (see below).

H 2 *specificity.* This is the most important feature of the products. The specificities of the materials prepared from C3H-, C57BL- and Balb/c-specific ascitic fluids and from the relatively non-specific LAN-tumour fluids, tested against various H 2 antisera, has already been described (Davies & Hutchison, 1961; Davies, 1962*b*; 1962*c*). Some of these results are summarized qualitatively in Table 3. All the specificities detected are those expected from the known distribution of H 2 alleles and the several specificities of each mouse strain are carried on the products described.

Lability. Ability to inhibit haemagglutination is destroyed by heating at 60° for 20 min., and also by exposure to pH 5·5 or pH over 9 for 10 min. at about 20° followed by readjustment to neutral pH. Activity is lost on ultrasonic treatment, as shown in Fig. 3, the half-life being about 15 sec.

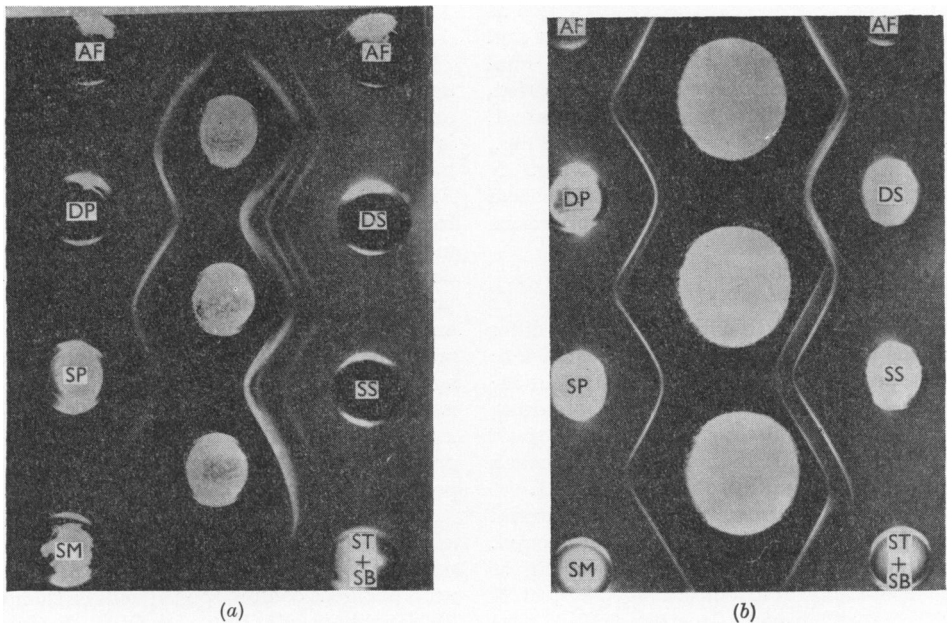

Fig. 2. Agar diffusion patterns of ascitic fluid and its products tested against (*a*) rabbit antiserum against normal mouse serum (central wells); (*b*) rabbit antiserum against mouse cells, after absorption with normal mouse serum (central wells). Peripheral wells contain 330 μg. of antigen samples; AF, original ascitic fluid; DP, dialysis precipitate; DS, dialysis supernatant; SP, Spinco precipitate; SS, Spinco supernatants; SM, active middle band from sucrose gradient; ST+SB, pool of top and bottom fractions from sucrose gradient centrifuging.

Table 3. *Specificities of the products in haemagglutination inhibition*

Experimental details are given in the text. The symbols C, D, D^b, D^k, E, E^d, F and K represent specificities determined by the H2 locus and for which antibodies are expected to be present in the antisera as indicated for each column (see Davies & Hutchison, 1961). —, No inhibition at 2 mg./ml.

	Specificities of test system						
Source of inhibitor	D^kEK	D^bE	DE^dF	C	D	F	K
C3H ($H2\text{–}CD^kEfK$)	+	+	–	+	–	–	+
Balb/c ($H2\text{–}CDE^dFk$)	–	–	+	+	+	+	–
C57BL ($H2\text{–}cD^bEFk$)	+	+	+	–	–	+	–
LAN(A) (H2–? nil)	–	–	–	+	–	+	–

Substantial but incomplete loss of activity occurs after freeze-drying, or freezing and thawing, but the material redisperses poorly after these treatments. Extraction of freeze-dried material with ether at $-15°$ does not further impair activity, but extraction with chloroform–methanol, even in the cold, gives an inactive residue. Emulsions of lipid extracts in lecithin–cholesterol are inactive although intact material emulsified in the same way remains fully active. Solutions in sodium dodecylsulphate or deoxycholate are inactive, and the addition of the non-ionic detergent Tween 20 (at 1%) and subsequent recovery of the material by centrifuging, also showed complete loss of activity. Potassium periodate (5 mM, pH 7·2 at room temperature) also destroys the ability to inhibit haemagglutination.

Antigenicity. When the antigens were injected into appropriate strains of mice, haemagglutinins were produced; for example, the C3H product injected subcutaneously with no adjuvant into groups of Balb/c mice induced antibody which

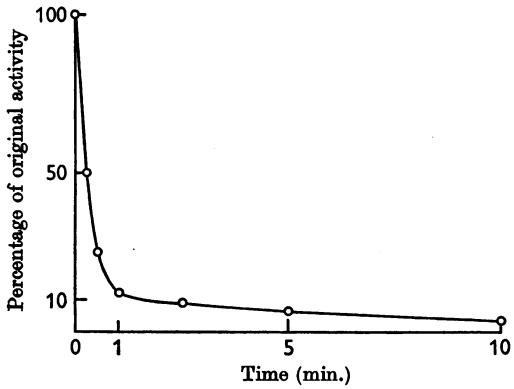

Fig. 3. Effect of ultrasonic vibration on the haemagglutination-inhibiting activity of H2 antigen (M.S.E.–Mullard ultrasonic disintegrator, 60w, 20 kcyc./sec.). Activity is shown as a percentage of that of the starting material.

agglutinated C3H erythrocytes (H2–D^{k}EK reaction) and could be absorbed out specifically by the homologous antigen.

Rabbits were immunized by weekly injections (0·5 mg. twice intramuscularly in Freund's adjuvant and subsequently intravenously) and sample bleedings taken each week immediately before each injection. After two injections weak reactions, on agar diffusion, for two normal-mouse-serum components appeared when the sera were tested against ascitic fluid, but the extent of contamination of the product with serum components is already known to be very small. The specificity of the H2 protein appeared after the third injection (sera absorbed with normal mouse serum and tested against ascitic fluid). Precipitins against other cell-antigens were not subsequently found and this is regarded as rather good evidence, of a kind not given by other homogeneity tests described, for lack of contamination from this source.

DISCUSSION

Rapid advances have been made in recent years in the field of tissue transplantation in mammals (see, for example, Converse & Rapaport, 1960; Brent & Medawar, 1961). It would be very helpful if purified antigens could now be made available; some of the troubles which have been encountered in attempts to do this have been inadequate numbers of inbred animals, difficulty in obtaining large amounts of suitable cells from the animals, lability of the material and its association with the structural parts of the cells, and its property of non-specific adsorption.

Large numbers of mice have been obtained by random mating inside each colony for up to three generations, where it is considered that the products of any aberrant mice would be diluted out. Ascitic fluid was chosen as the starting material because of the large amounts obtainable and because activity appeared to be present in soluble form, though the product proves to be insoluble nevertheless. However, the ability to remove 98% of the inactive constituents in the first step makes it a convenient source, and a knowledge of the properties of the substance might suggest better means of extraction from cells, which may really be a more productive source. In any case ascites tumours are not available except for laboratory rodents.

Inability to freeze-dry the material without impairing its biological activity is very inconvenient, but a reproducible product, lipoprotein in nature, can be obtained by the simple procedure described.

Ascitic fluid has a composition approximating to that of a 1:2 dilution of normal mouse serum, but with the addition of various products of the tumour cells. The H2 antigen is undoubtedly a cell product and probably arises by cell lysis; the ascitic fluid recovered at first is much less active than that obtained at the number of days after tumour inoculation recommended. The amount of material is more nearly related to the yield of cells than to the volume or solid content of the fluid, but relatively larger yields are obtained from the leukaemic cells, probably due to their greater fragility (Haughton & Davies, 1962).

Demonstration of homogeneity proves to be rather troublesome. Only trace contamination with serum components has been detected, and, since no component of normal mouse serum survives the purification procedure, the remaining traces are no doubt due to adsorption. It seems unlikely that a trace constituent is the active fraction although the possibility cannot be ruled out until the nature of the specific determinant groups is established. Apart from this possibility it cannot be assumed that the least entity able to carry specificity has necessarily been obtained. However, no degradation products have yet been obtained which retain biological activity; the component detected on agar diffusion is the protein moiety which has species specificity. Since rabbits immunized with the purified product 'recognize' no cell specificity other than this one, as tested by agar diffusion, only non-antigenic impurities can be present and this possibility is not supported by the physical properties which have so far been revealed.

The results would seem to agree with the views of Herzenberg & Herzenberg (1961) that H2 activity is associated with cell membranes, because, although no structure is revealed by the electron microscope, the protein component of the H2 antigen has been recognized as a structural component of mouse tumour cells and stroma derived

from them (Haughton & Davies, 1962). But whereas H2 specificity can be detected on cell surfaces by many methods (see, for example, Möller, 1961), the protein specificity is not agglutinogenic. Indeed the dominant agglutinogen of mouse tumour cells is a heat-stable substance (W. Boyle, unpublished work) which does not contaminate the H2 preparations.

Though all the properties associated with activity are consistent with a lipoprotein structure and by no means suggestive of carbohydrate, except, for example, as a glycolipid component, some carbohydrate remains in the solvent-extracted residue and its importance cannot yet be assessed. Inactivation by periodate under mild conditions, as has been recorded for T antigens (Billingham *et al.* 1958), could be an attack on sugars in the glycolipid fraction or elsewhere, or on lipid components other than the glycolipid fraction. In any event this might give information about the nature of the specific determinant groups. Some loosely-bound ether-soluble lipid is present but appears to be unnecessary for activity, but unfortunately its removal impairs the solubility or emulsion properties of the substance.

The H2 specificities of the products are very satisfactory as they are just those expected from the known distribution of H2 alleles in the mouse strains used. Since cross-overs occur between H2 alleles (pseudo-alleles) one might expect specificities to be carried on different molecules; it is possible that the preparations are mixtures of closely similar families of molecules. But for a cell structural component one can imagine a number of genes contributing to the control of its synthesis, so that the specificities would be due to different structural features of the complex. There is an analogy for this in the Enterobacteria where the 'O' 'antigens' of, for example, a *Salmonella* are just different structural features of one polysaccharide molecule (Davies, 1960). For the H2 antigen, however, any interference with the structural integrity of the main lipid component (sodium dodecyl sulphate, deoxycholate, lysolecithin, non-ionic detergent, ultrasonic treatment, organic solvents, freeze-drying etc.) impairs activity; unless this is a reflexion of some particular feature of the test system, it suggests the possibility either that the lipid itself carries the specificity, or that the lipid moiety stabilizes some particular configuration in the protein component.

The relationship of transplantation, enhancing and histocompatibility antigens should become clear when purified materials are available for study. It already seems likely that they are the same: for example, T-antigen preparations will absorb haemagglutinins (Brent *et al.* 1961) and ascitic fluid has been shown to carry T activity (Hašková & Hilgert, 1961). Ability to maltreat E-antigen preparations by freeze-drying, or even some exposure to organic solvents, may only mean that the test system (humoral antibody response in the mouse) is not sensitive to any changes thus induced. The stability of T antigens to ultrasonic treatment, compared with the lability of H antigen, may be no different from the situation, admittedly unexplained, where human blood-group mucopolysaccharides treated with ultrasonics lose the ability to inhibit haemagglutination without losing specificity as determined by precipitation with antisera (Morgan, 1960). The test system for T antigen is induction of a cellular immunity in mice.

SUMMARY

1. Ascitic fluid, induced in mice by tumour cells, has been used as a source of antigens carrying H2-histocompatibility specificity.

2. Activity, measured by inhibition of haemagglutination induced in mouse anti-mouse sera with dextran and normal human serum, shows a 500–1000-fold increase on purification.

3. The product is a lipoprotein, in which only 1–2% of impurity has been detected although a variety of tests have been used.

4. The substance is insoluble and is believed to be a structural component of cell membranes.

5. Material isolated from different inbred mouse strains carry the H2 specificities expected from the H2 antigenic characters associated with these mouse strains.

6. Analogous material isolated from non-specific tumour-cell products grown in inbred mice do not carry the specificities of that mouse. No analogous substance is present in normal serum.

I wish to thank Mr V. S. G. Baugh and Mr J. A. Manstone for excellent technical assistance, Dr J. S. Paterson and his staff for breeding all the mice and Mr F. C. Belton and his staff for recovering ascitic fluids. I am also indebted to Dr K. A. Cammack for analytical ultracentrifuge runs, Mr W. J. Harris for examining material in the electron microscope, Dr J. Pirt for providing 'L' cells grown in continuous culture and Dr M. Gray for samples of lysolecithin. I am most grateful to Dr D. W. Henderson, C.B., F.R.S., Director of the Microbiological Research Establishment, for encouragement and providing the necessary facilities.

REFERENCES

Amos, D. B., Gorer, P. A. & Mikulska, Z. B. (1955). *Proc. Roy. Soc.* B, **144**, 369.

Billingham, R. E., Brent, L. & Medawar, P. B. (1956). *Nature, Lond.*, **178**, 514.

Billingham, R. E., Brent, L. & Medawar, P. B. (1958). *Transplant. Bull.* **5**, 377.

Brent, L. & Medawar, P. B. (1961). *Proc. Roy. Soc.* B, **155**, 392.

Brent, L., Medawar, P. B. & Ruszkiewicz, M. (1961). *Brit. J. exp. Path.* **42**, 464.
Castermans, A. (1961*a*). *Nature, Lond.*, **189**, 504.
Castermans, A. (1961*b*). *Transplant. Bull.* **27**, 98.
Converse, J. M. & Rapaport, F. T. (1960). *Ann. N.Y. Acad. Sci.* **87**, 6.
Crumpton, M. J. & Davies, D. A. L. (1956). *Proc. Roy. Soc.* B, **145**, 109.
Davies, D. A. L. (1957). *Biochem. J.* **67**, 253.
Davies, D. A. L. (1960). *Advanc. Carbohyd. Chem.* **15**, 271.
Davies, D. A. L. (1961). *Abstr. 5th int. Congr. Biochem., Moscow*, **20**, p. 415.
Davies, D. A. L. (1962*a*). *Nature, Lond.*, **193**, 34.
Davies, D. A. L. (1962*b*). In *Ciba Foundation Symp., Transplantation.* Ed. by Wolstenholme, G. E. W. & Cameron, M. P. London: J. and A. Churchill Ltd.
Davies, D. A. L. (1962*c*). *Ann. N.Y. Acad. Sci.* (in the Press).
Davies, D. A. L. & Hutchison, A. M. (1961). *Brit. J. exp. Path.* **42**, 587.
Dische, Z. & Borenfrend, E. (1951). *J. biol. Chem.* **192**, 583.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Earle, W. R. (1943). *J. nat. Cancer Inst.* **4**, 165.
Gorer, P. A. (1956). *Advanc. Cancer Res.* **4**, 149.
Gorer, P. A. & Mikulska, Z. B. (1954). *Cancer Res.* **14**, 651.
Gorer, P. A. & Mikulska, Z. B. (1959). *Proc. Roy. Soc.* B, **151**, 57.
Hašková, V. & Hilgert, I. (1961). *Folia biol., Prague*, **7**, 81.
Hašková, V. & Hrubešová, M. (1958). *Nature, Lond.*, **182**, 61.
Haughton, G. (1962). *Ann. N.Y. Acad. Sci.* (in the Press).
Haughton, G. & Davies, D. A. L. (1962). *Brit. J. exp. Path.* (in the Press).
Herzenberg, L. A. & Herzenberg, L. A. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 762.
Kaliss, N. (1958). *Cancer Res.* **18**, 992.
Kandutsch, A. A. (1960). *Cancer Res.* **20**, 264.
Kandutsch, A. A. (1961). *Transplant. Bull.* **27**, 135.
Kandutsch, A. A. & Reinert-Wenck, V. (1957). *J. exp. Med.* **105**, 125.
Ma, T. S. & Zuazaga, G. (1942). *Industr. Engn Chem. (Anal.)*, **14**, 280.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
Medawar, P. B. (1958*a*). *Proc. Roy. Soc.* B, **149**, 145.
Medawar, P. B. (1958*b*). *Nature, Lond.*, **182**, 62.
Möller, G. (1961). *J. exp. Med.* **114**, 415.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Morgan, W. T. J. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 1591.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Snell, G. D. (1957). *Annu. Rev. Microbiol.* **11**, 439.
Snell, G. D. (1958). *J. nat. Cancer Inst.* **20**, 787.
Snell, G. D., Winn, H. J., Stimpfling, J. H. & Parker, S. J. (1960). *J. exp. Med.* **112**, 293.
Synge, R. L. M. & Wood, J. C. (1958). *Biochem. J.* **70**, 321.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.

Biochem. J. (1962) **84**, 317

Production of Aminoacetone by *Rhodopseudomonas spheroides**

BY A. NEUBERGER AND G. H. TAIT
Department of Chemical Pathology and M.R.C. Research Group in Enzymology, St Mary's Hospital Medical School, London, W. 2

(*Received* 13 *February* 1962)

Shemin (1955) and Nemeth, Russell & Shemin (1957) were the first to suggest a possible metabolic role for aminoacetone. On administration of amino-[1-^{14}C]acetone to rats the label appeared in respiratory carbon dioxide, in urinary formate and in the ureido groups of uric acid. These findings were analogous to those obtained with δ-aminolaevulic acid, on the basis of which the succinate–glycine cycle was postulated (Shemin & Russell, 1953). Mauzerall & Granick (1956) detected a small amount of an aminoketone, with properties similar to those of aminoacetone, in normal human urine. Gibson, Laver & Neuberger (1958) and Kikuchi, Kumar & Shemin (1959) showed that δ-aminolaevulate synthetase was not specific for succinyl-coenzyme A, but would catalyse the condensation of glycine with various acyl-coenzyme A compounds, including acetyl-coenzyme A. Elliott (1958, 1959, 1960*a*) reported that an aminoketone is produced when *Staphylococcus aureus* is incubated aerobically in a medium containing glycine and glucose or in one containing threonine. He characterized the aminoketone produced from threonine as aminoacetone by the preparation of derivatives, but the one from glycine and glucose is produced in very small quantities and has only been identified as aminoacetone on the basis of its chromatographic properties. Elliott (1959) postulated that aminoacetone could be metabolized by a cyclic pathway similar to the succinate–glycine cycle for δ-aminolaevulate, and supported this

* Some of this work forms part of a Ph.D. Thesis submitted to the University of London in July 1961 by G. H. Tait.