Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

1. ENCEPHALOMYOCARDITIS VIRUS IN KREBS II MOUSE-ASCITES-TUMOUR CELLS

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Many attempts have been made during recent years to define the relationship between protein and nucleic acid biosynthesis. There is now adequate evidence that soluble proteins such as ribonuclease, insulin or haemoglobin have fully-defined chemical structures, each with a defined amino acid sequence. These sequences appear to be under genetic control and numerous hypotheses have been advanced to explain the control mechanism. In essence, they are all variants of one proposition, that protein synthesis takes place upon a template. Only three types of molecule have been considered as serious candidates for this role, deoxyribonucleic acid, ribonucleic acid and protein. Although deoxyribonucleic acid is closely associated with the genetic apparatus, current evidence favours ribonucleic acid as the macromolecule most closely associated with protein synthesis. There seems no doubt that ribonucleic acid can carry the necessary information for control of protein synthesis; thus, for example, ribonucleic acid from tobacco mosaic virus can induce the synthesis of strain-specific tobacco mosaic protein (Gierer & Schramm, 1956; Fraenkel-Conrat & Singer, 1957), and various ribonucleic acid preparations obtained from virus-infected mammalian cells or from mammalian viruses can induce synthesis of new virus (Colter, 1958). It seems quite likely therefore that ribonucleic acid could act as a template for protein synthesis, but no direct demonstration of this function has been achieved.

If a template exists, it is essential that a purified preparation of the template material should be isolated, and for this material to be recognized it is essential that it should have a measurable biological activity. It seemed possible that encephalomyocarditis virus grown in Krebs II mouse-ascites-tumour cells by the methods developed by Sanders (Sanders, 1957; Sanders, Huppert & Hoskins, 1958) might provide a system that would meet our requirements. The present series of papers represent studies designed to define the biological and biochemical characteristics of this system.

In particular, attention was directed to the effects of virus infection on the protein and nucleic acid metabolism of the host cell, with the object of determining the site within the cell and the time in the infectious cycle at which the protein and nucleic acid components of the virus are synthesized.

The advantages of the encephalomyocarditis–Krebs II cell system may be defined as follows. The host cell can be propagated on a large scale without technical complication and, moreover, the cell suspension can be maintained in a simple synthetic medium. The 'take' rate of virus in the host cell approaches 100% in a single growth cycle so that biochemical analyses of a cell population are unlikely to be vitiated by random variation throughout that population. In addition, the host cell contains an infective ribonucleic acid (template?), which may be isolated by phenol extraction (Huppert & Sanders, 1958), and the pure virus may be obtained as a crystalline ribonucleoprotein (Faulkner, Martin, Sved & Work, 1960). It is unlikely that a direct demonstration of the template function of ribonucleic acid could be achieved in intact cells, but the Krebs II cell gave good cell-free preparations capable of protein synthesis (Martin, Malec, Coote & Work, 1961). Since viral protein may be readily assayed or, if necessary, isolated by immunological or haemagglutination techniques, the system seems to offer the possibility of the direct induction of synthesis of a specific protein by a specific ribonucleic acid in a cell-free preparation.

The present paper describes the standard methods that have been used throughout these investigations for the propagation of Krebs II cells and for the growth of encephalomyocarditis virus in these cells. The effects of virus infection upon the nucleic acids and proteins of the host cell have been measured both by direct quantitative analyses and by measurement of turnover rate with [^{14}C]valine and [^{14}C]orotic acid as markers.

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METHODS

Buffers and nutrient solutions

Abbreviations. EMC, Encephalomyocarditis; PBS, phosphate-buffered saline.

The following solutions were used throughout the investigations reported in successive papers of this series.

Phosphate-buffered saline. Three stock solutions were prepared. Solution *A* contained NaCl (40 g.), KCl (1 g.), Na_2HPO_4 (5·75 g. or equivalent of hydrated salt), KH_2PO_4 (1·0 g.) and water (4 l.). Solution *B* contained $MgCl_2,6H_2O$ (0·5 g.) in water (500 ml.). Solution *C* contained $CaCl_2$ (0·5 g.) in water (500 ml.). Each solution was autoclaved and stored at 0°. For use solutions *A*, *B* and *C* were mixed in the proportions 40:5:5 and benzylpenicillin (100 i.u./ml.) and streptomycin–$CaCl_2$ (60 μg./ml.) (Glaxo Laboratories) were added. This solution is referred to throughout as PBS.

Earle's medium. Four stock solutions were prepared. Solution *A* contained NaCl (68 g.), KCl (4 g.), $MgSO_4,7H_2O$ (2 g.), phenol red (0·1 g.) and water (400 ml.). Solution *B* contained $NaH_2PO_4,2H_2O$ (1·58 g.), glucose (20 g.) and water (400 ml.). Solution *C* contained $CaCl_2$ (2·0 g.) in water (200 ml.). Solution *D* contained $NaHCO_3$ (4·4 g.) in water (100 ml.). These solutions were autoclaved and stored at 0°. For use solutions *A* (4·0 ml.), *B* (4·0 ml.) and *C* (2·0 ml.) were mixed and diluted to 100 ml. with sterile water; 5 ml. of this mixture was discarded and replaced by 5 ml. of solution *D* previously saturated with CO_2. Antibiotics were added as above. Where the Earle's medium was to be used for plaque assay the phenol red was omitted. This medium is similar to that of Earle (1943) and will be referred to as Earle's medium.

Phosphate-buffered gelatin. For assay of virus haemagglutinin, gelatin (0·2 g.; Difco Laboratories, Detroit, Mich., U.S.A.) was dissolved in boiling water (about 25 ml.), cooled, mixed with glucose (4·5 g.) and made up to 100 ml. with water. This solution was stored at 0°. It was mixed with an equal volume of PBS just before use.

Agar. Bacto-agar (150 g.; Difco Laboratories) was washed by decantation with water (20 times in all with 4 l. of water each time) and thrice with acetone (A.R. grade; 500 ml. for each wash). The agar was dried on open trays and dissolved in water as required (3 g. of agar in 100 ml. of water autoclaved for 10 min. at 10 lb./sq.in.).

Glassware. All glassware to be used in experiments on virus growth was washed by a method recommended by Dr F. K. Sanders (personal communication). This method was an essential requirement for reproducible results. Calgon (Albright and Wilson Ltd., 20 g.) was mixed with water (2270 ml.) and sodium metasilicate (180 g.) and, after 24 hr. the mixture was filtered. All glassware was cleaned in a neutral detergent (e.g. Lissapol; Imperial Chemical Industries Ltd.), rinsed and soaked overnight in the diluted silicate solution (1 part to 99 of water). The solution was heated to boiling and the glassware was removed, rinsed in tap water, soaked in 0·1 N-HCl (30 min.), rinsed in distilled water and steam-sterilized. Pipettes were cleaned in H_2SO_4–HNO_3 mixture and treated as above.

Krebs II mouse-ascites-tumour cells. The tumour-cell line used in this work originated as a mammary carcinoma and was converted into the ascites form by the method of Klein & Klein (1951). Dr J. Craigie, Imperial Cancer Research Institute, Mill Hill, kindly supplied mice bearing these Krebs II tumour cells and this cell line was used throughout. In order to obtain reproducible results it was necessary to propagate the cells in female mice. The strain of mouse was not critical and three strains of hybrid albino mice were used, Parkes, T.O. and Schneider. As judged by the ability to produce large yields of blood-free ascites fluid the Parkes strain was, however, preferable.

Production of a standard ascites-tumour-cell preparation. A cell line constantly propagated through mice might be subject to mutational change over a large number of successive generations and it was therefore considered advisable to maintain a stock of frozen cells as a standard preparation and to build up fresh experimental stock from these cells as required. In this way it has been possible throughout these investigations to use cells that were not more than five passages removed from the parent stock. Since, however, Krebs II cells are subject to temperature shock, some attention had to be paid to the conditions of preparation and storage of the standard cells.

Female Parkes mice aged 7–8 weeks and weighing 20–22 g. were used. This narrow range is specified because younger mice had a high mortality rate and older mice produced ascitic fluid containing few Krebs cells and large amounts of blood and fatty material. The mice were injected intraperitoneally with washed tumour cells (see later). About 1×10^7 washed cells gave a high yield of ascitic fluid in 7–8 days and a median survival time of 12 days. Smaller inocula produced solid infiltrative growths and bloody peritoneal exudates. After 7 days the ascitic fluid was withdrawn under sterile conditions. The usual yield of fluid was within the range 5–10 ml. and the cell count about 1×10^8/ml.

Aqueous glycerol helps to protect frozen tissues (Smith, 1954), and addition to the fresh ascitic fluid of glycerol to a concentration of 6% (v/v) increased the resistance of Krebs cells to freezing (12% of glycerol was less effective). A number of experiments were conducted to determine the optimum conditions for freezing. A suspension of cells plunged into ethanol–CO_2 at about −65°, then thawed and injected into mice, gave a 55% take rate (200 mice), whereas cells cooled during 5–7 min. to −70° showed a 65% take rate and cells cooled over 1 hr. a 75% take rate as compared with fresh cells. An apparatus was constructed (cf. Mathews, Maida & Buthala, 1959) that facilitated controlled cooling of cell suspensions. Ampoules of ascites-tumour-cell suspensions were prepared as follows. Ascitic fluid was collected from Parkes mice about 8 days after inoculation and all samples discoloured by blood were discarded. The pooled ascitic fluid was mixed thoroughly with glycerol (final concentration, 6%, v/v), dispensed into ampoules (4 ml.), flame sealed, and stored in a cold room at −4° for 1 hr. The ampoules were then transferred to a cold bath and the temperature was lowered 1·5°/min. for 10 min. to −15° and more slowly to −25° (15 min.). As soon as the ampoules had reached −25° they were cooled rapidly to −70° and maintained at this temperature.

When cells were required for the propagation of the tumour a single ampoule was thawed rapidly by agitation in water at 40° for about 2 min. Slow thawing (15 min.) gave a cell preparation with about one-quarter of the potency of rapidly thawed material. When cells were prepared, frozen and thawed as described above, 0·1 ml. of

cell suspension produced large ascitic tumours in 7 days in over 80% of the injected mice and the number of mice dying before 7 days was not more than 5%.

The first passage from the original tumour supplied by Dr Craigie was used to prepare a batch of the standard stock of cells. This stock was used either to replenish the standard stock or for the production of the large batches of ampouled material used to produce tumour for experimental purposes. Cells prepared in this way and stored at −70° have retained their viability unaltered for periods up to 8 months.

Preparation of Krebs II ascites-tumour cells for experimental use

Fresh ascitic fluid obtained as described above was transferred to 50 ml. sterile centrifuge tubes, diluted with about twice its volume of ice-cold PBS, and mixed at once. The cells were spun down at 400 rev./min. (60*g*) for 10 min. and the supernatant was discarded. Cells were rewashed at least twice in the same way or more often if necessary until free from erythrocytes and fat clumps. A sample of the cell suspension was diluted about 50-fold with 0·2% of nigrosin in PBS (water-soluble nigrosin, E. Gurr Ltd., London, S.W. 14) and the number of stained (dead) and unstained (live) cells counted in a Neubauer haemocytometer (Kaltenbach, Kaltenbach & Lyons, 1958). The number of dead cells did not exceed 10% and was usually below 5%. Nigrosin was found preferable to eosin (Sanders, 1957), since with the latter dye the proportion of dead cells rises rather rapidly with time as a result of the toxic action of the dye.

Growth of virus

Origin of virus. The strain of EMC virus used in these studies was obtained from Dr F. K. Sanders, M.R.C. Laboratories, Carshalton, Surrey. The virus was derived from the original strain isolated by Helvig & Schmidt (1945). A number of mice were injected intraperitoneally with the suspension of virus, and 36–40 hr. later those mice that were partially paralysed were killed and their brains removed under sterile conditions. The brains were homogenized in 0·9% NaCl, centrifuged and injected intraperitoneally into a second batch of mice. The virus was passaged in this way once more and an homogenate of two infected mouse brains was then added to a suspension of 1×10^8 Krebs II ascites-tumour cells in 5 ml. of Earle's medium and incubated under the standard conditions set out below. When the proportion of dead cells was 80% the cells were spun down and the supernatant was used to infect a further series of ascites-cell suspensions. The virus produced by this second cycle of growth in Krebs cells was harvested, ampouled and stored at −70°. This material provided the primary virus culture. Stocks of secondary cultures, built up from this primary culture, were used for experimental purposes.

Incubation conditions. Krebs II cells were washed in cold PBS as described above, transferred to Earle's medium, spun down and resuspended in fresh medium in a stoppered flask at a known cell concentration (2×10^7/ml.). A standard preparation of virus was added (3 plaque-forming units/host cell), and, after sufficient time to allow for adsorption of virus (30 min.), the flask was transferred to a mechanical swirler, which swirled the flask gently at about 70 rev./min. at 36°. The shape of the flask used probably was not critical; however, in the experiments described here, all incubations were performed in Erlenmeyer flasks containing one-tenth of their volume of cell suspension.

Assay of virus. The methods used were those developed by Sanders (1957). The plaque assay involved addition of 0·1 ml. of a suitable dilution of virus to a fresh preparation of washed ascites cells ($1{\cdot}2 \times 10^8$ cells/1·2 ml.) in Earle's medium without phenol red. The cells were then mixed with warm agar (1·8 ml. of 1·5%, w/v, in Earle's solution at 44°). The infected cells were layered upon a disk of preset agar (about 12 ml. of 1·5% agar in Earle's solution in a Petri dish), spread evenly and allowed to set. Plates were incubated for 2½ days at 37°, flooded with neutral red (0·0001% in 0·9% NaCl) and left for 1 hr. at 37°, the dye solution was discarded, and the plaques were counted. It was essential to maintain the plates, both during preparation and during incubation, in 5% CO_2, or alternatively to incubate in the presence of Pardee buffer as described by Bellett (1960). A stock virus preparation was always assayed along with each set of plaque assays.

As an alternative to plaque assay we have at times used cell-death titration (Sanders, 1957). In this method serial dilutions of virus were added to freshly washed Krebs II cells and incubated under the standard incubation conditions. After 15 hr. samples were withdrawn and mixed with nigrosin, and the percentage of dead cells was counted as already described. The relationship of this assay to the plaque is given by Sanders. The stock virus assayed by plaque count gave 3×10^9 plaque-forming units/ml. The same virus preparation assayed by cell-death titration gave 2×10^9 infective particles/ml. and also gave a haemagglutinin titre (see below) of 12 000–25 000.

Estimation of haemagglutinin. EMC virus agglutinates sheep red cells. Jungeblut (1958) has summarized earlier evidence that the haemagglutinating activity is part of and not just associated with the infective agent. This view is amply confirmed by our own studies on crystalline virus (Faulkner, Martin, Sved, Valentine & Work, 1961) since we have found that the ratio of haemagglutinin to infectivity remains constant throughout purification. The standard haemagglutination technique used throughout the present series of papers is given below.

Fresh sheep red cells (defibrinated sheep blood without preservative; Burroughs Wellcome Ltd., Beckenham) were washed in three changes of 0·9% NaCl and suspended in Alsever's solution (1 ml. of packed cells/10 ml. of solution as described by Bukantz, Rein & Kent, 1946). In this state cells could be stored at 3° for 7 days. When required, cells were washed in fresh 0·9% NaCl by three successive cycles of centrifuging (800*g* for 5 min.) and suspended in PBS–gelatin to a cell concentration of $2{\cdot}5 \times 10^7$ cells/ml. A suitable dilution of virus was prepared, also in PBS–gelatin (usually 1 in 100) and serial one-half dilutions of this stock solution (0·5 ml.) were made in standard M.R.C. agglutination plates; PBS–gelatin was used for serial dilution. A sample (0·25 ml.) of the red-cell preparation was added to each dilution of virus and the plates were left for 18 hr. at 4°. The end point was taken as the highest dilution of virus able to prevent formation of a complete ring of red cells. The titre, expressed as haemagglutinin units/ml., is defined as the reciprocal of this dilution. The stock virus had a titre of 12 000–25 000 haemagglutinin units/ml.

Rapid haemagglutinin method. At times, it was advantageous to have available a method of rapid haemagglutinin assay requiring minutes rather than hours. This was especially important in virus purification as it enabled several steps in the purification procedure to be carried out during 1 day (cf. Faulkner *et al.* 1961). The method adopted gave an assay in about 30 min. Serial dilutions of virus (1 ml.) were made in 15 ml. glass centrifuge tubes. The tubes were cooled in ice and 1 ml. of the standard sheep-red-cell preparation (also cooled at 0°) was added to each tube. After 15 min. at 0° the tubes were centrifuged for 3 min. at 1500*g* (swing-out buckets). The tubes were removed singly, held against a good light and agitated gently by slow swirling. In the absence of virus the cells swirled up evenly from the small hard button at the bottom of the tube. Starting at the highest dilution of virus, the first tube in which the cells failed to be resuspended evenly was taken as the end point (1/8000 with the stock virus). With progressively larger quantities of virus, the red cells became resuspended as progressively larger clots.

Extraction and estimation of nucleotides, nucleic acids and proteins

Preparation of cells. Much of the analytical work was carried out on Krebs II cells that had been incubated either with or without virus in Earle's medium. Before analysis these cells were washed as described below. The suspension of cells in the incubation flask was cooled to 0°, mixed with an equal volume of ice-cold PBS and transferred to capped centrifuge tubes. The flasks were rinsed with cold PBS, the washings mixed with the cells, and the whole was centrifuged at 120*g* for 5 min. at 0°. The supernatant was discarded. The cells were resuspended in cold PBS and the volume was made up to twice the original incubation volume. After centrifuging a second time the cells were rewashed in the same way, transferred to analytical homogenizer tubes (Kamphausen & Morton, 1956) and centrifuged at 2200*g* for 5 min. at 0°, and the cell pellet was stored at −20°.

Acid-soluble nucleotides. The cell pellet was thawed, homogenized with water (2 ml. for 2×10^7 cells) at 0°, mixed with cold M-$HClO_4$ (1 ml.) and rehomogenized. Insoluble material was collected (2200*g* for 5 min.) and washed first with 3 ml. and then with 2 ml. of 0·25M-$HClO_4$. The pooled acid extracts contained the acid-soluble nucleotides.

Removal of lipid. The acid-insoluble residue was washed with 5 ml. of methanol at 0° and the washings were discarded. After a second methanol wash at room temperature the residue was extracted with 4 ml. of methanol–$CHCl_3$ (1:1, v/v) for 30 min., then with 4 ml. of ether–$CHCl_3$ (1:1, v/v) for 30 min. and then washed twice with 4 ml. of ether. On each occasion the solids were homogenized with the solvent and removed by centrifuging.

Total nucleic acid. The lipid-free residue was homogenized with 3 ml. of 0·5M-$HClO_4$, heated at 70° for 30 min. and cooled to 0°. Cooling was necessary otherwise an $HClO_4$-soluble protein was precipitated at a later stage. The cold $HClO_4$ extract was separated by centrifuging and the residue re-extracted at 70° with $HClO_4$ (1 ml.) for 10 min. The residue from the second extraction was washed once with 0·5M-$HClO_4$ (1·0 ml.), and the combined acid extracts were pooled and diluted to 5·0 ml. This material is referred to as total nucleic acid.

Nucleic acid was estimated by extinction measurement at 264 mμ in $HClO_4$. For direct estimation of P the method of Griswold, Humoller & McIntyre (1951) was used and for conversion of extinction into μg. of P the relationship was $E_{1\,cm.}/0{\cdot}290$ = μg. of nucleic acid P/ml. (cf. Ogur & Rosen, 1950).

Total protein. The residue from the nucleic acid extraction was homogenized with water (1 ml.) and 0·2N-NaOH (3·0 ml.) was added. The mixture was heated to 50° for 30 min. and centrifuged. The residue was re-extracted twice, first at 50° as above and then at room temperature. The insoluble residue was mainly powdered glass from the homogenizer but a small residue of alkali-insoluble protein was always obtained. The pooled alkaline extracts were diluted with 0·2N-NaOH as required and the extinction at 290 mμ was measured. The relationship between extinction and total protein N was determined after Kjeldahl digestion of a standard sample of tumour-cell protein, either by the standard distillation method or by use of Nessler's reagent as described by Lang (1958). If the amount of protein was too small for estimation by ultraviolet absorption, the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used. For calibration of the colour yield/μg. of N, a standard sample of tumour-cell protein was used. This standard protein was prepared by extracting a large batch of washed Krebs cells by the methods given above to remove acid-soluble material, lipid and nucleic acids. The residue was washed free of acid with methanol–water (90:10, v/v), dried and stored in a vacuum desiccator.

Separation of ribonucleic acid and deoxyribonucleic acid. The method was based on that of Davidson & Smellie (1952). The lipid-free cell pellet (see above) was homogenized with 10% (w/v) NaCl (10 ml.) buffered at pH 6·0 with sodium acetate (0·1M) and heated at 90° with constant shaking for 12 hr. The extract was mixed with 95% ethanol (2·5 vol.) and left overnight at −20°. The supernatant from the ethanol precipitation was separated and discarded. It contained no measurable quantity of DNA and only small amounts of RNA, less than 5% of the total. The solids from the ethanol precipitation were digested with 0·33N-NaOH for 18 hr. at 37°. Glacial acetic acid was added to pH 4·0 followed by 95% ethanol (2·5 vol.). After 2 hr. at −20°, the suspension was spun at 2500*g*. The pellet was dissolved in 0·1N-NaOH and heated to 80° for 30 min. The DNA was again precipitated with acetic acid and ethanol, and the two supernatants were combined. The DNA precipitate was washed with 70% (v/v) ethanol and dried.

The supernatants from the alkaline digestion of DNA were evaporated to dryness and the hydrolysed RNA was separated from the sodium acetate by adsorption on Dowex 2 resin in tris–HCl buffer (pH 7·8; 0·1M) and eluted with N-HCl (de Deken-Grenson & de Deken, 1959). This eluate was used for the assay of radioactivity and for the electrophoretic separation of the nucleotides, as described below.

Estimation of deoxyribonucleic acid. DNA was estimated by the method of Burton (1956). A reference preparation of calf-thymus DNA was used as standard. This preparation contained 7·31% of P and after hydrolysis in $HClO_4$ (0·5N at 70° for 30 min.) had $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 232 mμ

and $E^{266}_{1\,cm.}$ for 1 μg. of P/ml. was 0·285. Perchloric acid extracts of whole cells gave the same value for DNA as did ethanol precipitates obtained after separation of RNA from DNA. Both methods were used for the determination of DNA.

Estimation of ribonucleic acid. The orcinol method of Hurlbert, Schmitz, Brumm & Potter (1954) was used. A preparation of yeast RNA was used as standard. This material contained 7·50% of P. After hydrolysis with $HClO_4$ as above the $\lambda_{max.}$ of the standard preparation was 260 mμ, $\lambda_{min.}$ 230 mμ and $E^{260}_{1\,cm.}$ for 1 μg. of P/ml. was 0·335. Except for experiments on the fate of the radioactive precursor (Table 7), all estimations of RNA concentration and radioactivity were performed on the hot $HClO_4$ total nucleic acid extracts. DNA, allowed to react with orcinol in the same manner, gave an extinction at 670 mμ 9·2% of that of an equivalent amount of RNA. Estimates of RNA in total nucleic acid extracts were corrected for the amount of DNA present, measured by Burton's method, by using this factor.

Estimation of nucleotide concentration. The concentration of nucleotides in the cold $HClO_4$ (acid-soluble) extract was estimated from the extinction at $\lambda_{max.}$ (257 mμ), assuming the $E^{257}_{1\,cm.}$ for 1 μg. of nucleotide P/ml. was 0·330. When cold $HClO_4$ extracts from Krebs II cells, previously incubated for 30 min. with [^{14}C]orotic acid, were treated with charcoal, and the nucleotides eluted and subjected to two-dimensional chromatography (Tsuboi & Price, 1959), they were found to contain predominantly the mono- and di-phosphates of adenosine and uridine, and guanosine monophosphate. Uridine-containing nucleotides accounted for 18–20% of the total ultraviolet-absorbing material in the extract. No orotic acid was detected, even after very brief incubations with the precursor.

Measurement of radioactivity

Protein turnover rates were measured by following the rate of incorporation of uniformly labelled L-[^{14}C]valine (specific activity 8·0 mc/m-mole). As a precursor of nucleotide and nucleic acid [6-^{14}C]orotic acid (specific activity 9·7 mc/m-mole) was used. Its radiochemical purity was checked by paper chromatography (Leone & Scale, 1950). Both labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of cells for extraction of radioactive components. Ascites-tumour cells, incubated with [^{14}C]orotic acid (or [^{14}C]valine) for the required period, were diluted with 10 ml. of ice-cold carrier (1 mM-[^{12}C]orotic acid or 4 mM-[^{12}C]valine in PBS) and transferred to 50 ml. centrifuge tubes with a further 10 ml. of carrier. The tubes were centrifuged at 120g for 5 min. at 0°, the supernatant was discarded, and the procedure for the preparation of cells for analytical studies, described above, was applied. When the final washing fluids were examined, they were free of radioactivity and unlabelled precursors.

Radioactive proteins. The cell material which remained after removal of acid-soluble substances, lipids, RNA and DNA as already described was dissolved in NaOH (0·2N) mixed with excess of [^{12}C]amino acid and reprecipitated with trichloroacetic acid. The precipitated protein was washed successively with acetone, acetone–ether and ether and dried. Plates were prepared for counting either by packing the solid on weighed polythene planchets for infinitely thick samples, or by dissolving the protein in 100% formic acid and spreading it upon aluminium planchets for counting at zero thickness. In order to avoid the troublesome curling of dry protein films, the aluminium planchets were coated with silicone adhesive (Cold-Cure Silastomer; Midland Silicones Ltd., London, W. 1) before use.

Radioactive deoxyribonucleic acid. The DNA extracted by the hot NaCl method (see above) was dried, powdered, spread evenly on Perspex disks of 0·3 cm.^{2} area and counted with a standard thin-window Geiger counter. Some samples of DNA were also hydrolysed in $HClO_4$, adsorbed on charcoal as described below, and counted in the same way as RNA. The two methods gave the same result within the limits of the counting error.

Radioactive ribonucleic acid. In most methods for the extraction of total nucleic acids from tissues, the nucleic acids are obtained as a hydrolysate in $HClO_4$ or trichloroacetic acid. Most of the $HClO_4$ may be removed as $KClO_4$ but even the small amount of residual $KClO_4$ that separates when solutions are dried for counting is sufficient to cause large errors in the radioactive assay. Hurlbert & Potter (1952) neutralized trichloroacetic acid extracts with NH_3 and evaporated them to dryness, but this method also gave variable results (cf. Reid & Stevens, 1957). An alternative method was suggested by Kruh & Borsook (1955). They adsorbed nucleotides on activated charcoal and counted the dried samples of charcoal. According to these authors the charcoal had to be saturated with nucleotide and this placed a severe limitation on the usefulness of the method. In the present investigations the charcoal method could be made completely reliable without recourse to nucleotide saturation.

A stock solution of hydrolysed radioactive nucleic acid was prepared in 0·5N-$HClO_4$. This material had a specific activity of 1·060 μc/m-mole of nucleic acid P. A series of samples of Norit A (British Drug Houses Ltd.) were dispensed into centrifuge tubes (2–40 mg.) and portions of the standard radioactive nucleic acid solution were added (211 μg. of nucleic acid P in 1 ml. of 0·5N-$HClO_4$ to each). In a second series of tubes a fixed amount of Norit (40 mg.) was mixed with various quantities of radioactive nucleic acid hydrolysate. The specific activity of the hydrolysates was varied by dilution of the standard radioactive preparation with various amounts of non-radioactive hydrolysate, and the $HClO_4$ volume was varied between 3 and 8 ml. All samples of charcoal were left for 1 hr. at 0° with occasional mixing and then centrifuged at 2200g for 5 min. at 0°. The supernatant was removed and the unadsorbed nucleotide estimated by ultraviolet absorption. The charcoal was washed at 0° with ice-cold water and then with acetone–ether (1:1) followed by two washes with ether. It is essential to avoid ethanol during washing as this elutes some of the adsorbed nucleotide. The dry charcoal samples were plated on 2 cm.^{2} planchets and counted on a thin-window Geiger counter. The results of this experiment (Table 1) demonstrate that the method gave reliable results over a wide range of specific activities and over a wide range of ratios of nucleic acid to charcoal.

The particular advantage of this method was that no limitation was placed on volume of extract that could be taken for radioactivity assay; 80% of the total extract was usually used for this purpose. The procedure adopted throughout the remainder of this investigation was to adsorb the nucleic acid hydrolysate on about 40 mg. of

Table 1. *Determination of specific radioactivity of a nucleic acid hydrolysate by adsorption on charcoal*

The standard preparation of nucleic acid had a specific activity of 1·06 μc/m-mole of nucleic acid P and this was diluted with various quantities of a non-radioactive hydrolysate before mixing with charcoal. Full details are given under Methods.

Charcoal (mg.)	Total nucleic acid used (μg. of P)	Dilution* factor	Amount of standard† radioactive nucleic acid adsorbed (μg. of P/mg. of charcoal)	Specific radioactivity of standard (μc/m-mole of P)
24·9	412	100	15·98	1·110
25·5	206	100	8·00	1·045
25·0	103	100	4·11	1·085
25·1	41·2	100	1·62	1·095
39·8	206	100	5·17	1·050
60·5	206	100	3·39	1·050
26·0	360	57·3	7·77	1·000
40·7	360	57·3	5·03	1·140
60·2	360	57·3	3·42	1·085
25·1	333	30·9	3·99	1·060
40·9	333	30·9	2·50	1·000
25·8	286	80·1	11·73	1·030
40·3	286	80·1	7·59	1·035
25·6	180	57·3	3·99	1·080

* Expressed as the percentage of the standard radioactive preparation in the diluted solution used for assay (column 2).
† Calculated from the difference in extinction of the solution before and after treatment with charcoal.

charcoal, and to measure the extinction of the solution at 264 mμ both before and after treatment for 1 hr. at 0° with charcoal, which was then washed and counted as above. Originally, a sample of nucleic acid of known specific radioactivity was always carried through the same process and its radioactivity assayed concurrently with the unknown samples. However, experience showed that this precaution was unnecessary. The specific activity of the unknown nucleic acid, expressed as μc/m-mole of nucleic acid P, was calculated from the formula:

$$\text{Specific activity} = \frac{\text{Observed counts/min.} \times \text{wt. of charcoal (mg.)}}{\text{Standard counts/min.} \times \text{area of sample (cm.}^2\text{)} \times \mu\text{mole of P adsorbed}}.$$

'Standard counts/min.' refers to the counting rate of a 1 cm.² disk of poly[^{14}C]methyl methacrylate of specific activity 1·0 μc/g. (supplied by The Radiochemical Centre); this was normally 900–1000 counts/min. in the counting equipment used for these studies. This method was also used to measure radioactivity in soluble nucleotide extracts.

It is shown below (see Table 7) that the ratio of specific activities of RNA to DNA when [^{14}C]orotic acid is incubated with Krebs II cells under the standard conditions is approx. 19:1. As the ratio of RNA to DNA in these cells was normally about 1·67:1 (see Table 2), the specific activity of RNA in total nucleic acid extracts was estimated on the assumption that 97% of the total radioactivity measured represented incorporation into RNA.

RESULTS

Growth cycle of encephalomyocarditis virus in Krebs II ascites-tumour cells

The growth cycle of EMC virus in Krebs II ascites-tumour cells has already been described by Sanders (1957) and Sanders *et al.* (1958). We have obtained essentially similar results and these are given here only to demonstrate the characteristics of the system. Under the conditions of incubation described in the paper and with an infectivity ratio of 3 plaque-forming units/host cell there is no detectable haemagglutinin or virus until after 3 hr. at 36°. Thereafter there is a steady increase in intracellular haemagglutinin and in the number of intracellular virus particles up to about 8 hr. (Fig. 1). After this time free virus is released into solution and the host cells die.

In some experiments, some degree of synchrony of virus growth was induced by adding the virus at 0° and leaving it at this temperature for 3 hr., when the temperature was rapidly increased to 36° immediately before incubation. The method was suggested to us by Dr Sanders and when it was used for a particular experiment this is stated in the appropriate table. The effect of synchrony was to increase the rate and shorten the time of virus growth.

Effect of virus infection upon total deoxyribonucleic acid, total ribonucleic acid and total protein of Krebs II cells

No previous results have been reported on the effect of EMC virus infection on the level of DNA, RNA and protein in the Krebs cell, but two previous investigations (Ackermann, 1958; Salzman, Lockart & Sebring, 1959) on the effect of polio-

myelitis-virus infection in HeLa cells suggested that quite substantial changes might be found. A series of infected suspensions of Krebs cells were therefore incubated at 36° for periods of from 1 to 7 hr. After the appropriate time, the cell suspension was cooled to 0°, together with a similar uninfected control, and the cells were collected, washed and analysed as described under Methods. One infected and one control flask were incubated for 20 hr. At this time 95% of the infected cells were dead (as shown by staining with nigrosin) while the control flask contained 14% of dead cells. From Table 2, it is evident that there is no change in total RNA, total DNA or total protein as a result of virus infection.

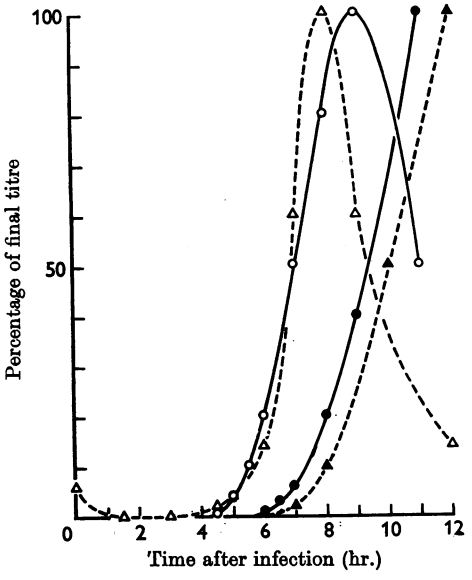

Fig. 1. Growth cycle of EMC virus on Krebs II ascites-tumour cells suspended in Earle's medium. △, Intracellular haemagglutinin; ▲, extracellular haemagglutinin; ○, intracellular infective particles (plaque-forming units); ●, extracellular plaque-forming units.

Effect of virus infection on penetration of [^{14}C]orotic acid into Krebs cells

Radioactive orotic acid was used as tracer in order to compare the rate of turnover of nucleic acid in normal and in infected tumour cells. Since the values for turnover rates measured in this way would be affected by the rate of penetration of radioactive tracer into the intracellular pool of nucleotide, it was essential to ensure that virus infection did not prevent free penetration of the tracer into the cells.

The choice of orotic acid rather than a nucleotide as tracer for nucleic acid turnover was dictated by considerations of cost. It has been suggested, however, that orotic acid is poorly converted into uridylic acid and into RNA in HeLa cells (Pileri & Ledoux, 1957), and it was therefore necessary to examine this point in Krebs II ascites-tumour cells. A series of flasks, each with 1×10^8 cells in Earle's medium, were incubated at 36° for 3 hr. At the end of this time [^{14}C]orotic acid was added and flasks were removed at intervals of 30 min. during the next 2 hr. The intracellular nucleotide pool and the total cell RNA were isolated as described under Methods and their specific radioactivities were determined. From the results given in Table 3, it is evident that orotic acid is a satisfactory label for nucleotides and for RNA in the Krebs cell, and that equilibration between added orotic acid and the intracellular nucleotide pool required less than 30 min.

In another experiment, bulk suspensions of virus-infected and control cells were incubated under the standard conditions for 7 hr. At hourly intervals suitable portions of each cell suspension were transferred to smaller flasks and incubated with [^{14}C]orotic acid for 30 min. At the end of this time ice-cold carrier was added and the cells were washed as already described. Each acid-soluble nucleotide pool was measured quantitatively and its specific radioactivity determined (Table 4). For the first 4 hr., virus infection caused only a slight

Table 2. *Effect of virus infection on ribonucleic acid, deoxyribonucleic acid and protein content of Krebs II cells*

The ascites-tumour cells were suspended in Earle's medium (2×10^7 cells/ml.) and infected with 3 plaque-forming units/cell. Each flask contained 5 ml. of cell suspension. Flasks were incubated at 36° and removed at hourly intervals, and the cells analysed as described in the text.

Time after infection (hr.)	RNA P/10^8 cells (μg.)		DNA P/10^8 cells (μg.)		Protein/10^8 cells (mg.)	
	Infected	Control	Infected	Control	Infected	Control
1	367	375	230	229	30·5	31·9
2	374	371	232	234	30·1	29·4
3	363	373	228	229	29·7	29·8
4	359	356	230	226	30·6	29·6
5	359	368	227	229	30·1	29·1
6	372	376	234	233	30·8	29·1
7	366	386	228	229	30·2	30·9

increase in the size of the nucleotide pool, but during the last 2 hr. there was some loss of nucleotide from the infected cells. The specific activity in the control cells remained almost constant during 6 hr. incubation, but at 4 hr. the specific activity of the pool in infected cells began to rise, and this rise continued steadily until it was more than 100% above the initial value. The very slight lowering in specific activity of nucleotides in the infected cells 1 and 2 hr. after infection is due to dilution of the pool and not to any effect on the rate of precursor incorporation, as the total radioactivity found in the pool is not decreased by infection. It is evident from these results that orotic acid is an adequate precursor of soluble nucleotides in both normal and infected cells and that infection far from causing any inhibition of the rate of conversion of orotic acid into nucleotide actually appeared to stimulate conversion. The significance of these observations became more apparent as further information was collected on the behaviour of infected cells (see below).

Table 3. *Radioactive orotic acid as a precursor of soluble nucleotides and of ribonucleic acid in Krebs II ascites-tumour cells*

The cells (10^8) in Earle's medium (5 ml.) were incubated at 36° for 3 hr. before addition of [6-^{14}C]orotic acid (5 μc). Incubation was continued for the times indicated, and the acid-soluble nucleotides and RNA were then separated and assayed as described in the text.

Time of incubation with orotic acid (min.)	Total radioactivity in nucleotide pool (μmc)	Total radioactivity in RNA (μmc)
30	15·7	15·0
60	13·2	25·1
90	16·4	41·8
120	16·6	58·7

Effect of virus infection on penetration of valine into Krebs cells

To examine the rate of penetration of radioactive valine into the intracellular amino acid pool, a series of suspensions of infected cells were incubated for different times in the presence of [^{14}C]-valine. The cells were then cooled quickly at 0°, washed once with ice-cold 0·9% sodium chloride and homogenized in cold ethanol–water (4:1). The radioactivity of the ethanol-soluble material was taken as a measure of the rate of penetration of valine into the intracellular amino acid pool. From the results in Table 5, it is evident that the added [^{14}C]valine equilibrated with the intracellular valine in about 5 min. In further experiments, virus-infected cells were incubated under the standard conditions and, at various times after infection, [^{14}C]valine was added to an infected flask and to a corresponding control. After a further 5 min. at 36°, the radioactivity of the intracellular pool was measured as above. From the results given in Fig. 2, it is evident that virus infection had little if any effect on uptake of valine into the cell during the first 4 hr. (replicate flasks gave values differing by about 10%). From 6 hr. onwards, however, the cells had lost much of their capacity for the concentration of valine. There seems no doubt that this is a concentrative process since after 5 min. 14% of the ^{14}C added was found in the intracellular pool, whereas the total cell volume only represented about 3% of the total volume (cf. Christensen & Riggs, 1953).

Effect of virus infection on the turnover of ribonucleic acid in Krebs II ascites-tumour cells

In a preliminary experiment, tumour cells were incubated with [^{14}C]orotic acid for 2 hr. under the conditions given in Table 6. At the end of this time

Table 4. *Effect of virus infection on the size and on the radioactivity of the nucleotide pool during incubation with* [^{14}C]*orotic acid*

A large batch of cells (16×10^8 in Earle's medium) was cooled to 4° and divided into two lots, and one lot was infected with virus (3 plaque-forming units/cell). After 3 hr. at 4° the cells were collected, resuspended in fresh Earle's medium (2×10^7 cells/ml.) at 36° (synchronized infection) and incubated in the usual way. At the times indicated samples (5 ml.) were withdrawn from each flask and incubated with [^{14}C]orotic acid (7·5 μc) for 30 min. After this time excess of ice-cold [^{12}C]orotic acid was added and the cells were washed with cold buffer. The intracellular acid-soluble nucleotides were isolated and assayed for specific radioactivity as described in the text.

Time after infection (hr.)	Nucleotide pool size (μmoles of nucleotide P/10^8 cells)		Specific radioactivity of nucleotide pool (μc/m-mole of P)		Infected / Control (specific radioactivity)
	Infected	Control	Infected	Control	
1	1·98	1·88	9·33	9·80	0·95
2	2·19	1·83	8·12	8·71	0·92
3	2·18	1·85	8·59	7·51	1·14
4	2·03	1·82	11·1	8·38	1·32
5	1·99	1·81	14·2	8·08	1·76
6	1·50	1·69	19·5	8·01	2·44
7	1·12	1·76	20·3	6·54	3·10

Table 5. *Rate of equilibration of added [^{14}C]valine with the intracellular amino acid pool during incubation of Krebs II ascites-tumour cells in Earle's medium*

Each flask contained 4×10^7 cells in 2 ml. of Earle's medium. Cells were incubated for 1 hr. at 36° before adding valine (0·1 μc; 0·17 μmole). After the stated time cells were rapidly cooled to 0°, washed with cold 0·9% NaCl and homogenized in ethanol–water (4:1, v/v).

Incubation time with [^{14}C]valine (min.)	Radioactivity in ethanol-soluble fraction (counts/min.)	Calculated percentage of added ^{14}C in intracellular valine pool
0	146	0·78
0·5	1240	6·5
1·0	1710	9·0
2·0	2180	11·5
5·0	2680	14·1
10·0	2680	14·1
15·0	2390*	12·6

* The drop in the amount of [^{14}C]valine found in the ethanol-soluble fraction at 15 min. is probably caused by its rapid incorporation into protein.

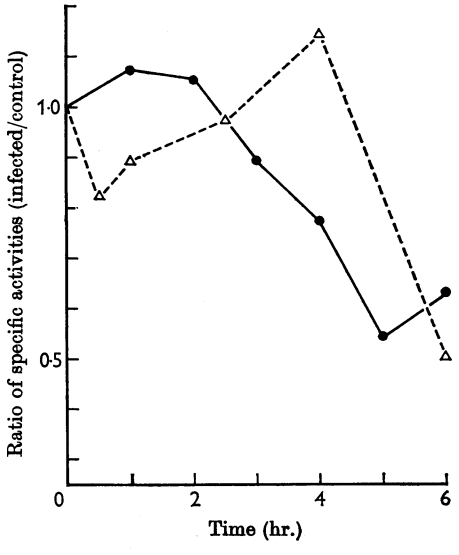

Fig. 2. Effect of virus infection on rate of penetration of valine and on protein turnover in Krebs II ascites-tumour cells. Experimental conditions were as described in the text. Results are expressed as the ratios of specific activities (infected/control) for protein (●), and as ratio of the total radioactivities (infected/control) of free valine in the intracellular pool (△). Intracellular haemagglutinin could not be detected until 4 hr. after infection. At 5 hr. there were 1500 haemagglutinin units and at 6 hr. 56 000. At 7·5 hr. the rate of penetration of valine into infected cells was less than 20% of the control rate and at 8 hr. it was about 7% of the control.

Table 6. *Distribution of radioactivity in the ribonucleic acid and deoxyribonucleic acid of Krebs II tumour cells after incubation with [^{14}C]orotic acid*

The Krebs cells (in two flasks, each with 10 ml. of a suspension of 4×10^7 cells/ml. in Earle's medium) were incubated for 2 hr. at 36° with 20 μc of [^{14}C]orotic acid in each flask. RNA and DNA were isolated as described in the text. Both were hydrolysed (see Methods) and the bases separated as described. The only radioactive products were uridine monophosphate from RNA and thymine from DNA.

Material	Radioactivity (μc/mole of P or μc/mole of thymine) Flask 1	Flask 2
RNA	9·9	9·15
DNA	0·547	0·485
UMP	—	33·1
Thymine	—	1·6
RNA/DNA	18·1	18·9
UMP/thymine	—	20·6

RNA and DNA were isolated from the cells by the modified Davidson–Smellie procedure (see Methods) and their specific radioactivities were determined. A sample of the DNA was hydrolysed with formic acid (Wyatt & Cohen, 1953) and the bases were separated in propan-2-ol–hydrochloric acid (Wyatt, 1951). A sample of the hydrolysed RNA was purified as already described and separated into nucleotide components by electrophoresis (Davidson & Smellie, 1952). Each component was assayed for radioactivity with the double-window paper scanner described by Piper & Arnstein (1956). The results (Table 7) showed that the uridine monophosphate from RNA accounted for 97% of the total nucleic acid radioactivity; the remainder was as [^{14}C]thymine in DNA.

In further experiments the rate of RNA turnover in normal cells was compared with that of virus-infected cells. Cells were incubated either with or without virus for various times before addition of [^{14}C]orotic acid. Incubation was continued for a further 30 min. to allow equilibration between the added ^{14}C and the intracellular nucleotide pool. At 30 min. the cell suspensions were cooled to 0° and the acid-soluble nucleotides and nucleic acids were isolated. As shown in Table 7, the rate of incorporation of orotic acid into RNA of control cells fell slowly throughout the incubations. In infected cells, the fall was at first more rapid but at about 5 hr. after infection the rate of incorporation began to increase. By 7 hr. after infection the amount of [^{14}C]orotic acid converted into RNA was more than twice as large in the infected as in the control cells. Much of this increased conversion is due to the increase in specific activity of the soluble nucleotide pool, but even when the results are expressed as the ratio of specific activities of RNA to soluble nucleotides (i.e. relative specific activity),

Table 7. *Effect of virus infection on the rate of turnover of ribonucleic acid in Krebs II ascites-tumour cells*

Specific activity of the RNA from cells used in the experiment described in Table 4 was measured as described in the text. Since there is no net change in the amount of RNA (Table 2) the results are a measure of the rates of turnover.

Time after infection (hr.)	Specific activity of RNA (μc/m-mole of P)		Total uptake of orotic acid (μmc/10^8 cells)		Relative specific activity of RNA*	
	Infected	Control	Infected	Control	Infected	Control
1	2·07	2·57	35·9	39·7	0·222	0·262
2	1·10	1·51	26·9	28·4	0·137	0·173
3	0·575	1·12	23·5	23·2	0·067	0·149
4	0·450	1·04	26·2	23·9	0·041	0·124
5	0·715	0·945	34·0	22·4	0·051	0·116
6	1·13	0·990	40·1	21·7	0·068	0·123
7	1·74	0·795	37·1	18·1	0·086	0·122

* Relative specific activity is calculated and is the ratio between the specific activity of RNA and the specific activity of the nucleotide pool from the same cells.

Table 8. *Effect of infection on the rate of amino acid incorporation into ascites-tumour-cell protein*

Flasks containing normal and infected (3 plaque-forming units/cell) suspensions of tumour cells (2·0 ml.; 2×10^7 cells/ml.) in Earle's medium were incubated at 36° for the periods indicated, and [^{14}C]valine (0·3 μc; 0·17 μmole) was added. Trichloroacetic acid (30%, w/v; 2·0 ml.) was added 30 min. later, the protein isolated, and its radioactivity was determined as described under Methods. Results are expressed as a ratio (infected/control) of specific activities.

Time of incubation with virus (hr.)	Specific activity of protein $\left(\frac{\text{infected}}{\text{control}}\right)$
0·5	0·96
1	1·10
2	1·06
4	0·93
6	0·75
7·5	0·68
8	0·31

a marked stimulation during the 4–7 hr. period in the rate of RNA synthesis is evident. The rate of increase of the relative specific activity during this period was proportional to the increase in intracellular virus titre. However, the amount of new RNA synthesized, calculated from these radioactivity data, seemed to far exceed the total increase in viral RNA calculated from the haemagglutinin titre and the data given by Faulkner *et al.* (1960).

Effect of virus infection on protein turnover in Krebs II ascites-tumour cells

In the experiment described in Table 7, infection was synchronized by the technique developed by Sanders (see under Methods). In another similar experiment incubation was started 30 min. after addition of virus. In the synchronized culture nearly all the infected cells died after 8–8·5 hr. at 36° but in the unsynchronized culture cell death was spread over a 4 hr. period (8–12 hr.). As might be expected, the increase in RNA turnover during the later stages of infection was much less obvious but it was still apparent. In several similar experiments, protein turnover was also measured by incubation with [^{14}C]valine. There was a slow fall in the rate of protein turnover in control cells throughout the 8 hr. incubation period. The rate of protein turnover fell more quickly in the infected cells and at 8 hr. was about 30% of that of the controls (Table 8). We have already shown, however, that the capacity of the cell to concentrate valine is lost during the later stages of infection (Fig. 2) and it may well be that any synthesis of viral protein would be obscured by this factor. Even if no allowance is made for this factor, however, a plot of rate of protein turnover in infected cells against that in control cells showed that there was some stimulation of protein synthesis in the infected cells around the sixth hour after infection (Fig. 2).

DISCUSSION

Much attention has been devoted by Cohen and others to delineation of the biochemical events which accompany reproduction of bacteriophage within *Escherichia coli* (Cohen, 1957; Flaks, Lichtenstein & Cohen, 1959). Less attention has been paid to the related biochemical problems which arise during virus replication within mammalian cells. The slower rate of progress in the mammalian field has been occasioned by both quantitative and qualitative factors. With a virus such as EMC, the amount of virus material that is formed in the host cell in a single growth cycle is an extremely small percentage of the total mass of the host cell. For this reason quantitative changes in host-cell composition are difficult to detect, and in

fact there were no measurable changes in overall content of RNA, DNA and protein in Krebs II cells during a complete cycle of virus growth.

In addition, mammalian RNA viruses do not contain, so far as is known, any base residues such as 5-hydroxymethylcytosine, and the formation of viral RNA cannot therefore be followed by qualitative methods.

No comparable results are available from other Laboratories on Krebs cells but the related system of poliomyelitis-virus growth in HeLa-cell suspension has been studied in some detail. The results from different Laboratories are contradictory, probably because the HeLa cell deteriorates in a simple maintenance medium such as that used for Krebs cells and it is thus difficult to distinguish between damage caused by virus infection and natural deterioration of the host cell. Ackermann, Loh & Payne (1959) found that in HeLa cells infected by polio virus there was a large increase of RNA in the cytoplasm during the first 4 hr. after infection, but Salzman *et al.* (1959) found no change in total RNA, DNA or protein during the first 3 hr. and thereafter a loss of RNA from the infected cells. These effects are reproduced by incubation of cells in an amino acid-deficient medium and the loss of RNA is at least three times as great as would be required for virus synthesis. These results indicate the difficulty of working with a cell that has to be maintained in a complex medium and underline the advantages offered by the Krebs ascites-tumour cell.

Since no quantitative changes in RNA, DNA or protein were detected during the growth cycle of EMC virus in Krebs cells it became necessary to use radioactive tracers in order to detect changes in nucleic acid and protein metabolism. Although ^{32}P has been used in several investigations on the effect of poliomyelitis-virus infection (Miroff, Cornatzer & Fischer, 1957; Ackermann *et al.* 1959; Contreras, Tohá & Ohlbaum, 1959), we regarded it as unsuitable for such investigations since it is incorporated into a wide variety of cell components. It is thus difficult to know the nature of the radioactivity of the particular phosphate pool that will be used for synthesis of any particular type of molecule, and, moreover, it is notoriously difficult to remove adsorbed radioactive phosphates of various kinds from RNA and DNA (Davidson & Smellie, 1952). It is clear from our results, however, that [^{14}C]orotic acid is a suitable tracer, since virus infection does not inhibit uptake and the orotic acid is rapidly converted by the Krebs cell into uridine nucleotides. It is thus possible to measure the radioactivity of the nucleotide pool and to distinguish between effects of virus infection on nucleotide metabolism and on RNA metabolism. To illustrate the effect of virus infection upon nucleotide and upon RNA metabolism, the results of the experiments outlined in Tables 4 and 7 are presented graphically as Fig. 3. It may be suggested that the cycle of events after virus infection is roughly as follows. During the first 4 hr. after infection there is an overall inhibition of RNA synthesis with a consequent accumulation of small nucleotides. From 4 hr. onwards and almost coincident with the appearance of intracellular virus there is an increase in the rate of synthesis of new RNA. Since there is probably no overall increase in RNA this synthesis is likely to be accompanied by an accelerated loss of old RNA, probably cell RNA. The fall in the size of the nucleotide pool indicates that the new RNA is being synthesized direct from nucleotides, and the rapid rise in the specific activity of this pool when radioactive orotic acid is added to the incubation medium indicates that the permeability barriers within the cell have begun to break down so that orotic acid can enter the nucleotide pool more quickly than in the normal cell. Before a more detailed picture of these biochemical events can be presented it will be necessary to study the changes that occur within the various intracellular organelles and to distinguish

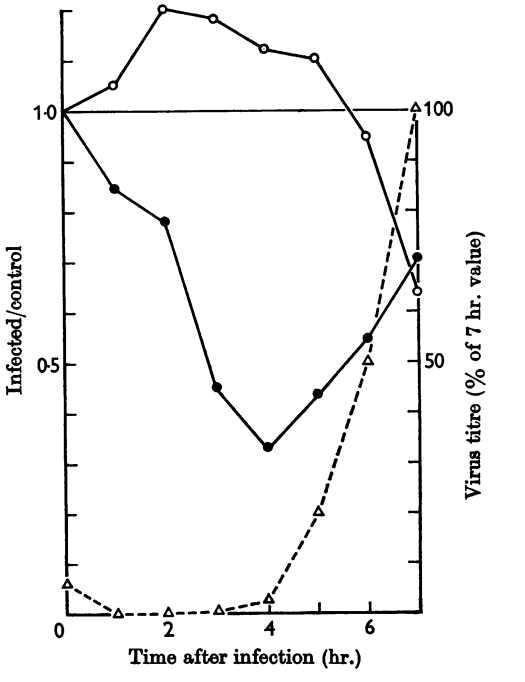

Fig. 3. Effect of virus infection on the rate of turnover of RNA in Krebs II ascites-tumour cells. Relative specific activity is defined as the ratio of specific activity of RNA to that of the nucleotide pool measured at the same time. △, Intracellular virus haemagglutinin titre; ●, RNA; ○, size of intracellular-nucleotide pool.

between synthesis of virus RNA and synthesis of other types of RNA.

The results obtained by the measurement of protein turnover in intact cells have been less conclusive than those for RNA. The mechanism for amino acid concentration by Krebs cells begins to break down some time between 4 and 6 hr. after infection and this breakdown probably serves to obscure the increase in protein turnover which would be expected to accompany virus synthesis. There is, nevertheless, some increase in the rate of protein turnover about 5 hr. after infection, which may represent synthesis of viral protein. With further development of our technique, virus has been isolated at various stages during the growth cycle and the results obtained in this way (E. M. Martin & T. S. Work, unpublished work) are in general agreement with our present interpretation.

In all the numerous experiments that were performed, a small increase in the rate of protein turnover in infected cells was observed between 1 and 2 hr. after infection (cf. Fig. 2). This increase was never more than about 20 % above that in the control, and we were at first inclined to regard it as insignificant. With constant repetition, however, we now believe that there is a genuine stimulation of protein turnover in infected cells during this period, and it is tempting to suppose that this may be related to the requirement for preliminary synthesis of new enzymes before virus reproduction can commence.

SUMMARY

1. Conditions are described for the propagation of Krebs II ascites-tumour cells and for measurement of the effects of infection by encephalomyocarditis virus on these cells in circumstances favourable for quantitative biochemical investigation.

2. The rate of penetration of orotic acid and of valine into Krebs cells was measured. Infection caused no alteration in the rate of penetration of orotic acid during the first 4 hr. During the later stages of the infectious cycle, orotic acid was taken up at a progressively increased rate, and cells lost their capacity to concentrate valine.

3. During the period of rapid virus synthesis there was a marked increase in the rate of ribonucleic acid turnover and a decrease in the size of the intracellular nucleotide pool.

4. Virus synthesis was accompanied by a small increase in the rate of protein turnover, but this effect was obscured by the loss of capacity to concentrate the radioactive tracer (valine) used to measure turnover rate.

5. In all experiments the infected cells showed a small increase in the rate of protein turnover during the first 2 hr. after infection and before any synthesis of new virus could be detected. This stimulation may be associated with the need for synthesis of new enzymes before virus reproduction can commence.

6. There was no change in the total deoxyribonucleic acid, ribonucleic acid or protein in infected cells.

7. A method of haemagglutinin assay was developed which gave results within 30 min. A method for the measurement of the radioactivity of nucleotides present in aqueous acid extracts of tissues is also described.

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REFERENCES

Ackermann, W. W. (1958). *Bact. Rev.* **22**, 223.
Ackermann, W. W., Loh, P. C. & Payne, F. E. (1959). *Virology*, **7**, 170.
Bellett, A. J. D. (1960). *Virology*, **10**, 285.
Bukantz, S. C., Rein, C. R. & Kent, J. F. (1946). *J. Lab. clin. Med.* **31**, 394.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Christensen, H. N. & Riggs, T. R. (1953). *Science*, **118**, 569.
Cohen, S. S. (1957). In *The Chemical Basis of Heredity*. p. 651. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Colter, J. S. (1958). *Progress in Medical Virology*, vol. 1, p. 1. Ed. by Berger, E. & Melnick, J. L. Basel: S. Karger.
Contreras, G., Tohá, J. & Ohlbaum, A. (1959). *Biochim. biophys. Acta*, **35**, 268.
Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 594.
de Deken-Grenson, M. & de Deken, R. H. (1959). *Biochim. biophys. Acta*, **31**, 195.
Earle, W. R. (1943). *J. nat. Cancer Inst.* **4**, 165.
Faulkner, P., Martin, E. M., Sved, S., Valentine, R. C. & Work, T. S. (1961). *Biochem. J.* **80**, 597.
Faulkner, P., Martin, E. M., Sved, S. & Work, T. S. (1960). *Nature, Lond.*, **186**, 908.
Flaks, J. G., Lichtenstein, J. & Cohen, S. S. (1959). *J. biol. Chem.* **234**, 1507.
Fraenkel-Conrat, H. & Singer, B. (1957). *Biochim. biophys. Acta*, **24**, 540.
Gierer, A. & Schramm, G. (1956). *Nature, Lond.*, **177**, 702.
Griswold, B. L., Humoller, F. L. & McIntyre, A. R. (1951). *Analyt. Chem.* **23**, 192.
Helvig, F. C. & Schmidt, E. C. H. (1945). *Science*, **102**, 31.
Huppert, J. & Sanders, F. K. (1958). *Nature, Lond.*, **182**, 515.
Hurlbert, R. B. & Potter, V. R. (1952). *J. biol. Chem.* **195**, 257.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Jungeblut, C. W. (1958). In *Handbuch der Virusforschung*, vol. 4, pp. 459–562. Ed. by Hallauer, C. & Meyer, K. F. Vienna: Springer Verlag.

Kaltenbach, J. P., Kaltenbach, M. H. & Lyons, W. B. (1958). *Exp. Cell Res.* **15**, 112.
Kamphausen, H. & Morton, R. K. (1956). *Biochem. J.* **63**, 647.
Klein, G. & Klein, E. (1951). *Cancer Res.* **11**, 466.
Kruh, J. & Borsook, H. (1955). *Nature, Lond.*, **175**, 386.
Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
Leone, E. & Scale, E. (1950). *Boll. Soc. ital. Biol. sper.* **26**, 1223.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Martin, E. M., Malec, J., Coote, J. L. & Work, T. S. (1961). *Biochem. J.* **80**, 606.
Mathews, J., Maida, F. T. & Buthala, D. A. (1959). *Exp. Cell Res.* **18**, 596.
Miroff, G., Cornatzer, W. E. & Fischer, R. G. (1957). *J. biol. Chem.* **228**, 255.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Pileri, A. & Ledoux, L. (1957). *Biochim. biophys. Acta*, **26**, 309.
Piper, E. A. & Arnstein, H. R. V. (1956). *Biochem. J.* **64**, 57P.
Reid, E. & Stevens, B. M. (1957). *Biochem. J.* **67**, 262.
Salzman, N. P., Lockart, R. Z. & Sebring, E. D. (1959). *Virology*, **9**, 244.
Sanders, F. K. (1957). *Proc. R. Soc. Med.* **50**, 911.
Sanders, F. K., Huppert, J. & Hoskins, J. M. (1958). *Symp. Soc. exp. Biol.* **12**, 123.
Smith, A. U. (1954). *Proc. R. Soc. Med.* **47**, 57.
Tsuboi, K. K. & Price, T. D. (1959). *Arch. Biochem. Biophys.* **81**, 223.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
Wyatt, G. R. & Cohen, S. S. (1953). *Biochem. J.* **55**, 774.

Biochem. J. (1961) **80**, 597

Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

2. THE ISOLATION, CRYSTALLIZATION AND CHEMICAL CHARACTERIZATION OF MOUSE ENCEPHALOMYOCARDITIS VIRUS

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Encephalomyocarditis virus is one of the large group of animal viruses, the Col SK group, rather closely related to poliomyelitis virus. They are generally pathogenic to rodents and occasional infection occurs in man (Jungeblut, 1958). The strain that has now been characterized was obtained through the courtesy of Dr Kingsley Sanders (cf. Hoskins & Sanders, 1957). It is highly pathogenic to mice but, after growth for many generations in Krebs II ascites-tumour cells (Martin, Malec, Sved & Work, 1961*b*), it was incapable of causing infection in rabbits even when massive doses of concentrated virus were injected intracerebrally.

Since we had studied the effect of infection on the nucleic acid and protein turnover of mouse-ascites-tumour cells, and had found that virus infection caused striking changes in the pattern of nucleic acid turnover (Martin *et al.* 1961*b*), we wished to extend these studies and to determine, if possible, the site of synthesis of virus nucleic acid and virus protein within the host cell. As part of this project it became necessary to characterize the virus more fully, and in the present paper we describe methods for large-scale culture of the virus and for purification and crystallization of the infective particle. The virus is, in the dry state, a spheroid of about 27 mμ diameter. Its nucleic acid and protein have been analysed. A convenient method has also been developed for the preparation of a precipitating antibody of high titre which may be used to flocculate or neutralize the virus. A preliminary report of some of this work has been published (Faulkner, Martin, Sved & Work, 1960).

METHODS

Abbreviations. EMC, Encephalomyocarditis; DEAE, diethylaminoethylcellulose.

Protein estimation. The method of Lowry, Rosebrough, Farr & Randall (1951) was scaled down to a total volume of 0·9 ml. and the extinction of the product was measured at 750 mμ ($E_{1\text{ cm.}}$) against a reagent blank. The method was satisfactory over the range 1–20 μg. of protein. Extinction measurements were made on 0·8 ml. of solution in normal 1 cm. cuvettes fitted with Perspex displacers, as described by Martin & Morton (1956). Since no standard preparation of virus protein was available the method was standardized on host-cell protein prepared as already described (Martin *et al.* 1961*b*).

Total nitrogen. The method of Jacobs (1960) was used. In this method, N is converted into NH_3 and estimated by interaction with ninhydrin. It is sensitive to 1 μg.