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

5. THE KINETICS OF SYNTHESIS OF VIRUS PROTEIN AND OF VIRUS RIBONUCLEIC ACID IN KREBS II MOUSE-ASCITES-TUMOUR CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS*

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Martin & Work (1961) have shown that during the course of a single growth cycle of encephalomyocarditis virus in Krebs II mouse-ascites-tumour cells there is a striking change in the pattern of ribonucleic acid metabolism as compared with the normal uninfected cell. In particular there was a 340% increase in the rate of ribonucleic acid turnover in the mitochondrial fraction coincident in time with the appearance of complete virus in this fraction. However, not more than 5–8% of this ribonucleic acid could be virus nucleic acid.

A simple method has now been developed for the isolation of complete virus from cell homogenates and from culture supernatants. The method depends on adsorption of the virus on sheep red cells and subsequent isolation of the sheep red-cell stroma with virus still attached to it.

By application of this method to virus grown for different times in Krebs cells in the presence of a suitable radioactive tracer it has been possible to follow the complete course of synthesis of virus ribonucleic acid and of virus protein. To do this, it was necessary, however, to measure the specific radioactivity of the precursor pool of amino acid and of ribonucleotide in the host cell. Phenylalanine and uridylic acid were the immediate radioactive precursors of virus protein and of virus nucleic acid and methods were devised to measure the specific radioactivity of the intracellular pools of these compounds and the effects of virus infection thereon. It has thus been shown that the synthesis of virus ribonucleic acid takes place early in the growth cycle and that it precedes the synthesis of virus protein by about 1 hr. The massive synthesis of ribonucleic acid in the cytoplasm of infected Krebs cells late in the growth

* Part 4: Martin & Work (1961).

cycle is not, therefore, a synthesis of virus ribonucleic acid. The results suggest that virus ribonucleic acid may provide the direct stimulus for the synthesis of virus protein.

METHODS

Preparation of host cells. Suspensions of Krebs cells were prepared in Earle's medium as described by Martin, Malec, Sved & Work (1961*b*). Since in kinetic studies it was essential that a standard growth curve should be obtained, any fresh cell preparation with more than 5% of dead cells was discarded.

Infection of cells. The suspension of Krebs cells at a concentration of about 3×10^7 cells/ml. in Earle's solution was kept at room temperature and the standard virus preparation (Martin *et al.* 1961*b*) added dropwise with gentle swirling. As soon as all the virus (5–10 plaque-forming units/cell) had been added the suspension was cooled in ice and kept at 0° (with occasional gentle swirling) for 3 hr. The cells were then centrifuged at about 60*g* for 5 min. at 0° and the supernatant was discarded. The cells were resuspended by adding warm Earle's solution so that the final cell concentration was 2×10^7 /ml. and the final volume 27 ml. in a 250 ml. flask. The temperature of the solution was raised to 37° as quickly as possible and incubation was continued at this temperature for the required time. This process of thermal shock produces a fair degree of synchrony in virus growth, so that the growth curve is steeper and more reproducible than it would otherwise be. The method was suggested to us by Dr F. K. Sanders. Its mechanism is not fully understood. Occasional batches of cells clumped partially after incubation for 4–5 hr.; these were discarded. In some experiments, where the above scale of operation was too large, the infected cells were incubated for 1–1.5 hr. in bulk and then divided into 12 portions of 2.5 ml. each. Incubation was then continued in 25 ml. flasks. In this way variation in behaviour between individual flasks was much less than when small batches of cells were infected individually.

Isolation of virus. According to the design of the experiment, virus had to be isolated at different times during the growth cycle, and the technique of isolation had to be adapted to the physical condition of the virus at the relevant time. Thus when infected cells were incubated for up to 9 hr. much of the virus was intracellular and the cells had to be disrupted before isolation of virus. In other experiments incubation was continued for 14 hr. after infection, by which time all the intracellular virus had been released and the cells could be discarded; virus was then isolated from the supernatant.

When the virus was intracellular the following technique was used. The cells were collected from the incubation medium by centrifuging at 700*g* for 5 min. and resuspended in ice-cold phosphate-buffered saline (Martin *et al.* 1961*b*) to which non-radioactive carrier (about 10 mg.) was added to dilute any isotope used in the experiment. The suspension was recentrifuged at 2300*g* at 0° for 3 min. and the packed-cell volume noted. The cells were resuspended in water (packed-cell volume $\times 5$) and disrupted with the Dounce homogenizer described by Martin, Malec, Coote & Work (1961*a*). When the homogenizer was used carefully it was possible to disrupt all the cells without disrupting more

than a small proportion of the nuclei. The nuclei were removed by centrifuging at 700*g* for 5 min. and the supernatant was diluted with about 3 times its own volume of phosphate-buffered saline again containing non-radioactive carrier. Cytoplasmic particles and virus were then sedimented together by centrifuging in a Spinco preparative centrifuge (100 000*g* for 90 min.). The pellet was resuspended in water (about 3 ml. for each 1 ml. of packed cells originally used) and homogenized vigorously at 0° with the Dounce homogenizer to detach virus from cytoplasmic granules. Sodium chloride soln. (9%, w/v) was added to a final concentration of 0.9% together with further non-radioactive carrier. At this stage the amount of virus present was estimated by the standard haemagglutinin technique (Martin *et al.* 1961*b*). The homogenate was stored overnight in the deep-freeze. This freezing step is essential since it coagulates any insoluble protein present in the homogenate.

Frozen homogenates were thawed and sufficient stock virus (Martin *et al.* 1961*b*) was added to each control (uninfected) homogenate to bring the haemagglutinin titre to the same value as that of the corresponding infected preparation. Sufficient 0.9% sodium chloride was added to each infected preparation to keep the volumes of infected and control preparations the same. All preparations were then centrifuged at 8000*g* for 10 min. at 0° and the pellets were discarded. At this stage the supernatants were only faintly opalescent.

To remove virus selectively from the solution, washed and packed sheep red cells (Martin *et al.* 1961*b*) were added and mixed with the ice-cold supernatants: 0.25 ml. of packed red cells adsorbed 5×10^4 haemagglutinin units of virus almost quantitatively. The mixture was kept at 0° for 30 min. and then centrifuged at 1500*g* for 5 min. The supernatant was discarded and the red-cell pellet resuspended in a few drops of 0.9% sodium chloride. The cells were lysed by adding ice-cold water (2.5 ml. for 0.25 ml. of packed red cells). After 15 min. the cell stroma were collected by centrifuging for 15 min. at 20 000*g*. No release of virus was detected during haemolysis. The stroma with attached virus were resuspended in water (by homogenizing) and perchloric acid was added to a final concentration of 0.3*M*. The precipitate was washed once with perchloric acid and once each with methanol-ether (1:1, v/v), methanol-chloroform (1:1, v/v), ether-chloroform (1:1, v/v) and ether. When the radioactive tracer was [¹⁴C]orotic acid the solid was dissolved in 98% (v/v) formic acid without further treatment and plated on a metal planchet (Martin *et al.* 1961*b*). When radioactive amino acid was used as tracer the defatted stroma residue was extracted with 0.5*M*-perchloric acid at 70°, dissolved in 0.2*N*-sodium hydroxide, mixed with carrier amino acid, reprecipitated with 30% (w/v) trichloroacetic acid, washed in ethanol-ether (3:1, v/v), dried and plated as above.

When the virus was extracellular (14 hr. after infection) it was unnecessary to disrupt the cells and it was sufficient to add non-radioactive carrier (amino acid or orotic acid as appropriate) and to remove the dead cells by centrifuging at 700*g* for 10 min. The cells were washed once with phosphate-buffered saline and the washings added to the main supernatant which was then frozen at -20°. From this stage onward the method was the same as that used for the frozen homogenates, except that unadsorbed virus was titrated in the supernatants from the red-cell adsorption.

This was considered necessary because rather large quantities of virus were being adsorbed. In fact about 10% of the virus did not adsorb. The amount of virus adsorbed was calculated from the difference between the haemagglutinin titre before and after adsorption.

Amino acid pool of Krebs cells. Krebs cells were isolated and washed with phosphate-buffered saline in the usual way (Martin *et al.* 1961*b*), resuspended in Earle's solution and counted. The cells (28×10^8) were diluted with Earle's solution to a final concentration of 2×10^7 cells/ml. and incubated for 30 min. at 37°. The cells were collected by centrifuging at 700g and were resuspended in 40 ml. of water. Trichloroacetic acid (10 ml. of 30%, w/v) was added immediately and the precipitate removed by centrifuging. The precipitate was washed with 5% (w/v) trichloroacetic acid (10 ml.) and the washings were added to the main extract. Trichloroacetic acid was removed by extraction into ether and the aqueous phase was concentrated. The extract was desalted by adsorption on Zeo-Karb 225 (H⁺ form) and elution with ammonia. The ammonia effluent was evaporated to a small volume and then freeze-dried. The amino acid composition of the freeze-dried material was kindly determined for us by Dr S. Jacobs by ion-exchange chromatography (Jacobs, 1956).

Effect of infection on the valine pool of Krebs cells. Infected and control cultures (5×10^8 cells/flask) were subjected to thermal shock as described above and then incubated at 37°. At appropriate times, according to the plan of the experiment (see Results section), [¹⁴C]valine (0.5 µC) was added and incubation continued for another 15 min. The cell suspension was then cooled as rapidly as possible to 0° and centrifuged for 5 min. at 700g. The cells were washed once by resuspension in cold phosphate-buffered saline, recentrifuged and suspended in 10 ml. of water. Trichloroacetic acid was added (2 ml. of 30%, w/v) and the precipitated protein removed. The aqueous supernatant was extracted with ether until free of trichloroacetic acid and a small sample was dried on a planchet for radioactive assay. The remainder of the aqueous solution was concentrated *in vacuo*, dissolved in water (1 ml.) mixed with sodium hydrogen carbonate (30 mg.) and ethanol (2 ml.), and allowed to react with fluorodinitrobenzene (1 drop). The ether-soluble dinitrophenyl-amino acids were fractionated on silica (5 g.) buffered with 2.5 ml. of sodium phosphate (pH 5.7, 0.5M). Provided that the silica had the right physical properties (cf. Askonas, Campbell, Godin & Work, 1955), dinitrophenylvaline could be obtained pure in a single column operation with propan-1-ol-cyclohexane (1:38, v/v) as solvent. Quantitative measurement of the dinitrophenylvaline and measurement of specific radioactivity were made as described by Askonas *et al.* (1955).

Uridine nucleotide pool of infected and uninfected Krebs cells. Cells were isolated, infected and incubated in the usual way. At the appropriate times, [¹⁴C]orotic acid was added (1.25 µC/2.5 ml. of cell suspension). After 30 min. the cell suspension was cooled to 0° and diluted with ice-cold phosphate-buffered saline (4 vol.) containing carrier orotic acid (mM). The cells were collected by centrifuging, washed once by resuspension in cold phosphate-buffered saline and recentrifuged. The acid-soluble nucleotide fraction was extracted and total acid-soluble nucleotide measured photometrically as described by Martin *et al.* (1961*b*). The extract was then heated to 100° for 10 min., cooled and adsorbed on charcoal. The charcoal was washed, dried and

assayed for radioactivity as described by Martin *et al.* (1961*b*). The nucleotides were eluted from charcoal by heating at 50° for 30 min. with aq. 50% ethanol containing 2% (v/v) of aq. ammonia (sp.gr. 0.880) and the extracts were evaporated to dryness. The residue was redissolved in water and 'spotted' on a strip, 2 cm. wide, of Whatman no. 1 paper. The paper was chromatographed in propan-2-ol-aq. ammonia (Markham & Smith, 1952) until the solvent reached the bottom of the paper, and was then dried. This step did not separate the nucleotides and was used only to remove coloured and fluorescent impurities extracted from the charcoal. The paper strip was then used directly for electrophoresis in sodium citrate buffer (pH 3.5) prepared by mixing 3 vol. of M-citric acid with 1 vol. of M-trisodium citrate and diluting 1:25 with water (cf. Burke, Isaacs & Walker, 1957). Electrophoresis was for about 2 hr. at 1 kv and 3-4 ma. Under these conditions uridine monophosphate was separated from the other nucleotides present in the nucleotide pool. The strips were scanned with a Geiger paper-strip scanner and the uridine monophosphate spot was cut out and eluted with 0.1 N-hydrochloric acid. The amount of nucleotide was estimated spectrophotometrically and identity was established both by the use of markers and by measurement of the complete absorption spectrum. A sample was also dried, plated on a metal planchet at infinite thinness and radioactivity was measured in the usual way.

RESULTS

During virus assay by the haemagglutinin technique, encephalomyocarditis virus is adsorbed on the red-cell membrane (cf. Jungeblut, 1958), and it seemed likely that this property could be used to develop a simple method for the isolation and assay of radioactive virus. In trial experiments known amounts of virus were added to Krebs-cell homogenates and then re-isolated by adsorption on red-cell stroma as described in the Methods section. Virtually all the virus was removed from the homogenate by adsorption on red cells at 0° and there was no measurable release of virus from the red-cell stroma during haemolysis. In further preliminary experiments virus was added to an homogenate prepared from highly radioactive Krebs cells and re-isolated on red-cell stroma. The Krebs-cell protein had a radioactivity of about 5000 counts/min./mg. due to [¹⁴C]phenylalanine whereas the re-isolated virus did not contain more than 50 counts/min./mg. when counted under the same conditions. Thus there is little cross-contamination with cell protein.

Previous results (Bellett & Burness, 1960; Martin & Work, 1961) had indicated that encephalomyocarditis-virus RNA was synthesized in the host-cell nucleus but that encephalomyocarditis-virus protein was synthesized in the host-cell cytoplasm. However, the rapid increase in the rate of synthesis of cytoplasmic RNA coincided almost exactly in time with the appearance of infective virus in the cytoplasm (Martin & Work, 1961). We

were therefore uncertain whether all or only a part of the synthesis of virus RNA was intranuclear and we were also uncertain as to whether virus RNA was synthesized before or at the same time as virus protein.

Time course of synthesis of virus ribonucleic acid

A series of Krebs-cell suspensions were infected and incubated in the usual way (Martin *et al.* 1961*b*) with the one modification that virus growth was carefully synchronized by thermal shock (see Methods section). At various times after infection [^{14}C]orotic acid was added, and virus growth allowed to proceed for one complete growth cycle (14 hr.). The virus was then isolated by the red-cell-stroma technique (see Methods section) and its radioactivity measured. Any virus RNA formed before the addition of orotic acid should appear at the end of the growth cycle in the completed virus as unlabelled RNA, and any RNA formed after addition of isotope should be labelled (cf. Darnell, Levintow, Thoren & Hooper, 1961). Over the same growth cycle the rates of production of intracellular virus and of cell death were also measured (Martin *et al.* 1961*b*). Repeated experiments showed that the relationship between synthesis of virus RNA and synthesis of complete virus was reproducible. The results of one such experiment are plotted in Fig. 1. Fig. 1 shows that nearly 65 %

Fig. 1. Course of synthesis of virus ribonucleic acid during a single cycle of virus growth in Krebs II ascites-tumour cells (for method see text). ●, RNA; □, complete virus; △, cell death.

of the virus RNA was synthesized before 10 % of the complete virus was formed, and also that about 60 % of the virus synthesis was completed before there was any significant increase in cell death.

Before such a Figure can be drawn with any degree of confidence, however, it must be established that addition of [^{14}C]orotic acid to the culture medium has resulted in rapid labelling of the uridine nucleotide pool, and, if necessary, corrections must be introduced to allow for changes in the rate of labelling of this pool. In fact such corrections were applied in the preparation of Fig. 1 and the basis of the method of correction which was used is described below.

Martin *et al.* (1961*b*) established that uridylic acid accounts for about 97 % of the radioactivity incorporated into RNA of Krebs cells after addition of [^{14}C]orotic acid to the culture medium. During the course of several experiments such as that described in Fig. 1, cells were collected at either 30 or 45 min. after addition of orotic acid to the incubation medium. The acid-soluble nucleotide pool was extracted into perchloric acid, and the total nucleotide was estimated as described by Martin *et al.* (1961*b*). The nucleotides were then hydrolysed to the monophosphates, adsorbed on charcoal, assayed for radioactivity, eluted from the charcoal and fractionated as already described (see Methods section). The uridine monophosphate was isolated and its specific radioactivity determined. The results of one such experiment are shown in Fig. 2. From 3 hr. onwards, the change in specific activity of the total nucleotide pool follows closely the change in that of the uridine nucleotide pool. It is permissible, therefore, in any experiment with [^{14}C]orotic acid, to measure the specific radioactivity of the total acid-soluble nucleotide and to multiply this figure by a constant factor (4.55) to obtain the mean specific activity of the uridine nucleotide pool.

Theoretically, at least, it would be desirable to have a continuous record of changes in specific activity of the nucleotide pool throughout the entire growth cycle, but this was not practicable. The compromise that we adopted (measurement of the specific activity of the pool at 30 or 45 min. after addition of orotic acid) seems justified by the demonstration by Martin *et al.* (1961*b*) that the specific activity of the nucleotide pool remains almost constant between 30 min. and 2 hr. after addition of isotope. In the preparation of Fig. 1 the specific activity of the nucleotide pool was measured 45 min. after the addition of isotope and the methods by which corrections were applied are set out in Table 1. For this table the specific activity of virus RNA was calculated from the analytical data given by Faulkner, Martin, Sved, Valentine & Work (1961) together with the haemagglutinin values obtained after incubation for 10 hr., since these give the

Fig. 2. Effect of virus infection of Krebs II ascites-tumour cells on the size of the nucleotide pool and on the specific radioactivity of both acid-soluble nucleotides and uridine monophosphate 30 min. after the addition of [^{14}C]orotic acid to the incubation medium (for methods see text). ●, Acid-soluble nucleotides; ○, specific activity of nucleotide pool; △, specific activity of uridine monophosphate.

amount of virus formed during the growth cycle. The results given in Table 1 show that, provided that [^{14}C]orotic acid is added to the incubation mixture before synthesis of virus RNA begins, the virus RNA has about half the specific activity of the uridine nucleotide pool. Theoretically, at least, if the virus RNA were synthesized from the nucleotide pool, it would be expected to have about one-quarter of the specific activity (per mg. atom of phosphorus) of the uridine nucleotide pool. Calculation of the specific activity of the virus RNA (Table 1) depends, however, on the accuracy of the haemagglutinin assay which is about $\pm 50\%$. The ratios of specific activities of virus RNA and uridine nucleotide pools given in Table 1 are therefore entirely consistent with the assumption that virus RNA is synthesized from the nucleotide pool of the host cell and not by any process of degradation and rearrangement of host-cell RNA, which has a much lower specific radioactivity than the nucleotide pool for some considerable time after the addition of [^{14}C]orotic acid (Martin *et al.* 1961*b*).

Time course of synthesis of virus protein

In choosing a suitable radioactive label for the amino acid pool, three factors had to be considered: the size of the intracellular pool which would dilute

Table 1. *Kinetics of synthesis of encephalomyocarditis-virus ribonucleic acid during a single growth cycle in Krebs-ascites-tumour cells*

Cells (6×10^8) were infected and incubated as described (see Methods section). After 1 hr. at 37° the cells were divided into 12 equal portions. At various times from 1.5 hr. onwards a sample (0.2 ml.) was taken from one flask and replaced by a solution of [^{14}C]orotic acid (1.25 μC) in Earle's solution. All flasks were incubated for 14 hr. and virus was then isolated on red-cell stroma as described (see Methods section). A second batch of cells prepared and infected at the same time was divided into 12 lots as above and [^{14}C]orotic acid was added at the same time intervals; 45 min. later the cells were collected, washed and used for isolation of the acid-soluble nucleotide pool as described in the text. Haemagglutinin (HA) units are as defined by Martin *et al.* (1961*b*).

Time after infection (hr.)	Total radioactivity of virus RNA (cell stroma) (μmC)	Specific radioactivity of virus RNA ($\mu\text{C}/\text{mg. atom of P}$)	Specific radioactivity of uridine nucleotide pool ($\mu\text{C}/\text{mg. atom of P}$)	Radioactivity of UMP pool (% of maximum)	Specific radioactivity of virus RNA corrected* for pool changes ($\mu\text{C}/\text{mg. atom of P}$)	Percentage of total virus RNA synthesized	Titre† (HA units)
1.5	1.52	22.9	45.4	63.0	36.3	0	—
2.5	1.52	25.2	48.6	67.5	37.3	0	—
3.0	0.922	28.2	—	—	36.5	2.2	600
3.5	0.866	25.3	65.8	91.3	27.7	24	—
4.0	0.954	18.0	—	—	19.2	48	800
4.5	0.775	17.9	69.6	96.3	18.5	50	3 600
5.0	0.657	12.8	—	—	13.0	65	6 400
5.5	0.290	7.0	71.8	99.6	7.0	82	16 000
6.0	0.239	6.35	—	—	6.4	84	32 000
7.0	0.199	3.2	72.0	100	3.2	93	45 000
8.0	0.071	1.15	68.0	94.7	1.2	98	64 000
10.0	0.016	0.29	40.6	56.4	0.5	100	64 000

* Corrections were taken from a graph drawn from the data of the preceding column. It is assumed that the specific radioactivity of the uridine nucleotide pool measured 45 min. after addition of orotic acid is a true measure of the availability of [^{14}C]uridine monophosphate for virus-RNA synthesis.

† Standard virus titrated with the same batch of red cells had a haemagglutinin titre of 51 200.

the added isotope, the amount of any particular amino acid present in the virus protein and the metabolic stability of each amino acid. The amino acid pool was extracted into trichloroacetic acid and analysed as described (see Methods section). The extract contained a variety of substances besides amino acids, so that there was incomplete resolution between some of the peaks on the ion-exchange column. Table 2 lists the amino acids that could be identified with confidence and the amount of each in the cell extract. From these results and from the data on amino acid composition of the virus (Faulkner *et al.* 1961), [^{14}C]phenylalanine seemed likely to be most effective as a label for virus protein. This was confirmed in preliminary experiments in which, under identical conditions of virus growth and isotope addition, [^{14}C]phenylalanine was at least four times more effective than [^{14}C]valine as a label for virus protein. For this reason all experiments designed to follow the kinetics of virus-protein synthesis were carried out with [^{14}C]phenylalanine.

The plan of each experiment was closely similar to that already used to measure RNA synthesis. A series of Krebs-cell suspensions were infected and incubated in the usual way and at various times after infection [^{14}C]phenylalanine was added. Incubation was continued for one complete growth cycle (14 hr.), after which the virus was isolated by the red-cell-stroma technique and its radioactivity determined. At the same time virus growth was followed by the haemagglutinin method. In another series of flasks with the same batch of cells, [^{14}C]phenylalanine was added as before, but 15 min. later the cells were cooled to 0° and the amino acid pool was extracted as described above. The total radioactivity of the amino acid pool was then measured. The results are set out in Table 3. A second similar experiment gave almost identical results.

In Table 3 the total radioactivity of the amino acid pool is listed rather than the specific radio-

activity of phenylalanine. Several attempts were made to measure the specific activity of phenylalanine, but the amount available was so small that the technical difficulties involved in purification defeated us. We have therefore assumed that the total radioactivity of the amino acid pool is proportional to its specific activity. This assumption was justified by further experiments in which the effects of infection on the valine pool were successfully measured: the valine pool is between three and four times as large as the phenylalanine pool (Table 2). Though this was disadvantageous so far as efficiency of labelling was concerned, the amount of valine was sufficient to permit isolation, purification and determination of specific activity after addition of [^{14}C]valine.

A large batch of cells was prepared in the usual way, divided into two and one lot kept as an uninfected control. Both batches of cells were then divided into four lots and incubated under standard conditions. At suitable intervals [^{14}C]valine was added. After a further 15 min. the cells were collected and washed, and the amino acid pool was extracted in the usual way. Valine was isolated as dinitrophenylvaline (see Methods section). The changes in the valine pool and in the specific activity of valine are shown in Table 4. Neither the size of the pool nor the capacity of the cells to concentrate added [^{14}C]valine varied greatly between 3.5 and 6.5 hr. after infection. By 8.5 hr. after infection, however, the pool size had changed substantially and the cells had also lost some of their ability to concentrate added [^{14}C]valine. The period between 3.5 hr. and 6.5 hr. after infection is the critical period so far as determination of the initiation of protein synthesis is concerned, and it is reasonable to assume that the behaviour of valine can be used as an index of the behaviour of phenylalanine. We are probably justified therefore in using the total radioactivity of the amino acid pool at 15 min. after addition of isotope (Table 3) as a measure of its initial specific activity, at least up to 6.5 hr. after infection. Beyond this time the course of virus-protein synthesis must remain in some doubt.

Alternative method for measurement of kinetics of synthesis of virus protein

In following the kinetics of RNA synthesis, we assumed that the specific radioactivity of the nucleotide pool in the Krebs cell was more-or-less constant between 30 min. and 2 hr. after addition of isotope. This assumption is justified by an observation by Martin *et al.* (1961b) on the specific activity of the uridylic acid pool. A similar assumption was made with respect to phenylalanine during determination of the kinetics of protein synthesis, but this assumption may not be

Table 2. *Amino acids extractable from Krebs-ascites-tumour cells with trichloroacetic acid*

The method of extraction and fractionation is described in the text (see Methods section). The total yield of nitrogen in the extract from 28×10^8 cells was $224 \mu\text{moles}$.

Amino acid	Amount present (μmoles of amino acid N)
Glutamic acid	7.45
Proline	10.25
Glycine	18.35
Alanine	24.63
Valine	1.36
Leucine	0.80
Tyrosine	0.55
Phenylalanine	0.40

entirely correct. The specific radioactivity of the amino acid pool remains constant between 2 and 15 min. after addition of isotope, but Martin *et al.* (1961*b*) have suggested that specific activity just begins to fall by 15 min. after addition of isotope. Synthesis of virus protein requires about 2 hr., however, from start to finish, and the latter stage of the process could be taking place in the presence of a pool of progressively decreasing specific activity. This would result in some flattening of the curve for protein biosynthesis determined by the above method. In view of this uncertainty, an alternative method of measuring the kinetics of protein synthesis was devised. In this method, the infected cells were exposed to a pulse of radioactive amino acid at various stages during the growth cycle and were then allowed to continue virus synthesis for only 30 min. The rate of incorporation of amino

acid into virus protein was thus measured over short segments of the growth cycle and the results were compared with those obtained by the other method.

Preliminary experiments with [^{14}C]valine showed that, although the method was practicable, it required rather large amounts of [^{14}C]valine. [^{14}C]Phenylalanine entered the amino acid pool equally quickly, and owing to the small size of the intracellular pool of phenylalanine it was a more effective label. Experiments were therefore designed in which a pulse of [^{14}C]phenylalanine was added to the suspension of infected Krebs cells at different times during the growth cycle. Instead of virus growth being allowed to go to completion as before (Tables 1 and 3), the host cells were homogenized 30 min. after addition of tracer and the virus which had been formed was isolated by the

Table 3. *Kinetics of synthesis of encephalomyocarditis-virus protein during a single growth cycle in Krebs-ascites-tumour cells*

The conditions of the experiment were essentially the same as in Table 1. [^{14}C]Phenylalanine (0.2 μC) was added to each of seven flasks (2.5 ml. of cell suspension) at the times indicated, and 15 min. later the cells were cooled to 0°, washed and the acid-soluble amino acid pool was extracted as described in the text. To another batch of ten flasks [^{14}C]phenylalanine was also added at the indicated times and virus growth was allowed to continue for one complete cycle (14 hr.). The rate of growth of virus was measured by sampling at intervals. At the end of the incubation complete virus was isolated by the red-cell-stroma technique.

Time after infection (hr.)	Specific radioactivity of virus protein ($\mu\text{C/g.}$)	Specific radioactivity of cell protein ($\mu\text{C/g.}$)	Total radioactivity in amino acid pool (μmC)	Radioactivity of amino acid pool (% of maximum)	Specific radioactivity of virus protein corrected for pool change ($\mu\text{C/g.}$)	Percentage of total virus protein synthesized	Titre* (HA units)
3.0	25.7	2.20	54.5	79	32.5	0	75
4.0	31.3	1.82	62.8	93	33.7	0	100
4.5	32.4	—	—	—	33.3	1.5	250
5.0	32.1	2.06	67.6	100	32.1	5	800
5.5	25.9	—	—	—	26.1	25	1 600
6.0	23.4	1.86	66.1	98	23.9	32	2 400
7.0	13.6	1.65	58.4	78	17.4	53	6 800
8.0	7.72	1.40	44.2	65.5	11.8	71	12 600
9.0	3.60	—	—	—	6.2	90	14 400
10.0	1.50	0.59	33.7	50	3.0	100	14 400

* Standard virus titrated at the same time gave a haemagglutinin value of 12 800.

Table 4. *Effect of infection with encephalomyocarditis virus on the size and specific activity of the free valine pool of Krebs-ascites-tumour cells incubated with [^{14}C]valine*

For conditions of incubation and isolation see Methods section.

Treatment	Time of incubation (hr.)	Total radioactivity in amino acid pool (μmC)	Specific activity of valine pool ($\mu\text{mC}/\mu\text{mole}$)	Size of valine pool (μmole)
Control	3.5	33.6	146	0.230
Control	5.0	38.4	149	0.250
Control	6.5	45.5	164	0.277
Control	8.5	35.3	150	0.235
Infected	3.5	32.7	125	0.261
Infected	5.0	32.2	151	0.213
Infected	6.5	42.3	165	0.256
Infected	8.5	18.1	200	0.091

Table 5. *Change in rate of [^{14}C]phenylalanine incorporation into protein of encephalomyocarditis virus during a single growth cycle*

A single batch of cells was divided into two lots and one lot infected. After synchronization, each lot of cells was divided into six equal portions (4×10^8 cells) and, after incubation at 37° for the indicated time, [^{14}C]phenylalanine ($2\mu\text{C}$) was added. After a further 30 min. the cells were cooled to 0° , washed and homogenized. Controls and infected cells were treated as described (see Methods section) and the radioactivity of each control was subtracted from that of the corresponding infected preparation.

Time of addition of [^{14}C]phenylalanine (hr.)	Virus titre at time of addition of tracer (% of final titre)	Radioactivity incorporation into virus in 30 min. (μC)
3	1	0.07
4	12	0.95
5	50	1.12
6	75	0.86
7	90	0.65
8	90	0.19
9	100	0.05

red-cell-stroma technique. The amount of isotope present in the virus was then a measure of the rate of virus-protein synthesis over a 30 min. period. The results obtained are given in Table 5.

DISCUSSION

In determining the time of synthesis of virus components, account must be taken of the variability of the growth curve in successive experiments. This can be most readily achieved by plotting the percentage of final haemagglutinin titre against time and then moving the haemagglutinin percentage values along the time axis until the 50% values for successive experiments are coincident. When this is done with the results given in Tables 1 and 3 it becomes evident that virus-RNA synthesis precedes virus-protein synthesis by about 1.5 hr. (Fig. 3). There appear to be two possible sources of error in this estimate. One, mentioned above, is the doubt about the constancy of specific activity of the amino acid pool, but this has been cross-checked by the alternative type of experiment described in Table 5. The addition of phenylalanine at various times throughout a complete growth cycle (Table 3) gives a protein-synthesis curve which is almost coincident with the haemagglutinin curve. This implies that the rate of synthesis of virus protein was likely to reach its maximum when approximately half the virus had been formed within the cell. The results from Table 5 have also been plotted in Fig. 3, again with adjustment of the haemagglutinin-production curve so that its 50% value coincides with the

Fig. 3. Course of virus-ribonucleic acid synthesis compared with the course of virus-protein synthesis and with the production of complete virus. Virus-protein synthesis was measured both as a percentage of total and as rate of synthesis over 30 min. periods (results from Tables 1, 3 and 5). Δ , RNA (%); $\square$, protein (%); $\bullet$, haemagglutinin (%); $\circ$, rate of synthesis of protein.

other two growth curves. Fig. 3 shows that the rate of virus-protein synthesis rises to a maximum at about the expected time. It seems fairly certain therefore that both methods give a true indication of the time of protein synthesis. Haemagglutinin can be equated with complete infective virus (Faulkner *et al.* 1961) and we deduce that protein is incorporated into virus almost as soon as it is formed.

The second possible source of error arises from the fact that, although [^{14}C]amino acids equilibrate with the intracellular pool in a few minutes, the rate of equilibration of [^{14}C]orotic acid with the uridine nucleotide pool is appreciably slower (Martin *et al.* 1961b). We have shown, however, that equilibration is approximately complete in 30 min., so that the time curve for synthesis of RNA cannot be in error by more than 30 min. This still leaves a gap of about 1 hr. between the times of synthesis of virus RNA and of virus protein.

This separation in time between the synthesis of virus RNA and virus protein supports the conclusion of Sanders (1960) that virus-RNA synthesis is largely completed before the appearance of infective particles begins. It is surprising therefore to find that in HeLa cells infected with the closely related poliomyelitis virus there appears to be no time interval between synthesis of virus RNA and of virus protein (Darnell *et al.* 1961).

The present results and our own earlier investigations (Faulkner *et al.* 1961; Martin & Work, 1961) correlate well with the results of Sanders and his colleagues (Sanders, 1960; Bellett & Burness, 1960). From the two groups of results taken together it is now possible to present at least a rough outline of the biochemical events involved in replication of encephalomyocarditis virus. It appears that the infecting virus enters the host cell during the eclipse phase and that at least the RNA of the virus enters the host-cell nucleus. It there catalyses the formation of new virus-specific RNA and for this synthesis it draws upon the acid-soluble nucleotide pool of the host cell. In all probability the synthesis of this new RNA in any one cell occurs rather rapidly (cf. Barski & Kishida, 1961), and the slope of the curve for RNA synthesis reflects variability in the competence of different cells in a population of cells that are, when collected, in various stages of their growth cycle.

After some degree of accumulation of virus RNA in the host-cell nucleus (Bellett & Burness, 1960; Martin & Work, 1961) there is a rather sudden change in the permeability of the nuclear membrane (Barski & Kishida, 1961) with release of virus RNA into the cytoplasm. The initiation of synthesis of virus protein in the cytoplasm is probably geared to this release of virus RNA from the nucleus, and the implication is that the virus RNA acts as messenger or template for the synthesis of virus protein. Presumably the number of available sites for adsorption of messenger on ribosomes is strictly limited. Thus any excess of virus RNA would remain free in the cytoplasm until sufficient virus protein was produced to combine with it and form complete virus. If this interpretation is correct, it should be possible to induce the synthesis of virus protein by the addition of excess of infective RNA to a cell-free protein-synthesizing system such as that described by Martin *et al.* (1961a).

The effect of nuclear degeneration is even more profound than is indicated by measuring virus-RNA and virus-protein synthesis since, more or less coincident with the production of virus protein, there is a dramatic increase in RNA synthesis in the cytoplasm. This new cytoplasmic RNA is apparently not virus RNA, since virus-RNA synthesis is complete at about 6 hr. whereas the rate of cytoplasmic-RNA synthesis is rising rapidly at this time (Martin & Work, 1961).

SUMMARY

1. The size and specific radioactivity of the nucleotide pool of Krebs II ascites cells has been measured 30 min. after addition of [6-¹⁴C]orotic acid to a suspension of cells in Earle's medium. The effect of infection with encephalomyocarditis virus on both pool size and specific activity was also measured.
2. The amino acid pool of normal Krebs cells was analysed. The effect of virus infection on the pool size and on the entry of [¹⁴C]valine into the pool was measured.
3. The rate of synthesis of virus protein in Krebs cells was measured over 30 min. periods of the virus growth cycle.
4. The relationship between time of synthesis of virus ribonucleic acid and virus protein was determined. Virus ribonucleic acid was found to be synthesized about 1 hr. before virus protein.
5. The formation of complete virus followed almost immediately after the synthesis of virus protein.

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