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

4. THE LOCALIZATION OF METABOLIC CHANGES WITHIN SUBCELLULAR FRACTIONS OF KREBS II MOUSE-ASCITES-TUMOUR CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS*

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Martin, Malec, Sved & Work (1961*b*) showed that, during a single growth cycle, infection with encephalomyocarditis virus caused no changes in the total deoxyribonucleic acid, ribonucleic acid or protein of the host ascites-tumour cell. However, by the use of [6-¹⁴C]orotic acid and [¹⁴C]valine, it was shown that there were substantial changes in the rates of turnover of ribonucleic acid and protein at different times during the cycle of virus growth. In particular, about 5 hr. after infection there was a striking increase in the rate of turnover of ribonucleic acid, and this increase coincided in time with the appearance of new virus. However, this stimulation in turnover appeared to be quantitatively greater than could be accounted for by formation of new virus ribonucleic acid.

Methods have now been developed for the well-defined separation of disrupted Krebs II ascites-tumour cells into nuclei, mitochondria, microsomes and cell sap (Martin, Malec, Coote & Work, 1961*a*). By the use of these methods we have been able to investigate the effect of virus infection on the turnover of the ribonucleic acid and protein of the subcellular components of the host cell, and thus to localize more exactly the sites of metabolic change within the infected cell. This paper describes the result of such a study.

METHODS

The origin and propagation of both the Krebs II mouse-ascites-tumour cells and the encephalomyocarditis virus have been described by Martin *et al.* (1961*b*).

Conditions of infection. Sufficient virus was added to suspensions of washed ascites-tumour cells in Earle's medium ($2-3 \times 10^7$ cells/ml.) to infect all cells (approx. 3 plaque-forming units/cell), and the cells were dispensed in 10 ml. portions into incubation flasks. The virus was allowed to adsorb for 30 min. at room temperature, followed by 15 min. at 36°. Suspensions of control (uninfected) cells were treated similarly. The cells were then incubated at 36° as described by Martin *et al.* (1961*b*).

For the study of protein and nucleic acid turnover, ¹⁴C-labelled precursors were added at appropriate intervals during the virus growth cycle to flasks containing infected and control cells; 30 min. later the flasks were removed and diluted with an ice-cold solution of the unlabelled precursor in phosphate-buffered saline (Martin *et al.* 1961*b*), and the cells sedimented by centrifuging at 120*g* for 5 min. They were then washed twice with buffered saline and stored in an ice bath until required for disruption.

Disruption of Krebs II cells and fractionation of subcellular components. Cells were prepared for disruption by washing with calcium- and magnesium-free buffered saline (Martin *et al.* 1961*b*), then with 0.125 M-sucrose-0.075 M-KCl solution. The cells were disrupted by a combination of double osmotic shock and Potter homogenizer, as described by Martin *et al.* (1961*a*). Nuclei, mitochondria, microsomes and cell sap were separated from the tumour-cell homogenates by differential centrifuging in 0.25 M-sucrose-0.1 M-KCl solution (Martin *et al.* 1961*a*). Preparations of isolated nuclei were usually examined microscopically after staining with nigrosin, and were always found to contain less than 3% of whole-cell contamination. The intermediate fraction that separated between nuclei and mitochondria, and which represented only a very small percentage of the cell contents, was discarded. Mitochondria were washed once with sucrose-KCl solution, but the microsomal fraction was not further treated after isolation except to rinse the surface of the pellet with distilled water.

Estimation of virus. Virus was estimated in tumour-cell homogenates and in the subcellular fractions derived from them by both haemagglutinin titration and plaque assay, as described by Martin *et al.* (1961*b*). To release any adsorbed virus, the nuclear fractions were incubated at 32° for 1 hr. with deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; 0.1 mg./ml.) before virus assay.

Estimation of protein and nucleic acids. Protein, RNA and DNA were isolated from tumour-cell homogenates, mitochondria, microsomes and cell sap and estimated as described by Martin *et al.* (1961*b*). The extraction methods were not considered ideal for the isolation and estimation of RNA from the nuclear fraction. Therefore the nuclei, free of lipids and material soluble in cold 0.2 N-HClO₄, were treated with 0.3 N-NaOH at 37° for 18 hr. to hydrolyse RNA, and protein and DNA were precipitated by addition of HClO₄. The DNA was removed from the acid-insoluble precipitate by extraction with 0.5 N-HClO₄ at 70° for 30 min. or, when required for the estimation of specific

* Part 3: Martin, Malec, Coote & Work (1961*a*).

Table 1. *Specific radioactivities of Krebs cell protein and nucleic acids after incubation with [^{14}C]valine and [$6\text{-}^{14}\text{C}$]orotic acid and a mixture of both labelled compounds*

Flasks containing 10^8 ascites-tumour cells in 5 ml. of Earle's medium and the indicated ^{14}C -labelled compounds were incubated for 30 min. at 36° (Martin, Malec, Sved & Work, 1961*b*). Total nucleic acid and protein extracts were prepared and their radioactivities determined, as described in the text.

^{14}C -compound added	Time of incubation (min.)	Specific radioactivities		Ratio of specific activities: Nucleic acid: Protein
		Protein ($\mu\text{mc/g.}$)	Nucleic acids ($\mu\text{C/mole of nucleic acid P}$)	
[^{14}C]Valine (0.1 μC)	30	276	27	2.59
[$6\text{-}^{14}\text{C}$]Orotic acid (5 μC)	30	3	714	
[^{14}C]Valine plus [$6\text{-}^{14}\text{C}$]orotic acid	30	266	665	2.50
[^{14}C]Valine plus [$6\text{-}^{14}\text{C}$]orotic acid	0	3	2	—

radioactivity, by extraction with 10% (w/v) NaCl solution (buffered at pH 6.0 with 0.1 M-sodium acetate). DNA was recovered from the hot saline extract by precipitation with 2.5 vol. of ethanol.

Measurement of radioactivity. Since the amount of work involved in cell disruption, in fractionation of the subcellular particles and in the separation of RNA and protein is rather considerable, it was thought best to study the incorporation of radioactive precursors (in this case [$6\text{-}^{14}\text{C}$]orotic acid and [^{14}C]valine) into both RNA and protein simultaneously. Before this could be done, however, it was necessary to show that cross-contamination of nucleic acid with protein, or vice versa, would not cause any significant error in the estimation of specific radioactivity. Ascites-tumour cells were therefore incubated in media containing either [$6\text{-}^{14}\text{C}$]orotic acid or [^{14}C]valine or a mixture of both labelled compounds. After incubation under the standard conditions, the appropriate ^{14}C carrier was added, and the protein and nucleic acid were assayed for radioactivity by the following method. The cells were resuspended in 0.1 M-DL-valine solution, HClO_4 was added to a concentration of 0.2 M, and the acid-insoluble material treated with an aqueous solution of trimethylamine (0.5 M) containing DL-valine (0.07 M) for 20 min. at 55° . The material did not completely dissolve, but the suspension became translucent. The suspension was cooled to 0° , HClO_4 added to a concentration of 0.2 M, and the precipitated material centrifuged off. The supernatant was examined for the presence of extracted nucleotides. Only about 3% of the total RNA was extracted during the trimethylamine treatment. The precipitate was washed twice with ice-cold 0.2 M- HClO_4 . Total nucleic acids and protein were then extracted, and their specific radioactivities determined as described by Martin *et al.* (1961*b*).

From the results of this experiment (Table 1) it was concluded that there was no contamination of protein by the labelled orotic acid, but that slight contamination (about 4%) of the nucleic acid with labelled valine may occur. In a further experiment, Krebs cells were incubated for 30 min. with a fixed amount of [$6\text{-}^{14}\text{C}$]orotic acid (5 μC /flask containing 10^8 cells in 5 ml. of medium) and various amounts of [^{14}C]valine (0.02–0.2 μC /flask). When the radioactivity of the nucleic acid and protein was measured it was found that the slight contamination of the nucleic acid extract with valine was directly proportional to the specific activity of the protein fraction. It was thus possible to apply a suitable correction factor to the measured specific activity of nucleic acid to account for any con-

taminating radioactive amino acid present in the extract. In experiments where both labelled precursors were used simultaneously, two control incubations were therefore included:

(1) A flask in which the cells were incubated for the full period with [^{14}C]valine, then [$6\text{-}^{14}\text{C}$]orotic acid added just before the carrier. The radioactivity of the nucleic acids from these cells gave a measure of valine contamination, and the extent of this contamination was assumed to be proportional to the specific radioactivity of the protein fraction.

(2) A flask in which cells were incubated with [$6\text{-}^{14}\text{C}$]orotic acid for the full period, then [^{14}C]valine added just before the carrier. The specific radioactivity of the protein from these cells was used as the zero-time control for estimates of protein radioactivity.

EXPERIMENTAL AND RESULTS

Effect of infection on incorporation of precursors into the protein and nucleic acids of subcellular components from tumour cells. A series of infected and control cultures of Krebs II ascites-tumour cells were incubated in Earle's medium under identical conditions. Exactly 30 min. before removing each flask from the incubation chamber, a mixture of [^{14}C]valine and [$6\text{-}^{14}\text{C}$]orotic acid was added. Flasks were removed at hourly intervals throughout the major portion of the virus growth cycle, ^{14}C carrier was added, and the cells were cooled at 0° , washed and disrupted by the double-osmotic-shock method (Martin *et al.* 1961*a*). Portions of the whole lysate were saved and the remainder was subjected to differential centrifuging in 0.25 M-sucrose–0.1 M-KCl. Protein and RNA were isolated from the nuclear, mitochondrial, microsomal and cell-sap fractions, as described under Methods, and their specific radioactivities were determined. Specific radioactivity measurements were also made on DNA from the nuclei. The results are summarized in Table 2. Difficulty was experienced in obtaining a representative sample of the nuclear protein by the method used (cf. Martin *et al.* 1961*a*), and no figures for the turnover rate of protein in the nuclear fraction are

given. As each subcellular fraction was isolated from the infected cells, part was set aside and its virus content assayed by the haemagglutination technique. The results of these assays are also given in Table 2.

Haemagglutinin is to a great extent concentrated in the mitochondrial fraction and appears in the microsomal fraction only in smaller amounts (Tables 2 and 4). At no time during the infection cycle were significant amounts of haemagglutinin found in the cell-sap fraction, and the trace of haemagglutinin found in the nuclear fraction was in part due to interference with the haemagglutinin reaction at low dilutions by DNA from the nucleus and in part to slight contamination with mitochondria.

The percentage of virus in the mitochondrial fraction of homogenates prepared by osmotic shock varied in successive experiments (67% of total in the experiment reported in Table 2, and 95% in the experiment of Table 4), but this probably reflects the impossibility of exact duplication of any method of cell rupture rather than biological variation.

The association of virus with the mitochondria was unexpected. It is certainly not caused by the sedimentation characteristics of the viral particle, since virus added to the cell homogenate before fractionation appears almost exclusively in the microsomal fraction after separation by differential centrifuging. As a further check, we have disrupted Krebs cells by an alternative method

Table 2. *Effect of encephalomyocarditis-virus infection on the incorporation of [14 C]valine and [6- 14 C]orotic acid into the proteins and nucleic acids of ascites-tumour subcellular fractions*

Portions (10 ml.) of control and infected (3 plaque-forming units of virus/cell) suspensions of ascites-tumour cells (3×10^7 cells/ml.) were incubated at 36° for the various times indicated under conditions described by Martin *et al.* (1961b). No attempt was made at synchronizing the infectious process. [14 C]Valine (0.5 μ c) and [6- 14 C]orotic acid (25 μ c) were then added as a solution in 1.0 ml. of buffered saline, and the flasks incubated for a further 30 min. The cells were washed, disrupted by osmotic shock and separated into the various subcellular fractions. Portions of each fraction were diluted with distilled water, stored overnight at 4° and assayed for haemagglutinating activity. Protein, RNA and DNA were isolated from the remaining portions and their specific radioactivities determined. All estimates of RNA specific activity have been corrected for contamination with valine, and of protein specific activity for contamination with orotic acid. The virus haemagglutinin titres are given in total haemagglutinin units/flask (3×10^8 cells), see Martin *et al.* (1961b).

Flask		Time after inoculation with virus (hr.)					
		1	2	3	4	5	6
Nuclear fraction							
RNA (μ c/m-mole of RNA P)	Control	55.8	31.4	32.1	30.1	30.3	31.7
	Infected	45.3	15.9	12.2	5.6	7.8	3.2
DNA (μ c/m-mole of DNA P)	Control	0.100	0.088	0.073	0.074	0.066	0.054
	Infected	0.108	0.083	0.058	0.043	0.042	0.019
Virus haemagglutinin titre	Infected	200	200	200	200	250	2050
Mitochondrial fraction							
Protein (μ c/g.)	Control	1.33	1.08	0.93	0.82	0.72	0.66
	Infected	1.48	1.01	0.91	0.57	0.46	0.65
RNA (μ c/m-mole of RNA P)	Control	1.22	0.910	0.775	0.595	0.565	0.610
	Infected	1.04	0.780	0.620	0.665	0.655	1.98
Virus haemagglutinin titre	Infected	0	0	0	1100	1100	36000
Microsomal fraction							
Protein (μ c/g.)	Control	1.16	1.04	1.03	0.99	0.99	0.76
	Infected	1.24	1.08	0.84	0.73	0.52	0.47
RNA (μ c/m-mole of RNA P)	Control	0.487	0.315	0.308	0.310	0.324	0.323
	Infected	0.448	0.206	0.140	0.165	0.189	0.545
Virus haemagglutinin titre	Infected	0	0	0	35	70	18000
Cell-sap fraction							
Protein (μ c/g.)	Control	0.84	0.68	0.62	0.49	0.53	0.43
	Infected	0.93	0.79	0.66	0.43	0.30	0.24
RNA (μ c/m-mole of RNA P)	Control	3.19	2.55	1.74	1.62	1.53	1.92
	Infected	2.68	1.98	1.07	1.22	1.38	2.05
Virus haemagglutinin titre	Infected	0	0	0	0	0	40

(Dounce homogenizer in 10 mM-MgCl₂; see Martin *et al.* 1961*a*), and again found most of the haemagglutinin in the mitochondrial fraction. Bellett & Burness (1960) have also reported the concentration of haemagglutinin in the mitochondrial fraction of Krebs II cells 4.5–6 hr. after infection with encephalomyocarditis virus.

When infected cells were disrupted by ultrasonic vibrations as described by Martin *et al.* (1961*a*), the virus was found largely in the microsomal fraction. In keeping with this observation it was found that virus could be released from mitochondria by treatment with ultrasonic vibrations.

When these experiments were designed it was assumed that haemagglutinin could be equated with virus. This assumption is supported by our demonstration (Faulkner, Martin, Sved, Valentine & Work, 1961) that the ratio of haemagglutinin to infectivity is the same in crystalline encephalomyocarditis virus as it is in crude virus preparation. This view is further strengthened by the results shown in Table 4, which indicate that haemagglutinating activity is associated with infective virus both in time of appearance during the growth cycle and in the site within the cell of maximum virus concentration. More precise measurements (E. M. Martin & T. S. Work, unpublished work) show that a slight delay occurs between the formation of viral protein and the appearance of an equivalent haemagglutinating activity, and this again suggests that haemagglutinating activity is a measure of the whole infective virus particle rather than of partially completed forms.

There are large differences in the rates of incorporation of orotic acid and valine into the different subcellular fractions of normal (control) Krebs II cells (Table 2). The rates of valine incorporation into the proteins of nuclei, mitochondria, microsomes and cell sap are in the proportions 0.28:1.0:1.2:0.67, whereas the rates of orotic acid incorporation into the ribonucleic acids of the same fractions are in the proportions 50:1.0:0.5:3.0. By using the data of Martin *et al.* (1961*a*) for the distribution of protein and RNA among these fractions, it can be calculated that turnover of RNA in the nucleus accounts for 88 % of the total cell RNA turnover, whereas mitochondria, microsomes and cell sap contribute 24, 33 and 39 % respectively to the total turnover of protein.

Infection with encephalomyocarditis virus caused an initial slight stimulation of valine incorporation into the proteins of all fractions. This stimulation was observed with whole-cell preparations (Martin *et al.* 1961*b*). The effect was most marked in the cell sap, thus supporting the suggestion that this initial stimulation in protein synthesis may represent the synthesis of new enzymes necessary for viral replication.

There then follows a period of general inhibition of protein turnover. The inhibition continued progressively during the course of the experiment in all fractions except the mitochondria, which showed marked increase in turnover rate from 5 hr. after infection. It is reasonable to suppose that this stimulation of protein turnover is associated with the appearance of virus in this fraction, and it is possible that it represents the incorporation of valine into the viral protein, as 6–6.5 hr. after infection is the period of maximal viral-protein synthesis under the growth conditions used in the present series of experiments.

Quantitative changes in ribonucleic acid in nuclei and mitochondria during virus infection

Martin *et al.* (1961*b*) showed that there was no significant change in the overall composition of infected Krebs cells as compared with normal controls. The marked fall in RNA turnover within nuclei of infected cells and the threefold increase in rate of turnover of RNA in the mitochondria of infected cells towards the end of the infectious cycle prompted the thought that there might have been quite substantial changes in the overall composition of subcellular fractions, but that, by chance, these had balanced one another and so produced the apparent overall constancy of composition observed earlier (Martin *et al.* 1961*b*). This view was strengthened by the results of the experiment described in Table 2, when it was observed that the net recovery of RNA from the mitochondrial fraction of cells in the later stages of infection was far higher than their corresponding controls, whereas the RNA to DNA ratio in infected nuclei appeared to fall. Accordingly, an experiment was set up to settle this point.

Krebs cells were harvested, washed and suspended in Earle's medium in the usual way (Martin *et al.* 1961*b*). The cell suspension was infected with encephalomyocarditis virus. All flasks were left at room temperature for 30 min. and then incubated at 36° under the standard conditions; one control and one infected flask were removed at 2 hr., another pair at 4 hr. and the last pair at 6.5 hr. The cells were collected, washed and disrupted by the double-osmotic-shock method (Martin *et al.* 1961*a*). The nuclear and mitochondrial fractions were isolated in the usual way, but the microsome fraction was not separated from the cell sap. The nuclear fractions were analysed for RNA and for DNA (see Methods), and the mitochondrial fractions were analysed for protein and for RNA (Table 3). In addition, the cytoplasmic fractions were assayed both for haemagglutinin and for viable virus (plaque count) (Table 4). The results of this experiment show that infection produces a substantial (39 %) increase in the amount of RNA

Table 3. *Effect of encephalomyocarditis-virus infection on the net amounts of nuclear and mitochondrial ribonucleic acids of Krebs II cells*

Suspensions of ascites-tumour cells (2×10^7 cells/ml.; 10 ml./flask) were incubated with encephalomyocarditis virus (3 plaque-forming units of virus/cell) for 2, 4 and 6.5 hr., together with uninfected controls. The flasks were removed, and the cells washed and disrupted by the double-osmotic-shock method. The lysates were fractionated to yield nuclear, mitochondrial and microsomal-plus-cell-sap fractions (Martin, Malec, Coote & Work, 1961a). The mitochondrial fraction was analysed for protein and RNA content, and DNA and RNA estimates were carried out on the nuclear fraction. All results have been calculated as the total amount of each constituent for 10^6 cells, assuming the distribution of protein and DNA to be that given in Table 5 [Martin *et al.* (1961a)]. The total nuclear-plus-cytoplasmic RNA content/ 10^6 cells was 230 μ g. of RNA phosphorus.

Cell fraction	Flask	Period of infection (hr.)		
		2	4	6.5
		RNA P (μ g./ 10^6 cells)		
Mitochondria	Infected	20.6	25.7	30.8
	Control	22.5	20.7	22.2
	Difference	-1.9	+5.0	+8.6
Nuclei	Infected	25.8	24.3	27.9
	Control	25.1	29.8	32.4
	Difference	+0.7	-5.5	-4.5
Net change in RNA P (% of total cell RNA P)		-0.5	-0.2	+1.8

Table 4. *Distribution of viral haemagglutinin and infective virus among cytoplasmic fractions from infected Krebs II ascites-tumour cells*

Portions of the mitochondrial and mixed microsomal-plus-cell-sap fractions from the experiment described in Table 3 were examined for their virus content by haemagglutinin titration and infective particle (plaque) count assay. Results are expressed as haemagglutinin units or plaque-forming units/ 10^6 cells.

Cell fraction	Virus assay method	Period of infection (hr.)		
		2	4	6.5
Mitochondria	Haemagglutinin	0	150	12300
	$10^{-6} \times$ Infective particles	1.75	15.5	650
Microsomes plus cell sap	Haemagglutinin	12	12	675
	$10^{-6} \times$ Infective particles	0.4	1.0	22

in the mitochondrial fraction 6.5 hr. after infection and that this is almost balanced by a corresponding decrease in the amount of nuclear RNA so that the overall change is negligible (1.8%). In a second similar experiment the percentage increase in mitochondrial RNA in infected cells at 6 hr. was even greater than that shown in Table 3.

DISCUSSION

Infection caused profound alterations in the RNA metabolism of the tumour cell. Since there was a slow fall in the rate of metabolism of the control cells throughout the course of the incubation, the results for the infected cells obtained in the experiment described in Table 2 have been expressed as a percentage of the corresponding control and plotted in Fig. 1, together with the figures for virus haemagglutinin titre in the mitochondrial fraction. It is evident that coincident with the appearance of virus in the mitochondrial

fraction there is an enormous increase (320%) in the rate of orotic acid incorporation into the RNA of this fraction. At the same time there is a similar, though smaller, increase in the rate of turnover of RNA in the microsomal fraction. A large increase in total cell RNA turnover at about the time of synthesis of complete virus was reported by Martin *et al.* (1961b). The present results indicate that this is accounted for largely by the increase in turnover of the RNA of the mitochondrial and microsomal fractions.

It has been proposed (Martin & Work, 1961) that the synthesis of RNA in ascites-tumour cells takes place entirely in the nucleus. This contention is supported by the results given in Table 2, which show a constant relationship between the rates of orotic acid incorporation into the ribonucleic acids of the nuclear and cytoplasmic fractions, in both normal cells and cells up to 4 hr. after infection with encephalomyocarditis virus. However, in cells infected for a greater period, the rate of cyto-

plasmic RNA synthesis, calculated from the ratios of specific activities of the uridylic acid in RNA and the acid-soluble pool (Martin *et al.* 1961*b*), far exceeds that which would be expected from the nuclear RNA turnover rate, assuming that the nucleo-cytoplasmic relationship had remained unaltered. Therefore it may be argued that this difference represents the synthesis of viral RNA. However, when the amount of this anomalous, newly synthesized cytoplasmic RNA is compared with the amount of virus-associated RNA expected to be formed during the same 30 min. period, as estimated from the increase in haemagglutinin titre by the data of Faulkner *et al.* (1961), the virus-associated RNA represented only 5–8% of the total RNA formed. Hence, the stimulation in mitochondrial and microsomal RNA turnover in the later stages of infection cannot be accounted for in terms of synthesis of virus-associated RNA.

From the beginning of infection the rate of precursor incorporation into the RNA of the nucleus was progressively inhibited (Fig. 1). As nearly 90% of the total cell RNA turnover takes place in the nucleus, and as it is probable that most or all of the cell's RNA is synthesized at this site, it is likely that the marked inhibition of RNA turnover in the whole cell (Martin *et al.* 1961*b*) and

the slight inhibition of turnover in the cytoplasmic constituents in the eclipse phase (Table 2; Fig. 1) can be ascribed to the effect of infection on nuclear RNA synthesis. Although this inhibition is doubtless a consequence of viral infection, there is little evidence to suggest that it is concerned with the replication of viral constituents.

Sanders, Huppert & Hoskins (1958) and Sanders (1960) have shown that the ability of encephalomyocarditis virus to kill Krebs II cells is independent of its power to multiply within them, and a similar separation between cell-killing properties and viral replication has also been observed in HeLa cells infected with poliomyelitis virus (Ackermann, Rabson & Kurtz, 1954). Hence it is possible that the inhibition of nuclear RNA synthesis is associated with the process, caused by infection but unrelated to the replication of viral RNA or protein, which leads to the death of the cell.

Huppert & Sanders (1958) showed that infective RNA could be extracted by cold aqueous phenol from Krebs II ascites-tumour cells that had been infected with encephalomyocarditis virus, although no RNA could be obtained from the virus particles by this treatment. Bellett & Burness (1960) have used the osmotic-shock method of Martin *et al.* (1961*a*) to localize the formation of infective RNA, and found it to be almost entirely confined to the nucleus during the first 4.5 hr. after infection, thus giving a direct demonstration that the nucleus is the site of infective RNA synthesis in this system.

Bellett & Burness (1960) found that the titre of infective RNA in the nucleus decreased sharply after 4.5 hr., and that this loss was accompanied by a rise in the infectivity of the RNA from the mitochondrial fraction. These results suggested a nucleo-cytoplasmic transfer of the viral RNA. The present results (Table 3) strongly support the idea of a transfer of RNA from nucleus to cytoplasm, but the amount of RNA transferred is much greater than would be required for the formation of virus particles. It may well be, however, that the cell produces considerably more virus-specific RNA than it can incorporate into virus. The appearance of free infective RNA in the culture medium at the end of the virus growth cycle suggests that this does in fact occur (Huppert & Sanders, 1958).

Although the present results demonstrate unequivocally that virus-protein synthesis takes place in the cytoplasm of the Krebs tumour cell, they are less definitive with regard to the site of RNA synthesis. We have emphasized elsewhere (Work, 1960; Martin & Work, 1961) that biological replication always requires the simultaneous presence of DNA and RNA and that neither DNA

Fig. 1. Effect of viral infection on rate of [6-¹⁴C]orotic acid incorporation into the RNA of nuclei, mitochondria and microsomes. Estimates of specific radioactivities of the RNA from the nuclei, mitochondria and microsomes of virus-infected Krebs II cells have been plotted as a ratio of the estimates of specific radioactivity of control uninfected cells. Experimental details are given in Table 2. ●, Nuclear RNA; ○, mitochondrial RNA; △, microsomal RNA; □, haemagglutinin titre (units/10⁸ cells) of the mitochondrial fraction.

nor RNA virus is capable of replication except in an environment that can supply the missing components. Such a requirement could well explain the apparent formation of infective RNA within the nucleus of the ascites-tumour cell and the transfer of this to the cytoplasm before synthesis of complete virus can begin.

SUMMARY

1. The effect of infection with encephalomyocarditis virus on the rate of incorporation of ^{14}C -labelled precursors into the protein and ribonucleic acid of the subcellular components of Krebs II ascites-tumour cells has been investigated.

2. The nucleus, which was the major site of ribonucleic acid synthesis in the cell, contained negligible amounts of virus. Infection caused a marked progressive inhibition of orotic acid incorporation into nuclear ribonucleic acid, and also some net loss of ribonucleic acid from the nucleus. It is suggested that this disruption of nuclear ribonucleic acid metabolism is related to the cell-killing properties of the virus.

3. Most of the virus sedimented with the mitochondrial fraction. The amount of mitochondrial ribonucleic acid increased progressively during infection by an amount approximately equivalent to that lost from the nucleus. Incorporation of orotic acid into mitochondrial ribonucleic acid was slightly inhibited for the first 3 hr. after infection; thereafter it was stimulated, reaching 320% of the control at 6 hr.

4. Less virus appeared in the microsomal fraction. The pattern of incorporation into microsomal

ribonucleic acid was similar to that of the mitochondria, but less pronounced. In both fractions, the increase in incorporation rate was apparently related to the amount of virus present, but estimates showed that only 5–8% of this newly synthesized ribonucleic acid could be ascribed to viral ribonucleic acid formation.

5. In all cytoplasmic fractions, infection caused an initial slight stimulation of valine incorporation into protein, which was most marked in the cell-sap fraction. This was followed by a period of moderate inhibition, until appreciable amounts of virus had accumulated intracellularly, when incorporation into mitochondrial protein was again elevated.

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A Study of the Kinetics of the Fibrillar Adenosine Triphosphatase of Rabbit Skeletal Muscle

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One of the most puzzling features of the kinetics of the adenosine-triphosphatase activity of actomyosin and of the myofibrils in which it is contained is the so-called explosive phase of hydrolysis which occurs immediately after addition of substrate and which is followed under certain special conditions by a 'linear' phase of lower, but constant, velocity. These features were originally

studied by Weber & Hasselbach (1954) in myofibrillar preparations at low ionic strengths (< 0.15), but later Tonomura & Kitagawa (1957) showed that they were also characteristic of the hydrolysis of adenosine triphosphate by myosin B in the presence of Ca^{2+} ions, at high ionic strength (> 0.5). Tonomura & Kitagawa (1960) have extended their observations on myosin B to include