PRELIMINARY DATA ON A VIRUS-SPECIFIC ENZYME SYSTEM RESPONSIBLE FOR THE SYNTHESIS OF VIRAL RNA

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The synthesis of Mengovirus RNA can be clearly distinguished from normal cellular RNA synthesis.[1] In normal cells, all RNA synthesis is DNA-dependent as shown by sensitivity to actinomycin, a drug which only inhibits DNA-dependent RNA synthesis.[2] On the other hand, the synthesis of Mengovirus RNA is completely refractory to actinomycin.[1,2] Autoradiographic evidence indicates that all normal RNA synthesis is localized in the nucleus, whereas viral RNA synthesis is cytoplasmic.[1,3] Lastly, cell fractionation studies have indicated that Mengovirus RNA first appears in the particulate fractions of the cytoplasm.[1] In this paper we will demonstrate the existence of a particulate system in cytoplasmic extracts of Mengovirus-infected L-cells which incorporates all four labeled ribonucleoside triphosphates into an acid-insoluble form.

The reaction mixture used to demonstrate the activity contained the following in a final volume of 0.5 ml: 20 µg pyruvate kinase (PK); 5 µmoles phospho(enol)-pyruvate (PEP); 60 µmoles Tris-HCl (pH 8.1 at 37°C); 2.5 µmoles magnesium acetate; 0.1 µC ATP-8-C^{14} (2.05 µC/mg, Schwarz BioResearch, Inc.); and 60 µg each of GTP, UTP and CTP. Reaction mixtures were incubated for 15 min at 37°C and rapidly chilled in an ice bath. One-half ml of chilled 0.1 M pyrophosphate was added to each tube followed by 4 ml of 0.5 M perchloric acid (PCA). The precipitate was washed three times with PCA, washed once with alcohol-ether (1:1), taken up in formic acid, transferred to planchets and counted in a windowless gas-flow counter

A cytoplasmic extract of Mengovirus-infected L-cells incorporates more than three times as much ATP-C^{14} per unit protein than an equivalent extract from

TABLE I

COMPARISON OF C^{14}-NUCLEOSIDE TRIPHOSPHATE INCORPORATION BY FRACTIONS OF UNINFECTED AND MENGOVIRUS-INFECTED L-CELLS

	μμmoles incorp/mg protein	
Cytoplasm	Infected	Control
ATP-C^{14} complete	94.3	24.6
Microsomes		
ATP-C^{14} complete	120	13.8
" less UTP, GTP	51.5	20.3
GTP-C^{14} complete	55	5.9
" less CTP, ATP	7.8	7.5
UTP-C^{14} complete	50.5	3.9
" less ATP, GTP, CTP	14.1	3.9
CTP-C^{14} complete	73.5	20.8
" less ATP, CTP, UTP	27.9	19.0

Cells were homogenized in a Virtis homogenizer as has been described.[5] The nuclei were centrifuged off at 600xg. for 10 min and the mitochondria at 5,000 - 8,000xg. for 10 min to produce the "cytoplasm". Microsomes were harvested at 105,000xg. for 1 hour from the "cytoplasm". All cell fractionation was carried out in 0.25M sucrose plus 10^{-3}M $MgCl_2$ at 4°C. The reaction mixture (see text) contained 1.5 - 3.0 mg of protein and was incubated for 15 min. Protein was determined by the Folin method.[5] All cpm's were corrected by subtraction of the counting rate of unincubated control samples. The control rate never exceeded 10 per cent of that of the incubated sample.

Reaction mixtures where the labeled compound was other than ATP-C^{14} contained the following complements of triphosphates: 0.25 μC of GTP-8-C^{14} (9.1 μC/mg) with 15 μg of the other triphosphates; 0.25 μC of UTP-2-C^{14} (21 μC/mg) with 15 μg of the other triphosphates; or 0.42 μC of CTP-2-C^{14} (5.0 μC/mg) with 30 μg of the other triphosphates. Assay of the microsomal activity in this experiment was done with 1.8 μmoles of Mg acetate and 1 μg actinomycin per tube.

uninfected cells when assayed under the conditions described above (Table I). The additional activity is dependent on added Mg^{++}, inhibited by added Mn^{++}, only slightly affected by actinomycin (probably due to some nuclear contamination), and depressed by leaving out the triphosphate generating system (Table II).

The activity appears to be localized in the microsomal fraction of the cytoplasm (Table I). The virus-induced enzyme incorporates all four triphosphates into an acid-insoluble form and uninfected cells have ten-fold lower activity.

TABLE II

PROPERTIES OF THE ATP-C^{14} INCORPORATING SYSTEM
IN CYTOPLASMIC EXTRACTS OF MENGOVIRUS-INFECTED L-CELLS

	cpm
Complete	129
-Mg	40
-Mg, +$MnCl_2$ (2.5 μmoles)	46
-PK, -PEP	63
+actinomycin (10 μg)	95
Complete	82
+$MnCl_2$ (2.5 μmoles)	18
+actinomycin	69

Procedures as described in Table I and text.

The incorporating activity in control cells is independent of the presence of other triphosphates while the virus-induced enzyme shows a clear dependence. Since the various labeled triphosphates are incubated at different concentrations, different amounts of each appears to be incorporated (Table I). When the concentration factor is taken into account, it can be seen that roughly equivalent amounts of the four triphosphates are incorporated by the enzyme.

The microsomal activity has identical properties to those seen in the crude cytoplasmic extract. It requires Mg^{++}, is inhibited by Mn^{++}, and shows a pH optimum of 8-9. Magnesium at 1.8 μmoles per 0.5 ml is optimal, larger amounts are slightly inhibitory. Although there is activity in the mitochondrial fraction (5,000xg, 10 min) of infected cells which is dependent on UTP and GTP and is insensitive to actinomycin, the specific activity of the mitochondrial incorporatic is only 11 cpm/mg protein. Since the cytoplasm has only been crudely fractionated in these experiments, contamination of the mitochondria with endoplasmic reticulum probably explains the occurrence of activity in this fraction.

Table III shows that the microsomal activity is not affected by actinomycin or DNase, and not stimulated by added DNA. It is therefore independent of DNA. Since hydrolysis of the product by 0.3N NaOH renders it completely acid soluble,

it appears to be ribonucleic acid. Addition of 10 μg of ribonuclease (RNase) to the reaction mixture causes only a 28% reduction of ATP-C^{14} incorporation and a 41% reduction in UTP-C^{14} incorporation. Addition of 100 μg of RNase inhibits virtually all incorporation. The relative insensitivity of the system to RNase is as yet unexplained.

TABLE III

PROPERTIES OF THE ATP-C^{14} INCORPORATION OF THE MICROSOMES FROM MENGOVIRUS-INFECTED L-CELLS

	cpm
Complete	165
-UTP, -GTP	32
+10 μg actinomycin	145
+150 μg calf thymus DNA	121
Complete	177
-UTP, -GTP	54
+50 μg puromycin	181
Complete	58
+2.5 μmoles $MnCl_2$	22
Complete	72
+10 μg DNase	72

Preparation of microsomes and assay conditions as described in Table I and text. The DNase hydrolyzed sample and the "complete" to which it is compared were assayed after a 10 min preincubation at 37°C with or without enzyme, respectively.

Although this has yet to be directly demonstrated, it appears likely that the triphosphate-dependent incorporation described here is the system which is responsible for the synthesis of viral RNA. The insensitivity of the system to actinomycin and the microsomal localization correlate well with *in vivo* studies.[1] Furthermore, the requirement for magnesium and the sensitivity to manganese clearly distinguish it from the DNA-dependent enzyme which requires manganese and is sensitive to actinomycin.[4,5] The RNA-dependent incorporation noted by Nakamoto and Weiss in extracts of *M. lysodeikticus*[6] also appears to be manganese stimulated and is therefore probably unrelated to the present activity. Although

no stimulation by added RNA has yet been achieved, it has been shown that ATP-C^{14} incorporation is greatly depressed by the omission of any one of the other triphosphates, as would be expected if the enzyme is copying a primer molecule.

The synthesis of infectious RNA in Western equine encephalomyelitis and poliovirus-infected cells is inhibited when puromycin or fluorophenylalanine is added.[7,8,9] Furthermore, RNA synthesis, as measured by incorporation of labeled precursor, is stopped by the addition of puromycin or fluorophenylalanine to infected cells which are in the process of synthesizing viral RNA.[10] The results of Table III indicate that the puromycin effect is not directly on the enzymatic system which synthesizes viral RNA. The reason for the effect is not yet clear, but the data presented here indicate that concomitant protein synthesis is not required for synthesis of viral RNA.

The microsomes of polio-infected He-La cells have incorporating activity at five hours after infection which appears to have similar properties to that found in Mengovirus-infected L-cells. Uninfected He-La cells are lacking the activity.[11]

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