sequence of events recorded photographically with an electronic flash. During this process, the *Entamoeba* moved rapidly and came in contact with *Acanthamoeba*, and when this happened the plasmalema of the 2 amoebae became interlocked with each other (Figure 1). The *Entamoeba* then gradually started to draw in the *Acanthamoeba* into its cytoplasm. Sometimes the whole amoeba was taken in completely intact and could be seen lying inside the food vacuole in *Entamoeba* (Figure 2).

For further study of this phenomenon, a mixture of *Entamoeba* plus *Acanthamoeba* which had been kept together for 10 min was processed for electron microscopy. These cells were fixed in buffered glutaraldehyde (pH 7.2) and post fixed in Osmium tetroxide. Following acetone dehydration, they were embedded in araldite, sectioned and stained with lead citrate and uranyl acetate according to the standard technique. Sections were examined on formvar coated grids in a Hitachi HS8 microscope.

The observations made in electron microscope confirmed the findings of the light microscope. Figure 3 shows the interlocking of the 2 amoebae before the ingestion of *Acanthamoeba*. The cell membrane of *Entamoeba* appears more electron dense than *Acanthamoeba* and at this stage it was not possible to say which amoeba will be able to ingest the other. Figure 4 shows *Acanthamoeba* lying inside the cytoplasm of *Entamoeba*. *Acanthamoeba* was easily recognized by the large contractile vacuole, a more homogenous cytoplasm, and the presence of mitochondria. The cytoplasm of *Entamoeba*, on the other hand, was granular, containing a large number of electron-dense particles, and had no mitochondria. The nuclei of both *Acanthamoeba* and *Entamoeba* are visible in the section.

The ultimate fate of the ingested *Acanthamoeba* is not known. Apparently they are broken down by *Entamoeba*, as in a number of cells nuclear remnants of *Acanthamoeba* were seen after a few hours of ingestion[2].

Résumé. L'ingestion de l'*Acanthamoeba* par l'*Entamoeba invadens* est observée pour la première fois. Elle a été étudiée par microscopie lumineuse et électronique. An cours de l'ingestion l'*Acanthamoeba* et l'*Entamoeba* se rapprochent; leurs membranes cellulaires s'entrelacent et l'*Entamoeba* achève l'ingestion de l'*Acanthamoeba*.

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2 The species of *Acanthamoeba* used in this study was obtained through the courtesy of Mrs. K. M. G. Adam, Department of Zoology, University of Edinburgh.

On the Synthesis of Poliovirus RNA at Supraoptimal Temperatures[1]

At certain supraoptimal temperatures, the incorporation of labelled precursors in poliovirus RNA is reduced to insignificant levels and virus multiplication completely prevented[2,3]. According to some authors[4], the phenomenon is due to an inhibition of viral RNA synthesis; according to others[5], it would depend upon an equilibrium between a normal synthesis of viral RNA and its parallel digestion by nucleases activated by supraoptimal temperatures. The question is of interest in that at supraoptimal temperatures, poliovirus proteins are synthesized[6], responsible for the early blockade of cell metabolism and for the late cytopathic effect[7,8]. If these events occur in the absence of viral RNA synthesis, supraoptimal temperatures could be indicated to evaluate the direct effect of antiviral compounds on the viral protein synthesis.

Materials and methods. Actinomycin D and neutral red were obtained from Merck; cristalline insulin from Lilly; peroxide-free phenol and ethyl ether from Mallinkrodt; Sephadex G 100 from Pharmacia; H^3 Uridine (5 T; 24,000 mC/m*M*) and H^3 Leucine (15,200 mC/m*M*) from Amersham. HeLa cells (American type culture collection) were grown in Eagle's MEM plus 10% calf serum, pH 7.3, supplemented with 0.01 U/ml of insulin to prevent a possible antipolio activity of actinomycin D[9]. Poliovirus type 1 (Brunhenders) was used throughout.

Completely confluent cell monolayers in small petri dishes (10^6 cells) were infected with 1 ml of viral suspension (30 plaque forming units – PFU/cell in Hank's BSS) at 4 °C for 1 h, washed 3 times, supplemented with Hank's BSS (3 ml, pH 7.3) and incubated at different temperatures, in water bath. At prefixed time intervals, the entire cultures were frozen and thawed 3 times, the debris removed by centrifugation (5000 × *g*, 5 min) and PFU titrated according to the Dulbecco technique[10]. For the evaluation of the overall viral RNA synthesis, cell monolayers, infected as above, were added with Hank's BSS containing actinomycin D (2 μg/ml) and H^3 Uridine (1 μC/ml) and incubated either at 37 °C or at 41.5 °C (for more details see results). At various time intervals cells were scraped from the glass with rubber policeman, washed 3 times in cold Hank's BSS supplemented with unlabelled uridine (50 μg/ml), precipitated twice at 4 °C in trichloroacetic acid (5% in H_2O) and dried at 37 °C for 20 h. Precipitates were dissolved in Soluene and radioactivity was determined in a Packard scintillation counter (scintillation fluid: 7 g PPO, 0.6 g dimethyl POPOP, 1000 ml Toluene).

RNAase sensitivity of viral RNA was determined in phenol extracts obtained with the technique of Gierer and Schramm[11] modified by Muntoni et al.[12] from cells

1 Work supported by a Grant of Consiglio Nazionale delle Ricerche, Rome.

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infected and supplemented with actinomycin D and H^3 Uridine as indicated above. One half of each extract (1 ml) was treated with cristalline RNAase (0.4 μg/ml) at 22 °C for 5 min, according to the method of PLAGEMANN and SWIMM[12]. Fractionation was performed in Sephadex G 100 columns (20 × 0.9 cm; bed 18 ml) in *Tris*-EDTA buffer (0.01 and 0.05 *M*, pH 7.3). 1 ml fractions were collected (flow: 8 ml/h) and used for radioactivity measurement (scintillation medium: 7 g PPO, 0.1 g dimethyl POPOP, 333 g Triton X 100, 667 ml Toluene).

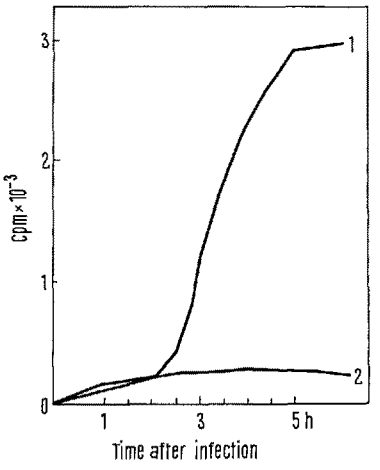

Fig. 1. Cumulative uptake of H^3 Uridine (1 μC/ml) in infected cells at 37 °C (1) and at 41.5 °C (2).

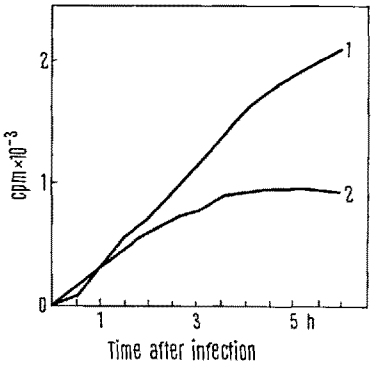

Fig. 2. Cumulative uptake of H^3 Leucine (1 μC/ml) in viral antigens at 37 °C (1) and at 41.5 °C (2).

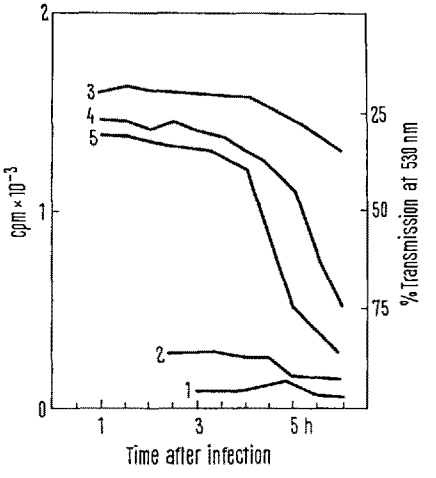

Fig. 3. 1. Cumulative uptake at 41.5 °C of H^3 Uridine (1 μC/ml) added 2.30 h after infection. 2. Persistence in infected cells at 41.5 °C of H^3 Uridine (1 μC/ml) incorporated within 2.30 h after infection. 3. Neutral red (100 μg/ml) incorporated in uninfected cells at 41.5 °C. 4. Neutral red in infected cells at 37 °C. 5. Neutral red in infected cells at 41.5 °C.

For the evaluation of virus-induced protein synthesis, infected monolayers in Hank's BSS (5×10^6 cells) were treated with H^3 leucine (1 μC/ml) immediately following infection. At prefixed time intervals, cells were washed 5 times with Hank's BSS, frozen and thawed 5 times in 0.5 ml of Hank's BSS and filtered through Millipore filters (0.45 μm). Filtrates were treated with 1000 neutralizing units (0.1 ml) of antipolio type 1 rabbit immune serum and incubated at 37 °C for 3 h and at 4 °C for 10 h. Then 0.1 ml of antirabbit γ-globulin serum was added and the incubation at 37 °C and at 4 °C repeated. Precipitates were collected by means of Millipore filters (0.45 μm), treated with 10% trichloroacetic acid at 4 °C, dried at 37 °C and counted in a scintillation counter as above.

The cytopathic effects (CPE) were evaluated on the basis of the intracellular uptake of vital stains. Infected cell monolayers (5×10^6 cells) were incubated with 10 ml of Hank's BSS at 37 °C. At various intervals neutral

Effect of supraoptimal temperatures on the growth of poliovirus 1 Brunhenders in HeLa cells (virus inoculum 30 PFU/cell × 10^6 cells)

(°C)	PFU produced after 6 h at different temperatures (°C)	PFU produced after 6 more h at 37 °C
37	8×10^7	1.2×10^8
39	2.5×10^7	6.3×10^7
39.5	6.6×10^6	7.1×10^7
40	7.2×10^5	5.4×10^7
40.5	1.5×10^5	8.2×10^7
41	6×10^4	4.2×10^7
41.5	$< 10^4$	5.1×10^7
42	$< 10^4$	2.3×10^6
42.5	$< 10^4$	4.1×10^5
43	$< 10^4$	8×10^4

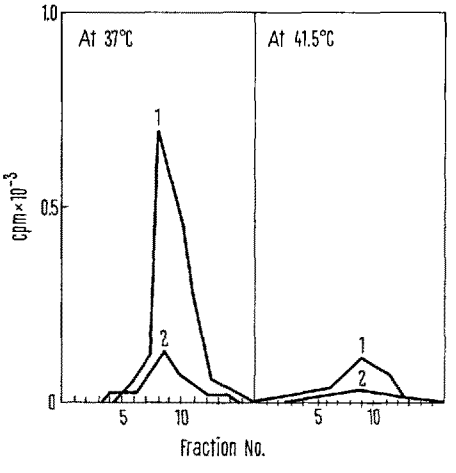

Fig. 4. RNAase sensitivity of viral RNA extracted from infected cells 3 h after infection. 1. Untreated viral RNA. 2. Viral RNA treated with 0.4 μg/ml of RNAase. Separation through Sephadex G 100 of phenol extracts from infected cells labelled with H^3 Uridine (1 μC/ml).

red was added (up to 100 µg/ml) and the cultures reincubated for 30 min. The medium was then discarded and the monolayers, after 5 washings with phenol red free Hank's BSS, were solubilized with 8 ml of natrium deoxycholate (2% in H_2O, pH 7.3). The neutral red incorporated was measured on the basis of percent transmission at 530 nm.

Results. Data of the Table show that the incubation of poliovirus infected cells at 41.5 °C causes a complete but reversible inhibition of virus growth. At lower temperatures only a partial inhibition is observed, while at higher ones virus recovery is not complete.

Figure 1 shows that the incorporation of H^3 Uridine in infected cells is very low, both at 37 °C and at 41.5 °C within $2^1/_2$ h after infection; thereafter a sharp increase is observed at 37 °C (up to a maximum at the 5th h) while no further increase can be detected at 41.5 °C.

Viral protein synthesis is, on the contrary, scarcely influenced by supraoptimal temperatures, at least in the early stages of infection (Figure 2). Both at 37 °C and 41.5 °C a significant amount of H^3 Leucine is incorporated in viral antigens within the first $3^1/_2$ h; only later at 41.5 °C the uptake ratio declines, to stop completely $4^1/_2$ h after infection.

The possibility of a continuous breakage of viral RNA at 41.5 °C has been investigated. Data of Figure 3 show that at 41.5 °C no H^3 Uridine is incorporated later than $2^1/_2$ h after infection and that the small amount of H^3 Uridine incorporated in acid-insoluble form within $2^1/_2$ h after infection is firmly retained for at least 2 more h, that is up to the first appearance of the cytopathic effect.

Data of Figure 4 finally demonstrate that both the large amount of viral RNA synthesized at 37 °C and the scarce quantity made at 41.5 °C within 3 h from the infection are mostly represented by RNAase sensitive (monohelix?) molecules; they therefore cannot be considered as a residual of an intracellular breakage by RNAases.

Discussion and conclusion. The results reported here can be summarized as follows: 1. At certain supraoptimal temperatures (41.5 °C) the incorporation of labelled precursors into poliovirus RNA is strongly reduced. This is not due to a continuous digestion of neosynthesized RNA molecules by nucleases but, rather, to an inhibition by supraoptimal temperatures of the de novo synthesis of viral RNA. 2. Under the same conditions the overall synthesis of poliovirus proteins is scarcely influenced, at least for $3^1/_2$ h after infection. It can therefore be assumed that most of the viral proteins made at 41.5 °C (including those responsible for metabolic blockade and cytopathic effect) have been built up in polyribosomes organized by the infecting virus RNAs. This gives the opportunity to use supraoptimal temperatures for studying the direct action of antiviral compounds on the synthesis of poliovirus proteins, ruling out a possible influence of the viral RNA replication.

Riassunto. La scarsa incorporazione di precursori dello ARN in cellule infette da poliovirus ed incubate a 41,5 °C è dovuta ad una inibizione della sintesi dello ARN virale più che non ad una sua digestione ad opera di nucleasi.

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Caffeine Removal from Growth Media by Microorganisms

The protean manifestations of the biological effects of caffeine (and other methylated xanthines) is exemplified by its toxicity to microorganisms[1], plants[2] and animals[3]; its roles as mutagen[4] and enzyme inducer[5]; its inhibition of cyclic AMP hydrolases[6] and the dimer excizing enzyme in repair of DNA[7]; and its ability to release Ca^{++} from the sarcoplasmic reticulum[8]. Its most well-known and pleasant attribute, the mild stimulation occasioned by intake of beverages, is not universally enjoyed. Thus, 10% of the coffee consumed in the United States and Europe is decaffeinated[9].

We have considered biological alternatives to the present use of chlorinated hydrocarbons for extraction of caffeine from coffee. There exists a paucity of information in this regard. *Fusarium oxysporum* apparently resists the toxicity of and grows, albeit poorly, on caffeine[10]. *Pseudomonas aeruginosa* appears to possess xanthine dehydrogenase and uricase of rather broad specificity capable of degrading methyl xanthines to methyl allantoins[11]. In the present communication we show that caffeine can be utilized as sole source of either carbon or nitrogen for growth of a bacterium, *Bacillus coagulans*, and two fungi, *Penicillium roquefort* and a *Stemphylium* species.

In addition to these three species, other colonies grew on our agar plates, some of which were identified as actinomycetes. However, prolonged exposure of the isolation medium was necessary to cause the appearance of relatively few colonies. Furthermore, neither the purine-utilizing *Sordaria brevicollis*[12] nor an authentic culture of *B. coagulans* (NCA 43P) grew in caffeine at the levels used in this investigation. Thus, although not restricted to a narrow group of microorganisms, the ability to utilize caffeine is not ubiquitous.

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