and purine bases and nucleosides. Considered together, these data led us to advance the working hypothesis that the substituted pyrimidine interacts with polipeptide chains soon after their synthesis in the polyribosomes, thus impairing the organization of these chains into normally active protein molecules. Glutamine and cysteine moieties, located in certain positions in the amino acid sequences of the same chain (or of different chains) would be the sites where this interaction occurs. The more frequent these amino acid sites are in the polypeptide chains (or the more favorable are their positions for interacting with the drug), the more intense will be the impairing effect of the drug on the chain structuring into active molecules. Viral capsids precursors would fulfill the above requirements, thereby representing a specific target for the drug action. Research is in progress to verify this hypothesis[5].

Riassunto. La 2-amino-4, 6-dicloropirimidina inibisce lo sviluppo del Poliovirus rendendo i precursori capsidici incapaci di partecipare alla formazione di particelle infettanti. Questo effetto è irreversibile in quanto la sostanza è avidamente incorporata e ritenuta dalle cellule infette. L'antagonismo esercitato dall'azione combinata di glutamina e cisteina fa ritenere che la pirimidina sostituita svolga il suo effetto interreagendo con questi due aminoacidi dopo la loro incorporazione nei precursori capsidici.

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23 May 1973.

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Irreversible Impairment Produced by Guanidine on the Functions of Poliovirus Proteins

It is still matter of controversy whether guanidine inhibits enterovirus growth by acting on RNA replication directly[1] or, rather, by impairing the functions of proteins[2]. To look into this question we have considered it of interest to study which effect guanidine has on the functions of capsid proteins of poliovirus. In detail, to establish whether capsid proteins synthesized in the presence of guanidine are able to assemble virus RNA, replicated later in a drug-free medium, into infectious virus.

In doing these experiments it was of crucial importance to rule out that any capsid proteins, synthesized after guanidine removal, could participate in virus assembly. To achieve this goal, use has been made of parafluorophenylalanine (FPA) and 2-amino-4, 6-dichloropyrimidine (Py11). Added in proper concentrations to cell cultures soon after infection, these substances prevent the assembly of poliovirus RNA into infections particles[3,4]. However, if drug treatment is delayed of some 2–3 h, capsid proteins made during that interval are able to assemble virus RNA into infectious particles[4]. As essential components for virus assembly, these capsid proteins can be used as 'targets' for evaluating the action of guanidine.

Material. Guanidine HCl was furnished by Eastman Kodak, parafluorophenylalanine (FPA) by Aldrich, actinomycin D (AMD) by Merck. 2-amino-4, 6-dichloropyrimidine (Py11) was synthesized by the Istituto

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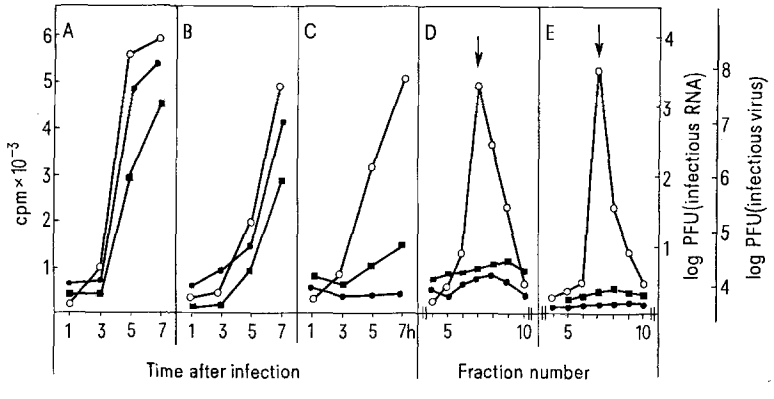

Fig. 1. Inhibition of poliovirus growth by early treatments with FPA or Py11 of infected cells. Infected cells were incubated at 37 °C in aminoacid-free Eagle MEM supplemented with AMD (2 μg/ml). FPA (25 μg/ml) or Py11 (100 μg/ml) were added to the cultures at time 0 after infection. A) Net synthesis of virus RNA, determined by the incorporation of [H³] uridine (0.2 μCi/ml from time 0); B) and C) synthesis of infectious virus RNA and production of infectious virus respectively, measured in PFU; D) and E) incorporation in virus particles of [H³] leucine and [H³] uridine, respectively (2 μCi/ml, cumulative pulses from time 0 up to 10 h after infection). ○— ○, untreaded cells; ■ — ■, FPA treated cells; ●— ●, Py11 treated cells. Arrows indicate maximum infectivity in the gradients.

Chemioterapico Italiano. [^{3}H] uridine (24 Ci/mM) and [^{3}H] leucine (15.2 Ci/mM) were obtained from Amersham.

Methods. HEp 2 cell monolayers (American Type Culture, Rockville) in Petri dishes were infected with 50 plaque-forming units (PFU) per cell of poliovirus 1 Brunenders. After 1 h at 4 °C, cell monolayers were washed 3 times in Hank's BSS and incubated at 37 °C in Eagle MEM (Hank's base, pH 7.3), lacking the aminoacid supplement and containing 2 μg/ml of AMD. The net synthesis of virus RNA was determined in 10^6 cell samples, by the incorporation, under acid insoluble form, of [H^3] uridine (0.2 μCi/ml, cumulative pulses from time 0). The infectivity of virus RNA, which was extracted by the phenol method of GIERER and SCHRAMM[5], and the production of infectious progeny, were measured in PFU, starting from 10^6 cell samples, according to the method of DULBECCO and VOGT[6]. To determine the participation in virus assembly of virus proteins and RNA, 2×10^7 cell samples were labelled with 2 μCi/ml of [H^3] leucine and [H^3] uridine. At 10 h after infection, the entire cultures were frozen and thawed (−70 and +20 °C) 3 times, treated with 1 volume of genetron at 4 °C for 5 min, deprived of cell debris at 5,000 rpm for 5 min and spinned at 30,000 rpm for 4 h at 4 °C in Spinco 30. Pellets thus obtained were dissolved in 1 ml of RSB[7] containing 1% sodium deoxycholate, treated with 20 μg/ml of crystalline DNA-ase and RNA-ase, supplemented with 0.2 ml of unlabelled poliovirus suspension, layered onto 10 ml of a 10–40% sucrose in RSB and centrifuged in Spinco SW 40 Ti (25,000 rpm for 2 h at 20 °C) 0.4 ml fractions were collected starting from the bottom of the tubes. Of these fractions, 0.1 ml were used to determine the infectivity. The remaining 0.3 ml were mixed with 1 ml of 1% bovine albumine, precipitated at 4 °C in TCA and dissolved in Soluene 350 (Packard). Radioactivity was determined in a scintillation counter (Tricarb; scintillation liquid: 667 toluene; 333 Triton X 100; 5 g PPO; 0.3 g dimenthyl POPOP).

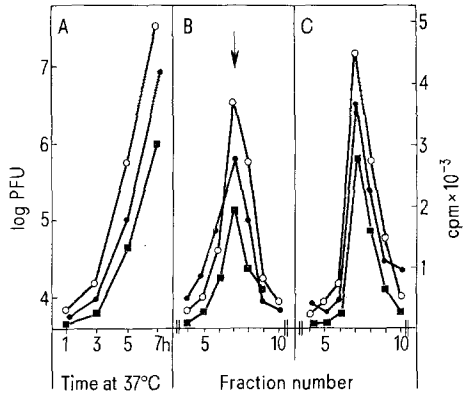

Fig. 2. Ineffectiveness on poliovirus growth of delayed treatments with FPA or Py 11 of infected cells. Infected cells were incubated at 37 °C in aminoacid-free Eagle MEM supplemented with AMD (2 μg/ml). FPA (25 μg/ml) or Py11 (80 μg/ml) were added 2.5 h after infection. A) Infectious virus yield (in PFU); B) incorporation in virus particles of [H^3] leucine (2 μCi/ml from 0 h up to 2.5 h after infection, then chased with 100 μg/ml of L-leucine); C) incorporation in virus particles of [H^3] uridine (2 μCi/ml from 2.5 h after infection up to 10 h after infection). O— O, untreated cells; ■— ■, FPA treated cells; ●— ●, Py 11 treated cells. Arrows indicate maximum infectivity in the gradients.

Results. At first the reliability of experimental procedures was verified. As shown in Figure 1, when FPA or Py11 are added to the cell cultures soon after infection, virus RNA and proteins are scarcely assembled into virus particles, and no infectious progeny is detectable. However (Figure 2), if a 2.30 h interval is allowed to elapse between infection and drug addition, virus proteins, which have been synthesized during that interval in a drug-free medium, assemble virus RNA, replicated later, into complete virus particles, and infectious progeny is produced. Thus, under these experimental conditions, capsid proteins synthesized before drug addition are essential for virus growth and can consequently be used as a tool for studying the action of guanidine.

Data in Figure 3 show that if cell cultures are incubated in the presence of guanidine for the first 2.5 h after infection and are then maintained in a drug-free medium, a noticeable amount of infectious virus is produced, but few of the proteins synthesized in the presence of guanidine

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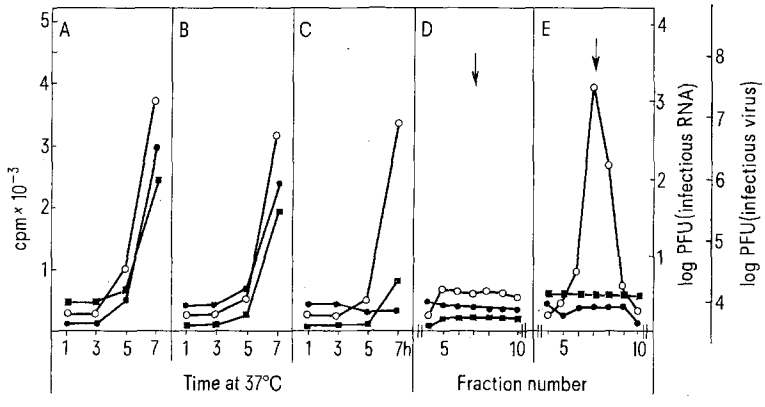

Fig. 3. Non-participation in virus assembly of virus proteins synthesized in the presence of guanidine. Infected cells were incubated at 37 °C in aminoacid-free Eagle MEM supplemented with AMD (2 μg/ml) and containing guanidine HCl (50 μg/ml). After 2.5 h, guanidine-medium was replaced by aminoacid-free Eagle MEM supplemented with AMD (2 μg/ml) (O— O), or by the same medium containing either FPA (25 μg/ml) (■— ■) or Py 11 (80 μg/ml) (●— ●). A) uptake of [H^3] uridine (0.2 μCi/ml from time 0) in virus RNA; B) and C) synthesis of infectious virus RNA and production of infectious virus progeny, respectively; D) incorporation in virus particles of [H^3] leucine (2 μCi/ml from time 0 up to 2.5 h after infection, then chased with 100 μg/ml of L-leucine); E) incorporation in virus particles of [H^3] uridine (2 μCi/ml from 2.5 h after infection up to 10 h after infection).

participate in the virus assembly. Furthermore, if after incubation in guanidine, infected cells are maintained in media containing FPA or Py11, neither virus particles nor infectious virus are produced, in spite of the remarkable synthesis of virus RNA.

These data lead to the conclusion that guanidine impairs the ability of capsid precursors to participate in the assembly of infectious virus particles[8].

[8] This work has been supported by a Grant of the Consiglio Nazionale delle Ricerche, Roma (Italy).

Riassunto. La guanidina altera irreversibilmente la capacità delle proteine capsidiche del poliovirus di partecipare alla formazione di particelle infettanti.

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Chromatographic Evidence for the Synthesis of Possible Sleep-Mediators in *Trypanosoma brucei gambiense*

Trypanosoma brucei gambiense produces a chronic disease in man characterized by prolonged sleep states, mental depression, and physical inertia. The onset of abnormal sleep periods corresponds to the penetration of trypanosomes into the cerebrospinal fluid[1]. One member of the protozoan family Trypanosomatidae, *Crithidia fasciculata*, as well as the ciliate, *Tetrahymena pyriformis*, have been reported to contain the indolamine, serotonin (5-hydroxytryptamine)[2]. This compound has been suggested to be a mediator of slow-wave sleep in mammals[3]. We have investigated the metabolism of tryptophan and 5-hydroxytryptophan, two immediate precursors of serotonin, in *T. b. gambiense* in an attempt to demonstrate the synthesis of serotonin or of other sleep-mediators.

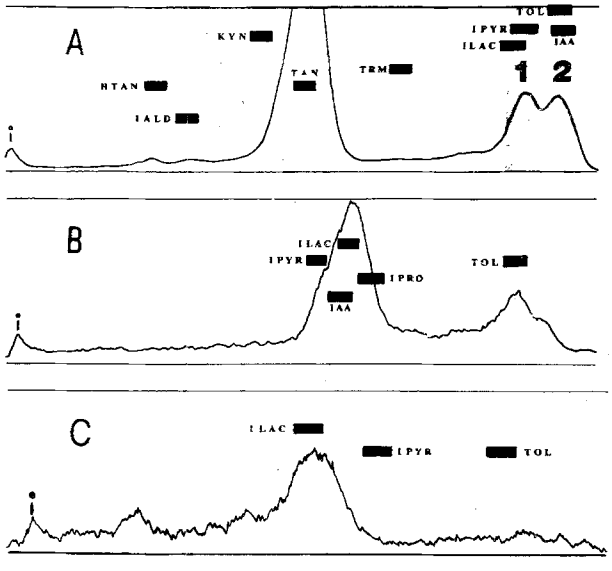

Fig. 1. (A) Radioactivity scan (10,000 cpm range) of chromatogram of supernatant from incubation of trypanosomes for 90 min with tryptophan-3-C^{14}, developed in butanol: acetic acid: water (4:1:1). Shaded areas 1 and 2 were eluted from the chromatogram and re-chromatographed in other solvent systems. (B) Scan (3,000 cpm range) of chromatogram of eluate from area 1 in A, developed in ethanol (95%): ammonia: water (16:1:3). (C) Scan (300 cpm range) of chromatogram of eluate from peak in B, developed in benzene: acetic acid: water (125:72:3). Abbreviations: HTAN, 5-hydroxytryptophan; IAA, indole acetic acid; IALD, indole acetaldehyde; ILAC, indole lactic acid; IPRO, indole propionic acid; IPYR, indole pyruvic acid; KYN, kynurenine; TAN, tryptophan; TOL, tryptophol: TRM, tryptamine.

Materials and methods. Bloodstream forms of this parasite were harvested from infected rats and separated from blood cells by column chromatography through diethyl amino ethyl (DEAE) cellulose (Sigma Chemical Co.)[4]. The trypanosomes were suspended in 5.0 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, containing NaCl (0.1 *M*), KCl (0.006 *M*), $MgSO_4$ (0.002 *M*), 1% D-glucose, vitamin mix (100× concentrated, 1.0 ml/100 ml buffer, designed for minimum essential Eagle medium, Microbiological Associates, Bethesda, Maryland), and enzyme cofactors (6,7-dimethyl-5, 6, 7, 8-tetrahydropterine, 1 m*M*; pyridoxal phosphate, 5×10^{-5} *M*; α-ketoglutarate, 8 m*M*; NADH, 1.4 m*M*; NAD^+, 0.6 m*M*; and NADPH, 6.7×10^{-4} *M*) and incubated for 90 min at 37 °C with 5.0 μCi of either L-tryptophan-3-C^{14} (3.82 mg/mCi) or DL-5-hydroxytryptophan-3-C^{14} (2.1 mg/0.05 mCi) (New England Nuclear). The concentration of trypanosomes was approximately 100 million/ml. Sonicated trypanosome preparations were also incubated with these C^{14}-substrates; sonication was carried out using a Branson S-75 sonifier at setting 5 for 45 sec.

Three volumes of absolute methanol were added to terminate the incubations, and after centrifugation and removal of precipitated protein, the supernatant extract was concentrated to 3.0 ml by evaporation. 100 μl aliquots of this were chromatographed in one dimension along with known, purified standards on 57 cm strips of Whatman 3MM chromatography paper using butanol: acetic acid: water (4:1:1). The developed chromatograms were scanned for radioactivity using a Packard radiochromatogram scanner, and radioactive peaks not identical with the substrate were eluted from the chromatogram and re-chromatographed in as many as 4 other solvent systems. Aliquots of the incubation extract were also subjected to two-dimensional paper chromatography using butanol: acetic acid: water (4:1:1) and ethanol (95%): ammonia: water (16:1:3). Control incubations were run simultaneously with the experimentals: these included a boiled preparation, a zero time incubation, an incubation of only buffer and substrate, and incubations of centrifuge-purified, sonicated platelets and erythrocytes.

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