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METHISOPRINOL* ENHANCEMENT OF NUCLEOCYTOPLASMIC TRANSPORT OF PUTATIVE MESSENGER RNA IN RAT LIVER

ITS RELATION TO HOST DEFENSE AGAINST VIRUS INFECTION

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Abstract—The experimental antiviral drug, methisoprinol, has been reported to increase the rate of rapid entry of RNA precursors into polyribosomes in diverse tissues. To further the understanding of its mechanism of action, in this paper methisoprinol was examined for effects on the rapidly labeled RNA metabolism of rat liver, in studies that distinguish between drug effect on RNA synthesis and on nucleocytoplasmic RNA transport. When ingested orally at a level of 500 mg/kg/24 hr, methisoprinol was found not to affect the incorporation of orotic acid[^{14}C] into cytoplasmic UMP, UDP or UTP, and not to affect the rate of labeling or the pool size of nuclear UTP. At this dose, methisoprinol was found not to affect the rate of labeling of extracted nuclear RNA, while at a dose four times greater methisoprinol depressed the labeling of nuclear RNA by 32 per cent. In contrast, methisoprinol was observed to markedly increase the early rate of incorporation of radioactive precursor into liver polysomal RNA at times between 15 min and 3 hr at both levels of drug ingestion, while not increasing the incorporation that occurs between 6 hr and 9 hr. Further, in the membrane-poor polyribosomal RNA fraction, methisoprinol treatment increased the proportion of RNA with sedimentation coefficient between 6S and 18S and increased the incorporated radioactivity in the 4–10S region, relative to standard incorporated nuclear counts. These changes were strongly suggestive of an increased presence of informosomal RNAs and of an increase in nucleocytoplasmic transport of rapidly labeled RNA. Also in the membrane-poor polyribosomal RNA fraction, drug treatment was found to increase the amount of polyadenylic acid (poly A)-bearing RNA by 50 per cent and to increase the amount of poly A attached to polyribosomal RNA by a far greater amount. Since poly A is found only as part of putative messenger RNA, these data permit us to conclude that methisoprinol increases the degree of nucleocytoplasmic transport of host messenger RNA (mRNA) in association with an increase in the poly A content of this mRNA, the latter change being especially evident in the membrane-poor polysome fraction.

In other studies, methisoprinol and cordycepin (3-deoxyadenosine), an inhibitor of poly A synthesis, were examined for effects on the development of cytopathology in herpesvirus-infected WI-38 cell monolayers, when added 48 hr after infection. When added alone, both methisoprinol and cordycepin inhibited the development of cytopathology during 100 hr; however, when added together, the same dosages provoked a mutual antagonism and no antiviral effect was observed. This observation suggests that methisoprinol's direct antiviral effect, as seen in tissue culture, is associated with an enhanced rate of poly A synthesis in the nucleus. Poly A added to the medium external to the cells also inhibited the antiviral effect of cordycepin, but did not exert an antiviral effect by itself. It is noted that lymphocyte-dependent methisoprinol effects on immune responses and virus infection have been observed; thus, methisoprinol may also exert indirect antiviral effects via the immune system. Since poly A is known to actuate nucleocytoplasmic mRNA transport and to contribute to the stability and activity of mRNAs in polysomes in diverse tissues, it is suggested that methisoprinol may enhance the synthesis of certain antiviral principles in both local tissues and lymphocytes through a poly A-related mechanism.

Methisoprinol* is a complex of dimethylamino isopropanol and inosine (molar ratio 3:1). It can be demonstrated by spectroscopy and through changes in the colligative properties of inosine, the parent compound, i.e. water solubility at pH 7·0, etc. [1].‡ This drug, developed in our laboratory,‡ exhibits antiviral activity both *in vivo* and *in vitro* against herpes, influenza and certain other virus infections [2–5], and exhibits no oral toxicity up to 5 g/kg [6].

In uninfected monkey kidney cells, methisoprinol has been found to increase the incorporation of [^{3}H]orotic acid and radioactive amino acids into polyribosomes [2]. In uninfected rat brain, methisoprinol increased the labeling of polyribosomes after the injection of [^{3}H]orotic acid [3]. In addition, spectrophotometric changes revealing an increase in relative hypochromicity occur in polyribosomes isolated from adult and aged rat brain after treatment with methisoprinol [3]. Protein synthesis, measured in a

* Until recently this was the generic name for Isoprinosine®, Newport Pharmaceuticals Inc., Newport Beach, Calif. After submission of this manuscript, the official generic name was changed from methisoprenol to inosiplex.

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‡ Gordon, Inosine Derivatives, U.S. Patent No. 3,646,007, issued in 1971.

cell-free system by the uptake of [^{14}C]phenylalanine into the nascent protein of polyribosomes from the brains of methisoprinol-treated animals, was increased over control. At the same time the addition of polyuridylic acid to the system *in vitro* decreased the incorporation of phenylalanine into polyribosomes from methisoprinol-treated rats, while increasing this incorporation in the controls [3].

Disaggregation of methisoprinol-treated rat brain polyribosomes by 0·01 M EDTA, after a short pulse labeling with radioactive orotic acid, revealed an increase in the labeling of ribonucleoproteins sedimenting in the range of 6–20S [3,7].

These effects suggested that methisoprinol exerts an enhancing influence over a group of coupled events that concern messenger RNA metabolism. In the present paper, we inquire into the basis of one such event: methisoprinol's enhancement of rapid cytoplasmic RNA labeling. We report on a group of experiments in normal rat liver which distinguish between drug effect on rapidly labeled RNA synthesis and on nucleocytoplasmic RNA transport.

This biochemical work will have three parts: (1) studies on the movement of radioactivity (added as orotic acid[^{14}C]) through precursor nucleotides, and nuclear and cytoplasmic RNAs; (2) development of chromatographic, ultraviolet and radioactivity profiles of extracted cytoplasmic RNAs; and (3) an assessment of drug effect on a component of cytoplasmic putative messenger RNA (mRNA) that is associated with, and may actuate, nucleocytoplasmic mRNA transport: polyadenylic acid.

Having obtained quite definitive results in these studies, it seemed not unreasonable to explore the possible relationship of the biochemical effects of methisoprinol to its antiviral action. Consequently, we performed experiments examining the interaction between methisoprinol and cordycepin (3-deoxyadenosine), an inhibitor of polyadenylic acid synthesis [8,9], and polyadenylic acid, as well, in a herpes simplex virus infection in tissue culture.

METHODS

Animals. Sprague–Dawley rats (female, 2–3 months old, obtained from ARS/Sprague–Dawley, Madison, Wis.) were housed six animals/cage and fed (Purina Laboratory Chow) and watered *ad lib.* The room was equipped with a light control device, giving alternating 12-hr periods of light (7:00 a.m.–7:00 p.m.) and dark.

Treatment. Methisoprinol (Lot No. 1032A) was given *ad lib.* in the drinking water for 3 days at concentrations of either 0·25% (approximately 500 mg/kg/day) or 1% (approximately 2 g/kg/day). Fluid consumption was monitored on a 24-hr schedule; volumes consumed were not changed by treatment. On day 3, all animals received a single intraperitoneal injection of 100 μCi [5-^{3}H]orotic acid (Amersham/Searle) or 5 μCi [6-^{14}C]orotic acid (Amersham/Searle). In each of four repetitions at each drug level, animals from control and treated groups were sacrificed in sets of three at 45 min after isotope or according to the following schedule: 15 and 45 min, 1·5, 3, 6 and 9 hr after isotope. Livers were excised, chilled and weighed. For each experiment, the three samples for a single time point were pooled.

Homogenate preparation. Homogenization was carried out using a glass-Teflon homogenizer, rotating the pestle at 1000 rev/min and completing 10 up-and-down strokes. The volume ratio of Tris–sucrose buffer (0·25 M sucrose, 0·025 M KCl, 0·05 M Tris, 0·03 M $CaCl_2$ at pH 7·5) to tissue was 4:1. The homogenate was filtered through six layers of cheesecloth, and the filtrate centrifuged at 2300 rev/min for 15 min in a Sorvall RC2-B centrifuge, SM-34 rotor, producing a crude nuclear pellet and a supernatant containing extranuclear organelles (see Fig. 1).

Extraction of crude nuclear pellet. Nuclei were recovered from the crude nuclear pellet by the method of Blobel and Potter [10] (I of Fig. 1). After removing an aliquot of the purified nuclear pellet for counting, the remaining pellet was washed with 95% ethanol, then with ether, and dried under nitrogen. RNA was extracted using the method of DiGirolamo *et al.* [11] (II of Fig. 1).

The pooled supernatants (alcoholic) remaining from the RNA precipitation were collected and evaporated to dryness on a Whatman No. 42 filter paper disc. The disc was cut into 1 × 5 cm strips and each strip eluted with 2 ml of distilled water. The effluents were collected and washed once with a half volume of water-saturated ether to remove traces of phenol left from extraction. Ether was removed under nitrogen and the remaining nucleotide solution was lyophilized and redissolved into 1 ml of distilled water (III of Fig. 1).

Quantification of nucleotides was carried out on a Varian Aerograph LCS-1000 high resolution, liquid ion exchange chromatograph. Fractions (0·8 ml) from the ion exchange chromatograph were counted for radioactivity.

Isolation of mitochondria and polyribosomes. The supernatant (Fig. 1) was made 0·01 M $MgCl_2$ by the addition of 0·1 M $MgCl_2$ in Tris–sucrose buffer, 1 ml/10 ml of supernatant. This was centrifuged at 12,000 rev/min for 15 min in a Sorvall RC2-B centrifuge, SM-34 rotor, resulting in a mitochondrial-rich pellet and S_1. S_1 was centrifuged at 16,750 rev/min for 20 min, resulting in a pellet of membrane-rich polyribosomes and S_2. S_2 was layered on a 1·2 M discontinuous gradient in volume ratio of 20 ml S_2

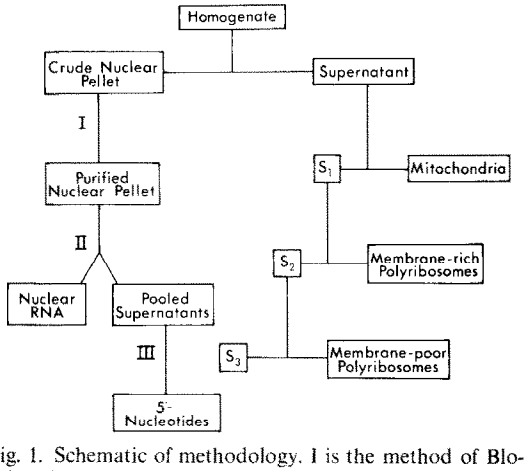

Fig. 1. Schematic of methodology. I is the method of Blobel and Potter [10] and II is the method of DiGirolamo *et al.* [11]. For a description of III, see Methods.

to 15 ml sucrose, and centrifuged at 40,000 rev/min for 90 min in a Beckman Spinco type Ti 50·1 rotor, resulting in a pellet of membrane-poor polyribosomes which was rinsed three times with sucrose-free Tris buffer and wiped clean. All pellets were dried with 95% ethanol and ether, weighed, and aliquots dissolved in hyamine and counted for radioactivity. Other aliquots were dissolved in acetate buffer (0·01 M NaCl, 0·005 M sodium acetate at pH 5·0) and assayed for RNA content by the orcinol procedure [12], for protein by the method of Lowry *et al.*[13], and DNA by the diphenylamine method [14].

Extraction of polyribosomal RNA. The membrane-rich and membrane-poor polyribosome pellets were gently homogenized into a buffer of 0·01 M Tris–HCl, pH 8·3, in a w/v ratio of 1:5. An equal volume of water-saturated phenol containing 0·1% 8-hydroxyquinoline was added to the homogenate and the mixture shaken for 40 min at 37°, centrifuged, and the aqueous (upper) fraction removed, reextracted as above and made 1% w/v with NaCl. RNA was precipitated by the addition of 2·5 times the aqueous volume of 95% ethanol and cooled to −10° overnight. The RNA was removed by centrifugation; the RNA pellet was rinsed twice with 95% ethanol and dried under nitrogen.

Analysis of polyribosomal RNA. Approximately 100 μg of the membrane-rich and membrane-poor polyribosomal RNA was layered onto a linear 5–20% (16 ml) sucrose gradient and centrifuged in a Beckman Spinco type 27·1 rotor at 21,000 rev/min for 18 hr. Gradients were eluted and fractionated using an ISCO model D fractionator equipped with an ISCO UA-2 spectrophotometer and strip chart recorder. Fractions from these gradients were examined individually for absorption at 260 and 280 nm, placed into scintillation counting vials and counted for radioactivity. Ratios of E_{260}/E_{280} were found to be not less than 1·9 for gradient fractions.

Estimation of polyadenylic acid content of polyribosomal RNA. Polyadenylic acid (poly A) was determined by adsorption of polyribosomal RNA onto polyuridylic acid (poly U)-treated glass-fiber filters following the method of Sheldon *et al.* [15]. The polyribosomal RNA was dissolved into a buffer of 0·05 M Tris–HCl, 0·1 M NaCl at pH 7·6. Multiple filtrations of RNA samples were carried out on a Millipore manifold filter apparatus using 25-mm diameter Gelman glass-fiber filters. Filters without poly U were used to determine non-specific adsorption. Filters were placed in scintillation counting vials, counted for radioactivity, and the results compared to the total radioactivity filtered.

Effects of methisoprinol, cordycepin and polyadenylic acid on herpes simplex virus infection in tissue culture. Experiments were carried out examining the interaction of cordycepin (3-deoxyadenosine), an inhibitor of poly A synthesis and nucleocytoplasmic mRNA transport, methisoprinol and poly A on the cytopathology developed during a herpes simplex infection of human embryonic lung fibroblasts (WI-38 cells) in tissue culture. For this work, WI-38 cells (passage 17) were obtained from American Type Culture Collection, Rockville, Md., passed twice and grown to monolayer in 25-cm² Falcon flasks employing Eagle's basal medium + 10% fetal calf serum. Monolayers were held for 2 days employing Eagle's minimum essential medium +2% fetal calf serum. Flasks were infected with 4 $TCID_{50}$ of a herpes simplex virus, type 1, that was two passages removed from a severe human oral infection. At 48 hr, drugs were added to the infected flasks. At this time, flasks were just beginning to evince viral cytopathology, the average number of foci/flask being 1·75. Cordycepin was added as 33 μg/ml at 48 hr, a dose found by Sarkar *et al.* [9] to inhibit poly A synthesis and mRNA transport in tissue culture. Methisoprinol was added at 50 μg/ml at 48 hr + 100 μg/ml at 72 hr, in recognition of its rapid metabolism in tissue culture [16]. Poly A was added at 500 μg/ml, a dose found by Nair and Owens [17] to inhibit cordycepin effects on rhinovirus. Flasks receiving both cordycepin and methisoprinol received a single addition of 33 μg/ml of cordycepin and the additions of methisoprinol cited. Flasks receiving both cordycepin and poly A had drugs added as above. All tissue culture incubations were carried out at 37°. There were 13 flasks/experimental group. Control herpesvirus cytopathology and drug effects thereon were evaluated at 100 hr as the average number of infectious foci/flask ± the standard error. Note that the particular herpesvirus strain employed produced lesions that were sufficiently discrete at 4 days to permit this procedure in the absence of an agar overlay.

RESULTS

Effect of methisoprinol on labeling of nuclear and cytoplasmic uridine nucleotides. Methisoprinol did not affect liver weight or liver DNA content. Liver weight averaged 5·9 g/rat for the control group and 5·6 g/rat for the drug-treated group. The average DNA content of both groups was 2·4 mg/g of tissue wet weight.

To evaluate the effects of methisoprinol on RNA metabolism, an examination was made of the size of the precursor uridine nucleotide pools in the cytoplasm and the nucleus, and on the rate of labeling of these pools. Figure 2 shows specific activities of the three uridine nucleotides in the post membrane-poor polysomal supernatant after a single injection of [5-^{3}H]orotic acid. It is apparent that, of the three nucleotides in cytoplasm, UTP is the most rapidly and most intensively labeled. The relative cytoplasmic concentrations were determined to be: UTP, 0·06 μmole/g of tissue wet weight; UDP, 0·10 μmole/g; and UMP, 0·45 μmole/g. As shown in Fig. 2, treatment with methisoprinol did not affect cytoplasmic uridine nucleotide labeling. The drug also did not affect cytoplasmic uridine nucleotide pool size.

It would appear that orotic acid is converted rapidly into uridine nucleotides, as the presence of labeled orotic acid or orotidine monophosphate was detected only at the 15-min determination. There was a paucity of orotidine nucleotides in liver, which is consistent with the findings of Fausto [18].

The specific activity of nuclear UTP at 45 min after isotope injection in the control and drug-treated groups was $1{\cdot}11 \times 10^7$ dis./min/μmole and $1{\cdot}07 \times 10^7$ dis./min/μmole respectively. The presence of UTP in the nuclei was quantitated for the 45-min samples and no reduction by drug treatment was observed.

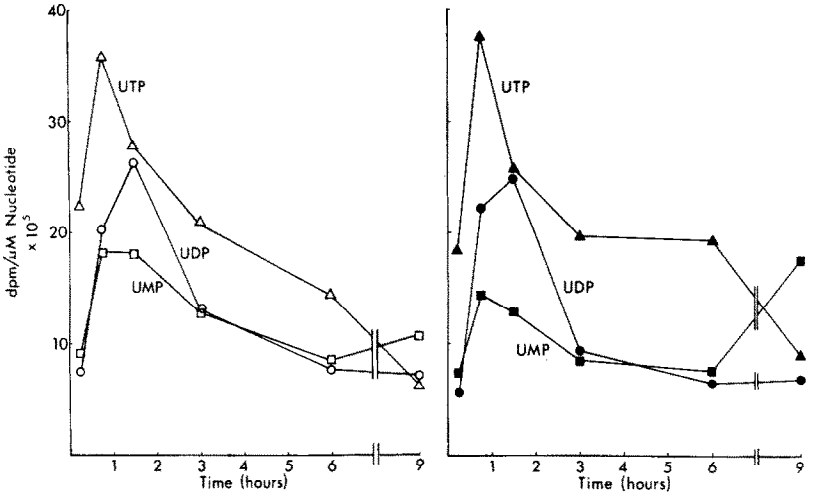

Fig. 2. Average specific activities over three experiments of cytoplasmic uridine nucleotides from livers of control (left) and treated (right) animals receiving a single intraperitoneal injection of 100 μCi [5-^{3}H]orotic acid at 15 or 45 min, 1·5, 3, 6 or 9 hr prior to sacrifice. Five ml of the post-membrane-poor polyribosomal supernatant (S_3) was precipitated with 5 ml of 20% cold perchloric acid. The perchloric acid was removed by adjusting the pH to 7 with 2 N potassium hydroxide and centrifuging. The resulting supernatant was desiccated to dryness under partial vacuum and reconstituted into 1 ml of deionized water. Thirty μl of the sample was injected onto a 0·04 in. × 10 ft stainless steel column packed with pellicular anion exchange resin (Varian Aerograph). The eluent, 0·01 M potassium dihydrogen phosphate, was prepared as a linear gradient with 2 M potassium chloride. The column flow rate was 0·5 ml/min; the retention times for the nucleotides were: UMP (square) = 12·9 min; UDP (circle) = 24·2 min; UTP (triangle) = 40·0 min. The effluent was monitored continuously at 254 nm and the absorption peaks were integrated using a Nester/Faust No. 1504 electronic integrator. The effluent was collected in 0·5-ml fractions, which were counted for radioactivity. The micromolar quantities were calculated from the elution of standards of known concentrations.

Regarding the relative importance of the uridine nucleotides in the labeling of RNA from orotic acid precursor, the specific activity of CTP was measured and found to be less than 5 per cent that of the uridine nucleotides at any time point, which would be compatible with the observation of Meisler and Tropp [19]. Note that nucleotides were 5′-nucleotides. In addition, we examined for 3′-nucleotides; these were not present in any samples.

Effect of methisoprinol on nuclear and polyribosomal RNA metabolism. When consumed at about 500 mg/kg/day, methisoprinol had no effect on the rate of incorporation of [5-^{3}H]orotic acid into RNA extracted from purified nuclei over 9 hr. When consumed at about 2·0 g/kg/day, methisoprinol exerted a small, but reproducible, reduction in incorporation at 45 min post-isotope, so that the average specific activity of treated nuclear RNA across all such experiments was 68 per cent that of control RNA. Contamination of nuclear RNA by protein and DNA were measured using the method of Lowry *et al.* [13] and the diphenylamine [14] method respectively; total contamination was less than 5 per cent of the RNA. Nuclei were examined by light microscopy and were essentially free of visible debris. At all time points, the amount of recoverable nuclear RNA from treated and control groups was similar, the mean value being 0·525 mg/g of liver wet weight.

The contamination of postmitochondrial supernatant (S_1) by nuclear RNA was calculated from the DNA content of this fraction and was found to be 3 per cent or less. Two fractions of polyribosomal RNA, the membrane-rich and the membrane-poor, were isolated by differential centrifugation techniques. The membrane-rich polysomal fraction sediments from the S_1 during centrifugation at 16,750 rev/min for 20 min and contains 15% RNA (dry weight). The membrane-poor polysomal fraction is isolated from the post-membrane-rich polysomal supernatant (S_2) by centrifugation at 40,000 rev/min on a discontinuous sucrose gradient and contains 42% RNA (dry weight).

As shown in Fig. 3, the rate of incorporation of [5-^{3}H]orotic acid into both membrane-rich and membrane-poor polyribosomal fractions is markedly increased by methisoprinol treatment. This effect is most pronounced in the membrane-rich fraction during the first 3 hr after isotope ($P < 0·001$). By 6 hr, drug enhancement of isotope incorporation into cytoplasmic RNA has disappeared, indicating an effect on rapidly labeled polyribosomal RNA, but not on the more slowly labeled polyribosomal RNA components. Data for the later time point suggest that methisoprinol treatment retarded the incorporation of radioactivity into the more slowly labeled polyribosomal RNA species, such as ribosomal RNA. The fact that total polyribosomal RNA was decreased by 20 per cent after methisoprinol treatment (control = 2·50 ± 0·07 mg/kg of liver wet weight) may be relevant to this observation.

Since the drug did not increase the rate of incorporation of radioactivity into nuclear RNA, it would appear that the increased labeling of polyribosomal

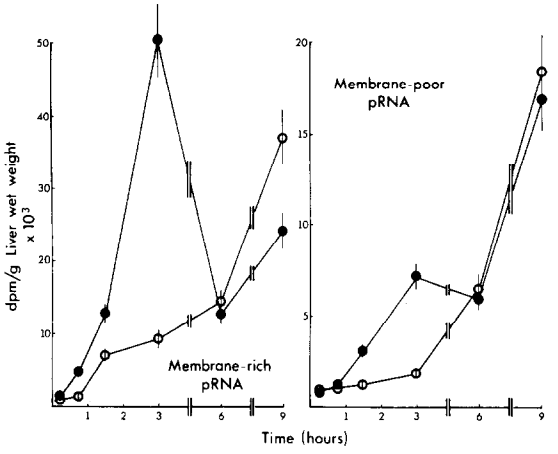

Fig. 3. Specific activity ± S.E.M. of polyribosomal RNA (pRNA) from livers of normal untreated (control) animals (open circles) and animals pretreated for 3 days with 500 mg/kg/day of methisoprinol (closed circles), after a single intraperitoneal injection of [5-^{3}H]orotic acid. pRNA was extracted from polyribosomes of the membrane-rich (left) and membrane-poor (right) fractions as described in Methods. Each point represents an average of four determinations employing three animals/determination.

RNA observed during the first 3 hr (Fig. 3) was the result of one or more post-transcriptional events affected by methisoprinol. The incorporation of labeled orotic acid into the mitochondria was found to be low in comparison to the incorporation into the nuclear or polyribosomal RNA fractions, and was unaffected by drug treatment. Consequently, mitochondrial RNA could not be involved in the changes in rate of labeling of polyribosomal RNA.

When animals consumed about 500 mg/kg/day of methisoprinol, the average degree of labeling of the polyribosomal RNA with [6-^{14}C]orotic acid increased by more than 75 per cent at 45 min. However, when 2 g/kg/day of methisoprinol was consumed, the average degree of labeling of polyribosomal RNA at 45 min was increased by only 34 per cent in the membrane-rich and by 23 per cent in the membrane-poor fraction. On the other hand, in the studies at the higher drug dose, average nuclear RNA labeling was only 68 per cent of control after drug treatment, whereas, at the lower drug dose, no effect was seen in the rate of labeling of nuclear RNA.

From the animals receiving the larger drug dose, polyribosomal RNAs extracted from the cytoplasmic fraction were chromatographed on 5–20% linear sucrose gradients. Elution of these gradients with continuous ultraviolet monitoring of the effluent produced the profiles shown in Fig. 4. These profiles reveal an effect of methisoprinol on the distribution of RNAs in the polyribosomal RNA fractions. In all experiments on membrane-poor polyribosomal RNA fractions, as shown in Fig. 4D, methisoprinol treat-

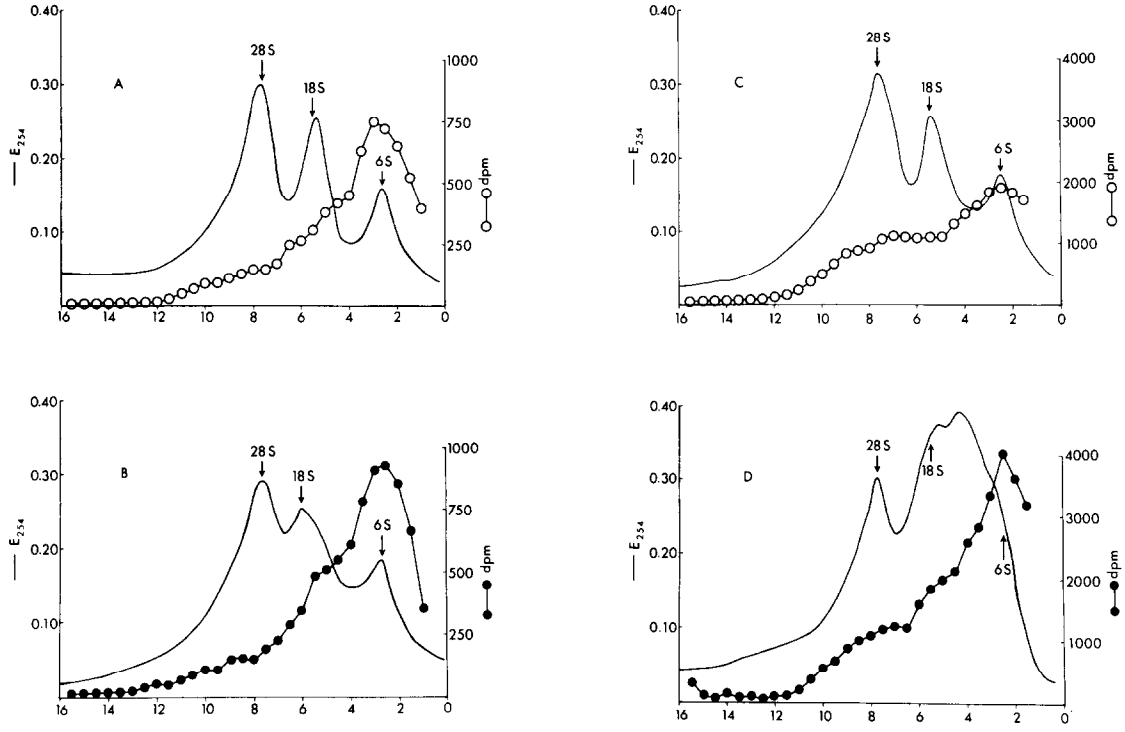

Fig. 4. Typical absorbance and specific activity profiles for polyribosomal RNA (pRNA) from livers of animals injected with [6-^{14}C]orotic acid (5 μCi/100 g body weight) 45 min prior to sacrifice. pRNA was layered on a 16-ml linear sucrose gradient as described in Methods. (A) pRNA from the membrane-rich polysomal fraction from normal untreated (control) animals. (B) pRNA from the membrane-rich polysomal fraction from animals pretreated with 2 g/kg/day of methisoprinol for 3 days prior to isotope injection. (C) pRNA from the membrane-poor polysomal fraction from normal untreated (control) animals. (D) pRNA from the membrane-poor polysomal fraction from animals pretreated with 2 g/kg/day of methisoprinol for 3 days prior to isotope injection.

ment increased the proportion of RNA with sedimentation coefficient between 6S and 18S. Also, the incorporated radioactivity was increased in the 4–10S region, relative to standard incorporated nuclear counts, giving further evidence for an effect on the nucleocytoplasmic transport of rapidly labeled RNA.

Effect of methisoprinol on polyadenylic acid content of polyribosomal RNA. Aliquots of the same RNA fractions described in Fig. 4 were assayed for poly A content by adsorption to poly U-treated glass-fiber filters. The amount of radioactivity adhering to the filters was compared to the total radioactivity of the polyribosomal RNA passed through the filter. For the membrane-poor polyribosomal RNA from control and drug-treated animals, the amount of radioactivity adsorbed represented 0·95 ± 0·10 and 1·52 ± 0·10 per cent of the respective polyribosomal RNAs. That such results reflected an increase in the poly A content of the fraction from drug-treated animals was verified by the employment of a competition technique, accomplished by combining a standard amount of high specific activity tritiated poly A with (a) increasing amounts of non-radioactive poly A, or polyguanylic acid for standardization, or (b) increasing amounts of polyribosomal RNAs, and passing these through the poly U-treated glass-fiber filters. As anticipated, the method was specific, because, in contrast to poly A, increasing amounts of poly G displaced little tritium radioactivity from the filters, findings which are in agreement with those of Sheldon *et al.* [15].

Figure 5 illustrates the effects of increasing amounts

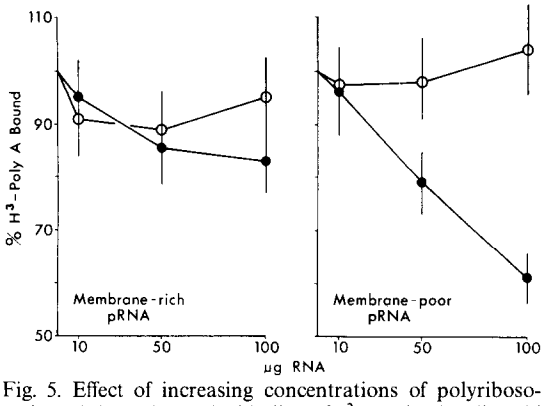

Fig. 5. Effect of increasing concentrations of polyribosomal RNA (pRNA) on the binding of [^{3}H]polyadenylic acid (poly A) to polyruridylic acid (poly U)-treated glass-fiber filters. Gelman glass-fiber filters (25-mm diameter) were pretreated with 0·15 ml poly U, 1 mg/ml, irradiated with u.v. light 20 cm from source for 5 min, and prewashed with 50 ml Tris–NaCl buffer. Increasing amounts of membrane-rich (left) or membrane-poor (right) pRNA from normal (control) rats (closed circles) or methisoprinol (2 g/kg/day for 3 days)-treated rats (open circles) were added to 3·85 nmoles [^{3}H]polyadenylate in 10 ml of 0·05 M Tris–HCl, 0·10 M NaCl, and the solutions passed through the pretreated filters. Filters were washed with 30 ml of Tris–NaCl buffer, 10 ml of cold 10% TCA, and a final wash of 20 ml of 95% ethanol. Filters were dried and placed into 22-ml scintillation vials with 15 ml of scintillation mixture and counted for radioactivity. Each point represents the average radioactivity ± S.E.M. for three determinations. Background counts were determined by passing equivalent amounts of [^{3}H]poly A through untreated filters; the maximum amount of [^{3}H]poly A binding was 2·2 nmoles or 57 per cent of the poly A.

Table 1. Effect of cordycepin, methisoprinol and polyadenylic acid on herpes simplex cytopathology in tissue culture

Treatment	Average FFU at 100 hr*
Control	81·5 ± 19·8†
Cordycepin	14·6 ± 3·8‡
Methisoprinol	24·6 ± 7·5§
Cordycepin + methisoprinol	75·3 ± 17·4
Polyadenylic acid	90·0 ± 18·1
Cordycepin + polyadenylic acid	100·2 ± 20·1

* FFU = focus-forming units, reflecting number of discrete infected foci.
† Data given in average FFU ± S.E.M.
‡ $P < 0.01$.
§ $P < 0.02$.

of polyribosomal RNAs on the binding of [^{3}H]poly A to the poly U-treated glass-fiber filters. As shown in this figure, the control polyribosomal RNAs exhibited only a small tendency to compete with radioactive poly A for adsorption to treated filters.

In contrast, polyribosomal RNAs from methisoprinol-treated animals competed successfully with [^{3}H]poly A for adsorption to treated filters. The capacity to compete with poly A was most pronounced in the membrane-poor polyribosomal RNA fraction. For the system including membrane-poor polyribosomal RNAs, the average per cent [^{3}H]poly A bound, as evaluated for four determinations each of three samples, is highly significantly less in drug-treated samplings than in controls ($P \leq 0.01$). For the membrane-rich samples, the absolute differences at 50 and 100 μg polyribosomal RNA do not reach significance. But, for all membrane-rich polyribosomal RNA samples from drug-treated animals, 100 μg/ml always displaced at least twice the amount of the [^{3}H]poly A as did 10 μg of the same sample. This was never true, however, for control samples. Drug effect, as calculated by this difference, is significant for both membrane-rich ($P < 0.05$) and membrane-poor ($P < 0.001$) polyribosomal RNA. These data provide evidence for a strikingly increased presence of poly A in cytoplasmic polyribosomal RNA after methisoprinol treatment.

Effects of cordycepin and methisoprinol on herpesvirus cytopathology. Both cordycepin and methisoprinol significantly reduced the rate of development of herpesvirus cytopathology in tissue culture between 48 and 100 hr post-infection. As shown in Table 1, while control infected foci rose from an average of 1·75 to 81·5 during this period, flasks receiving cordycepin or methisoprinol rose to an average of 14·6 and 24·6, respectively, which represents a reduction in cytopathology development of 82 and 70 per cent. When methisoprinol and cordycepin were added together, however, no significant reduction in cytopathology was observed relative to control, suggesting a mutual interference. In contrast to methisoprinol, poly A did not inhibit the development of herpesvirus cytopathology. Poly A, however, did prevent the reduction in cytopathology produced by cordycepin, when the two agents were added together. Such an interaction was also found on rhinovirus by Nair and Owens [17].

DISCUSSION

We have previously reported that methisoprinol enhances the incorporation of radioactive orotic acid into polyribosomes of both rat brain *in vivo* [3] and monkey kidney cells in a tissue culture system [2] during a 45-min labeling period. Here, we report that methisoprinol exerts a similar effect on the rate of precursor incorporation into the RNA of rat liver polyribosomes *in vivo*. This increase in the rate of penetrance of precursor radioactivity into the polyribosomal RNA of liver occurred in the absence of an increase in the rate of precursor incorporation into nuclear RNA and in the absence of a net change in the specific activity of both cytoplasmic and nuclear UTP, the immediate precursor of RNA synthesis. In addition, methisoprinol produced no measurable change in the relative concentrations of the uridine nucleotides in either the nucleus or the cytoplasm. We conclude from such considerations that methisoprinol increases the rate of appearance of rapidly labeled RNA in polyribosomes in liver, while not increasing the rate of RNA synthesis and, thus, is apparently affecting one or more post-transcriptional events. We note that mitochondrial RNA synthesis makes no contribution to the increased labeling of polyribosomes by methisoprinol.

In addition, we have presented data indicating that methisoprinol treatment strikingly increases the proportion of 6–18S RNA in the membrane-poor polyribosomal RNA fraction (Fig. 4). The unique pattern of optical density and incorporated counts observed during drug treatment was significant to us because of evidence that RNAs sedimenting in this region are likely candidates for messenger RNA [11, 20], and was of further interest for its specific resemblance to the pattern for the RNA components of the informosome as defined by Schumm and Webb [21] and by Samec *et al.* [22]. The informosome is, of course, the nucleoprotein unit associated with the transport of mRNA from nucleus to cytoplasm [23], whose RNAs include rapidly labeled putative messenger components sedimenting in the 6–15S region and a more slowly labeled 18S component. Note that we have previously reported the chromatographic appearance of an informosome-like unit in aged rat brain after methisoprinol treatment [24], giving further evidence that the methisoprinol effect on RNA metabolism is general.

Other workers have found poly A sequences to be associated with the transport of putative mRNA from nucleus to cytoplasm [8, 25] and with the binding of putative mRNA to ribosomes [26].

We were, of course, aware that poly A sequences occur not in other RNA species, but exclusively in messenger, where poly A is bound to the 3′-terminal [27]. Thus, it was to explore further the possible relationship of mRNA to the RNAs modified by methisoprinol that we examined for the increased presence of poly A in the polyribosomal RNA fractions.

When membrane-poor polyribosomal RNA fractions were examined for effects on their affinity to the poly U-treated glass-fiber filters, methisoprinol was observed to increase binding of counts by 50 per cent when compared to the control. In our present study, labeling with [^{14}C]orotic acid results in radioactivity appearing in the putative mRNA portion of the molecule rather than in the attached poly A sequences. Therefore, this increase in polyribosomal RNA binding can be held to reflect an increased number of putative mRNAs per unit of polyribosomal RNA.

We have concluded from our data that, in the membrane-poor polyribosomal fraction, the relative frequency of mRNAs per unit of ribosome increases in response to drug treatment. Whether the additional mRNAs locate in polyribosomes, reducing the average number of ribosomes per messenger, or whether they locate in informosomal units that contribute to the membrane-poor polyribosome fraction and are simply available to form polysomes is not yet known. The former circumstance would be a distinct anomaly, since polyribosomes by operational definition and structure are held to contain optimal concentrations of mRNA [28]. However, drug treatment has already been found to produce change in polyribosome structure [3]. Thus, as a possible result, change in the relative number of ribosomes in polyribosomes cannot be ruled out.

The examination made of competition between extracted polyribosomal RNA and high specific activity [^{3}H]poly A shows that methisoprinol treatment strikingly increases the poly A content of rat liver polyribosomal RNA. This increase is far greater than one of 50 per cent, which indicates that the polyadenylate nucleotides per unit of mRNA have increased in number in response to treatment.

It appears reasonably certain that, through a poly A-dependent mechanism, then, methisoprinol increases the nucleocytoplasmic transport of mRNA, which reaches cytoplasm in possession of an enhanced oligonucleotide poly A segment.

The significance of the observed effect on nucleocytoplasmic transport of mRNA may be best understood in the light of the natural fate of most mRNA transcripts in eukaryotic cells [21, 29]. Since most of these are produced and destroyed within the nucleus, transport becomes a step that specifically controls the efficiency of gene expression.

It has been reported by Schumm and Webb [30] that certain liver carcinogens that affect nucleocytoplasmic RNA transport increase the labeling of 18S and heavier RNAs by [^{14}C]orotic acid. While we have found methisoprinol to increase the rate of nucleocytoplasmic transport of rapidly labeled RNAs, the distribution of incorporated [^{14}C]orotic acid into 18S and heavier RNAs from methisoprinol-treated animals was unchanged by drug and was consistent with the findings of Schumm and Webb [30], for normal rat liver polyribosomal RNA.

Although we offer no data in this area, we wish to note that, because of chemical similarities between methisoprinol and other purines, it is possible that methisoprinol directly affects the synthesis of nuclear poly A and that the other findings reported here may be coupled to a primary increase in poly A. However, as indicated below, an effect on mRNA-poly A ligase activity is also possible.

We have previously reported [3] that methisoprinol increased the incorporation *in vitro* of radioactive phenylalanine into brain polyribosomes when this incorporation was directed by endogenous mRNA. In-

terestingly, the addition of poly U suppressed the incorporation of phenylalanine into polyribosomes of methisoprinol-treated animals as compared with the incorporation rate measured in the absence of poly U, while poly U did increase the incorporation of phenylalanine into the polyribosomes from control animals. In light of our present findings, this phenomenon may be explained by the increased content of poly A sequences in the polyribosomes from drug-treated animals. Poly U:A binding may produce an inhibition of the translation of mRNAs in this experimental situation.

We have hypothesized that an antiviral effect may arise from the methisoprinol enhancement of certain categories of host RNA utilization and protein synthesis, directly in infected tissues or indirectly through an enhanced immune response. We note reports that methisoprinol inhibits herpesvirus cytopathology, both in tissue culture [3, 4] and in herpes encephalitis in hamster brain [3, 5], and suggest that both effects exemplify a direct antiviral action, since Hirsch and Murphy [31] have shown that suppression of aspects of antiviral immunity with antilymphocyte serum does not modify the progression of encephalitic herpesvirus infection, while it does exacerbate herpes infection exterior to the brain.

Our study employing methisoprinol and cordycepin (3-deoxyadenosine) supports the contention that the enhancement of polysomal poly A and putative mRNA transport by methisoprinol is essential, at least for its direct antiviral effect. Note that cordycepin is an inhibitor of poly A synthesis [8] and, thus, also suppresses poly A-dependent processes, as nucleocytoplasmic mRNA transport [9].

The fact that cordycepin itself exerted an antiviral effect is compatible with the report of Bachenheimer and Roizman [32] that the RNA specified by herpes simplex virus contains poly A sequences that are not transcribed from viral DNA. They are performed and presumably come from the same pool of poly A that is utilized by host messenger. Cordycepin inhibits herpesvirus, then, by reducing the availability of poly A for viral RNA transport to cytoplasm.

Methisoprinol, in contrast, acts to increase this function for host messenger by enhancing the attachment of poly A to host putative mRNA and increasing the transport of this mRNA into cytoplasm. How this event produces an antiviral effect remains a question for which there are still several possible answers. These all concern competition between virus and host mRNAs for the translation apparatus. It may be that the enhanced attachment of poly A to host mRNA leads to a reduced availability of poly A for herpesvirus messenger; or, longer poly A segments on host mRNA may render them more able to form polysomes of greater half life that more effectively generate host proteins over time, including those that contribute to an antiviral state.

Certainly the difference between the effects of poly A and methisoprinol on herpesvirus infection tends to distinguish between biochemical effects of methisoprinol and a general increase in poly A levels.

It is of considerable interest, then, that despite this distinction between the actions of methisoprinol and added poly A, cordycepin and methisoprinol, when added together, produced no antiviral effect, i.e. they nullified each other. This certainly indicates a functional antagonism. From one point of view, we have enough information to understand this antagonism. Thus, an inhibitor of polyadenylic acid synthesis, by reducing nuclear levels of this agent, should reverse the pharmacological actions of an agent, if these depend on an enchanced poly A-host mRNA ligation. However, seen in reverse, the matter is not so clear. There is no reason that is evident at this time why such an effect on ligation should be associated with an inhibition of cordycepin action, unless the effect is secondary to an increase in intranuclear poly A levels. But, if this is the primary action of methisoprinol, then we must be concerned with why an increase in intranuclear poly A levels should be directed more to polyadenylation of host mRNA than to polyadenylation of the RNA of herpesvirus. At this stage, however, we can affirm that cordycepin and methisoprinol give evidence of being biochemical, and consequently functional, antagonists; the details of their interaction in the nucleus remain to be worked out.

The tissue culture experimentation reported here employed human embryonic lung fibroblasts and adds further data illustrating that effects of methisoprinol on poly A metabolism are very general.

Although we have not yet examined methisoprinol effects on the poly A metabolism of lymphocytes, having focused primarily on the study of direct antiviral effects, it appears clear that the effects of the drug are indeed general and that antiviral effects of methisoprinol may also emerge indirectly, from an impact of the drug on the immune system. Lynes [33] has reported that anti-lymphocyte serum inhibited antiviral effects of methisoprinol in mice, while Callaghan and Spitzer* have found that the homocytotropic antibody response to purified ragweed antigen is enhanced by methisoprinol. In unpublished data we have gathered, methisoprinol was found to decrease skin homograft rejection time in mice,† providing further evidence that methisoprinol does affect cell-mediated immunity.

Since poly A is held to actuate the nucleocytoplasmic transport of mRNAs in diverse tissues [9, 21] and to contribute to the stability and activity of mRNAs that are being translated in cytoplasm [34, 35], it is not unreasonable to suggest that the synthesis of certain antiviral principles, in local tissues and lymphocytes, may be enhanced during methisoprinol treatment through a poly A-related mechanism.

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* Personal communication.

† In a mouse study employing light yellow inbred 129/ReJ skin homograft donors and non-inbred Ha/ICR Swiss recipients, 500 mg/kg/day of methisoprinol, initiated at 48 hr, reduced the average control rejection time of 8 days to 5 days, while the immuno-suppressive agent, cyclophosphoramide, increased the average rejection time to 12 days.

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