Inhibition of Translation in Liver Polyribosomes by a New Substituted Thiopseudourea with Antitumour Action

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(Received 21 June 1973)

A new thiopseudourea, *S*-(10-undecen-1-yl)isothiuronium iodide (compound AHR-1911), was tested for antitumour action and shown to inhibit considerably the growth of the Walker carcinoma in rats. The mechanism of its effect on protein and nucleic acid synthesis was then studied with systems *in vitro* from rat liver. In incubations of liver slices, 1.4mm-compound AHR-1911 decreased by 96% the incorporation of [¹⁴C]leucine into microsomal proteins, and mitochondrial protein synthesis measured in the presence of cycloheximide was decreased by 44%. At lower doses, translation, as well as the incorporation of [³H]uridine into RNA, was also considerably impaired, compound AHR-1911 being the most active of all the thiopseudoureas tested whereas undecylenic acid and thiourea by themselves showed practically no inhibition. Protein synthesis by cytoplasmic ribosomes (microsomes and C-polyribosomes) was inhibited by compound AHR-1911 at different concentrations (72% at 0.42mM), and again the other related compounds were much less effective, with the exception of one antileukaemic thiopseudourea. The same occurred with the poly(U)-stimulated incorporation of phenylalanine. The puromycin reaction with pulse-labelled C-ribosomes was strongly inhibited, particularly when preincubation with compound AHR-1911 preceded the addition of puromycin, with no release of nascent chains by the thiopseudourea alone. In the presence of GTP and pH5 fraction, to induce translocation and transform all the ribosomes to the donor state, the percentage inhibition remained the same. The ribosomes incubated with the drug are aggregated, as shown by the polyribosome profile, but, when excess of inhibitor was removed, the activity in protein synthesis and the puromycin reaction was restored, indicating that the inhibition is not due to the polyribosomal aggregation. These results suggest that the effect on translation with both 55S and 80S ribosomes is derived from inhibition of chain elongation at the level of transpeptidation and not translocation, probably together with the interference with transcription playing a role in the antitumour activity.

The thiourea derivatives were initially introduced in pharmacology as antithyroid drugs blocking thyroid-hormone synthesis through the inhibition of iodide oxidation and the oxidative coupling of iodotyrosine residues (see Jorgensen, 1970). They are also antimycobacterial agents active against both tuberculosis and leprosy, and have radioprotective properties probably derived from interaction with cellular components, particularly proteins (Foye, 1970; Lewis & Shepherd, 1970). Recently, a new compound synthesized in our Institute, *S*-(10-undecen-1-yl)isothiuronium iodide (AHR-1911) was found to possess bactericide, fungicide and anti-inflammatory effects (Ercoli *et al.*, 1971).

As some substituted thiopseudoureas assayed by the National Cancer Institute, mainly *S*-(9,10-anthrylenedimethylene)-bisisothiuronium dichloride, have been shown to possess significant inhibitory activity against leukaemia L1210 (Carter, 1968;

National Cancer Institute, Drug Research and Development Chemotherapy, Screening Data Summary), we decided to test compound AHR-1911 for antitumour activity and, if positive, to study its mechanism of action at the molecular and subcellular levels. The present paper demonstrates that compound AHR-1911 effectively inhibits the growth of the Walker 256 carcinosarcoma and interferes with nucleic acid and protein synthesis in liver, the last effect probably at the level of chain elongation.

Materials and Methods

Materials

Chemicals. L-[U-¹⁴C]Leucine (sp. radioactivity 150mCi/mmol) and [5-³H]uridine (sp. radioactivity 5Ci/mmol), were obtained from the Commissariat à l'Energie Atomique, Gif-sur-Ivette, France.

L-3-[U-¹⁴C]Phenylalanine (sp. radioactivity 459mCi/mmol) and U-¹⁴C-labelled L-amino acid mixture (5.4mCi/mg-atom of C) were obtained from Amersham/Searle Corp., Des Plaines, Ill., U.S.A.

ATP (disodium salt, grade I), phosphoenolpyruvate (K⁺ salt), pyruvate kinase (type II), GTP (sodium salt, type III), D-chloramphenicol, sodium dodecyl sulphate, Tris as Trizma base, Triton X-100, cycloheximide, emetine and puromycin were all purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Poly(U) (ammonium salt) was from Miles Laboratories Inc., Kankakee, Ill., U.S.A. Cyclophosphamide (NSC-26271; Endoxan) is the product manufactured by Asta Werke AG., Brackevede, Germany; S-(10-undecen-1-yl)isothiuroniumiodide (AHR-1911), S-(2-propen-1-yl)isothiuronium bromide (TU-47) and S-(cyclopentyl)isothiuronium bromide (TU-40) were synthesized by Professor Nicola Ercoli of this Institute (Ercoli, 1968) and kindly given for the present work. Undecylenic acid was from E. Merck, Darmstadt, Germany, and thiourea from Schiaparelli, Torino, Italy.

S-(2,4,7-trichlorofluoren-9-yl)-isothiuronium bromide (NSC-114670), S-(deoxypodophyllotoxin-1-yl)isothiuronium bromide (NSC-35479) and S-(9,10-anthrylenedimethylene)-bisisothiuronium dichloride (NSC-56054) were kindly provided by Dr. Harry Wood, Jr., of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A.

The glass-fibre filter discs (2.4cm) were Whatman grade GF/A and purchased from H. Reeve Angel and Co. Ltd., London, U.K., or the equivalent grade 934 AH from Reeve Angel, Clifton, N.J., U.S.A. Fluoralloy was purchased from Beckman Instruments Inc., Fullerton, Calif., U.S.A.

Sucrose, toluene, 2-methoxyethanol, propylene glycol and most other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of analytical grade whenever commercially available.

The solutions of ATP, phosphoenolpyruvate, GTP and pyruvate kinase were prepared as described previously (González-Cadavid *et al.*, 1971) except that the enzyme was diluted freshly for each experiment. Double glass-distilled water was used throughout this work.

Animals. Male Sprague-Dawley rats were obtained from a closed colony bred at the Instituto de Medicina Experimental, Universidad Central, Caracas, Venezuela, and were starved for 17–19h before death. Body weights were 180 ± 5 g for the rats used in tumour transplantation, and 140–160g in all other cases.

Methods

Assays of antitumour activity. A strain of Walker 256 carcinosarcoma kept at the Instituto de Medicina

Experimental by subcutaneous transplantation in rats since 1962 was used throughout. The proportion of 'no takes' in our tumour was practically negligible. Rats were injected 24h after tumour transplantation, as described in Table 1, and killed after 12 days by ether anaesthesia followed by decapitation. Tumours were dissected, carefully freed from extraneous tissue and weighed to obtain the ratio treated tumour wt./control tumour wt. For other details see Table 1.

Incubation of liver slices. The liver of one rat was excised, cleaned of extraneous tissue, weighed, and cut into small rectangular pieces. Exactly 1g of tissue for each sample was accumulated on a thin layer of 0.9% NaCl in a beaker at 20–22°C. Slices were cut with a tissue slicer made of Lucite and then transferred to a 100ml conical flask containing 9ml of medium (Peters & Anfinson, 1950) supplemented with 20mM-glucose alone and prepared as described by González-Cadavid *et al.* (1970). The slices were washed at 37°C under a constant flow of water-saturated O₂+CO₂ (95:5) and shaking at 100 oscillations/min. After 20min the medium was decanted and replaced by fresh medium containing the drugs indicated in the respective experiments.

All thiopseudoureas and derivatives tested were dissolved immediately before incubation, either directly in the medium before mixing with NaHCO₃ solution, or first in 0.1ml of propylene glycol and then added to the medium (deoxypodophyllotoxin and trichlorofluoren derivatives). The undecylenic acid was dissolved in 0.1M-NaOH and adjusted to pH7.4 with 0.1M-HCl before dilution with the medium. Radioactivity was added 30min later to the samples and the incubation continued for another 60min. At the end, the incubated liver slices were chilled in an ice bath and separated from the medium by centrifugation at 300g for 10min at 0°C, and suspended in 30ml of 0.30M-sucrose with a Vortex mixer. After the suspension had been standing for 10min at 0°C the centrifugation and suspension procedures were repeated once more.

Subcellular fractionation of liver slices. The washed slices were homogenized in 13ml of 0.30M-sucrose as previously described (González-Cadavid *et al.*, 1970), and 0.5ml samples kept for protein and nucleic acid extraction. A postmitochondrial fraction was obtained in the SS-34 rotor of the Sorvall RC-2 centrifuge at 12000g for 10min. The microsomal fraction was then separated at 165000g for 40min in the rotor 50 of the Spinco L2-65 B ultracentrifuge.

In some instances, when a purified mitochondrial fraction was prepared, the following procedure was applied. The homogenate was spun at 600g for 10min and the sediment was resuspended in 8ml of 0.3M-sucrose (by Vortex mixer) and centrifuged again at 340g for 10min. Both supernatants were pooled and a crude mitochondrial fraction was

obtained by centrifugation at 6800g for 10min. The pellet was then washed with 0.5 and then with 0.25vol. of the original volume of 0.3M-sucrose under the same conditions, and used as 'mitochondrial fraction'. After a fraction that sedimented at 15000g for 15min had been discarded, the microsomal fraction was separated as described above. All fractions were stored at -20°C until further processing.

Preparation of protein and RNA from incubated slices for determination of radioactivity. The microsomal fraction was homogenized in 5ml of 0.5% sodium dodecyl sulphate-0.1M-NaCl-0.01M-Tris-HCl (pH 7.4)-0.001M-EDTA, and suitable samples were measured from this suspension and the whole liver homogenate for measurement of protein by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. Other samples (0.1ml) were adsorbed on top of glass-fibre discs (2.4cm diam.) and processed by a modification of the method of Mans & Novelli (1961), including precipitation with 10% (w/v) trichloroacetic acid, and successive washing with 5% (w/v) trichloroacetic acid (once at 90°C for 15min, and twice at room temperature), ethanol-ether (3:1, v/v), acetone, and ether (twice).

Portions (2ml) of the homogenate were measured and mixed with an equal volume of 1% sodium dodecyl sulphate-0.2M-NaCl-0.02M-Tris-HCl (pH 7.4 at 20°C)-0.002M-EDTA. RNA extraction was performed by applying the hot-phenol procedure of Penman (1966) at 56°C (Wagner *et al.*, 1967). The final precipitate in ethanol was centrifuged at 27000g for 15min and, after removal of the residual ethanol by vacuum, the residue was dissolved in 0.4ml of 0.2% sodium dodecyl sulphate-0.04M-Tris-HCl (pH 7.4 at 20°C)-0.002M-EDTA. A portion (10 μl) was used for RNA measurement at 260nm, by taking $E_{1\text{mg/ml}} = 20.0$ (Staehelin *et al.*, 1963), and the remainder was left for radioactivity counting.

Determination of the uptake of radioactive precursors into the liver slices. Incubations were carried out as described above, except for the following: (a) 400mg of liver slices was used; (b) total volume of incubation medium was 4ml, in 50ml conical flasks; (c) the concentrations of radioactive precursors were 0.40 μCi of [^{14}C]leucine/ml or 4 μCi of [^3H]uridine/ml; (d) the slices separated from the medium after incubation were directly homogenized in 0.3M-sucrose, without any washing, and treated with trichloroacetic acid (10%, w/v, final concn.). Samples (50 μl) were then taken from the incubation medium and the protein-free supernatant for radioactivity determinations.

Isolation of cytoplasmic ribosomes. The microsomal fraction was prepared from the liver of one rat by following essentially procedure 'B' described by González-Cadavid *et al.* (1971), except that the final centrifugation was at 165000g for 40min (rotor 50

of the Spinco L2-65B ultracentrifuge). A detergent-treated ribosomal fraction with a high polyribosome/monoribosome ratio ('C-ribosomes') was prepared from the liver of one or several rats by the method of Wettstein *et al.* (1963) as modified by Ragnotti *et al.* (1969). The pH5 fraction was obtained as described by González-Cadavid *et al.* (1971) and kept at -20°C until use. The microsomal pellets were kept, until incubation, in an ice bath for periods no longer than 1 h. The ribosomal pellets were either kept in a similar way, or stored at -60°C for several weeks, as indicated for each particular experiment. The fractions stored frozen were thawed only once.

Incubation of cytoplasmic ribosomes for protein synthesis. The pellets were gently suspended in the medium B used by Ragnotti *et al.* (1969), containing 0.15M-sucrose, 35mM-Tris-HCl (pH 7.8 at 20°C), 25mM-KCl and 10mM-MgCl₂, clarified by spinning at 2000g for 5min, and the RNA was measured in a 10 μl sample, at 260nm, by using the correction factors proposed by Ragnotti (1971). The RNA concentration was then adjusted to 1.25mg/ml. Incubations were performed in duplicate at 37°C in a medium based on that used by González-Cadavid *et al.* (1971), containing the following constituents: rRNA (250 μg /ml), pH5 fraction (1-1.5mg of protein/ml), sucrose (90mM), Mg²⁺ (6mM), Tris-HCl buffer (pH 7.8 at 20°C ; 21mM), K⁺ (25mM), pyruvate kinase (50 μg /ml), ATP (2mM), phosphoenolpyruvate (10mM), GTP (0.25mM), the inhibitors in the appropriate concentrations, and [^{14}C]leucine (0.25 μCi /ml), all in a total volume of 0.5ml. After 30min, the samples were chilled in an ice bath.

Measurement of binding of compound AHR-1911 by C-ribosomes. C-ribosomes were dissolved in medium B, clarified by centrifugation at 2000g for 5min, and incubated in triplicate in an ice bath in a medium containing rRNA (250 μg /ml), sucrose (90mM), MgCl₂ (6mM), Tris-HCl buffer (pH 7.8 at 20°C ; 21mM), KCl (15mM) and compound AHR-1911 (0.42mM), all in a total volume of 2.0ml. After 30min the ribosomal suspensions were centrifuged as described above and the sediments were suspended in medium B with a Vortex mixer. Thereafter, the procedures followed are explained in the appropriate figures. All spectra were recorded in a Beckman DBG-spectrophotometer with a 10in logarithmic recorder.

Density-gradient analysis of the polyribosome profile. Ribosomal suspensions in the medium described above were layered on a 12.5ml linear sucrose gradient (15-50%, w/v) in 5mM-MgCl₂-50mM-Tris-HCl buffer (pH 7.8 at 20°C)-25mM-KCl. Centrifugation was performed at 201800g in the SW-40 rotor of the Spinco L2-65B ultracentrifuge for 75min at 2°C . The E_{260} was recorded in a Beckman DU-2 spectrophotometer with a 10in logarithmic recorder, by pumping the gradient from the bottom

of the tube at 0.8 ml/min through a flow cell (10 mm light-path).

Incubation of C-ribosomes for polyphenylalanine synthesis. Ribosomal suspensions were prepared as described above for the experiments with protein synthesis. Preincubations for exhausting endogenous mRNA were performed in the same medium as used for protein synthesis, except that total volume was 0.4 ml, no inhibitors or [^{14}C]leucine were present, and all concentrations were 1.25-fold greater to compensate for further dilution. After 20 min the following were added: MgCl_2 to 10 mM, [^{14}C]phenylalanine to $0.5 \mu\text{Ci/ml}$ and poly(U) and compound AHR-1911 as specified in the experiment, so that the total volume was 0.5 ml and all the usual constituents (except MgCl_2) were in the normal concentrations. The incubations proceeded for 30 min more, and then unlabelled phenylalanine was added, to 2 mg/ml, and the samples were chilled in an ice bath.

Incubation of C-ribosomes for the puromycin reaction. A modification of the method of Cannon (1968) was followed. C-ribosomes stored at -20°C were thawed, suspended in medium B and pulse-labelled with [^{14}C]leucine for 5 min, exactly as described for protein synthesis. Eight samples were then pooled and the ribosomes sedimented at 165 000 g for 45 min. After resuspension in medium B and clarification for 5 min at 2000 g, the RNA concentration was adjusted to 1 mg/ml, the appropriate inhibitors were added (0.42 mM-compound AHR-1911 or 0.5 mM-puromycin, or both) and the incubation was performed in quadruplicate in 0.25 ml samples for 5 min at 37°C , followed, when indicated, by a second similar incubation with puromycin. The released radioactivity was separated from the ribosomes by centrifugation at 165 000 g for 60 min and the sediment dissolved in 0.5 ml of medium B containing 0.5% sodium dodecyl sulphate.

In certain instances the incubation with puromycin was performed in the presence of 0.25 mM-GTP and 0.05 ml of pH 5 fraction, in a total volume of 0.25 ml, to effect the translocation of those ribosomes that were in the acceptor state to the donor state. This allows all ribosomes to react with puromycin since the A sites are now totally free to bind the antibiotic.

Preparation of samples from incubations of cytoplasmic ribosomes for determination of radioactivity. In all cases (protein and polyphenylalanine synthesis, puromycin reaction) a portion (0.1 ml) was adsorbed on top of glass-fibre discs and processed as described above. The RNA measurements performed previously were used for protein and polyphenylalanine synthesis to calculate the specific radioactivity, whereas in the puromycin reaction a new RNA determination was performed on the suspensions of the recovered ribosomes in medium B containing 0.5% sodium dodecyl sulphate.

Radioactivity measurements. All the samples on glass-fibre discs were counted for radioactivity in vials with 2 ml of a diluted 'Fluorallory', containing 0.4% of PPO (2,5-diphenyloxazole) and 0.05% of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in toluene. The RNA dissolved in the sodium dodecyl sulphate buffer was counted for radioactivity by adding 10 ml of a scintillation liquid containing 0.4% of PPO and 0.05% of POPOP in 60% toluene-40% 2-methoxyethanol, to the remainder of the solution after the $10 \mu\text{l}$ sample for RNA determination had been removed. The samples for measuring the entrance of radioactive precursors into the liver slices were counted for radioactivity under similar conditions. All the vials were counted for radioactivity in an automatic liquid-scintillation spectrometer (Nuclear-Chicago, model 720) at an 80% efficiency for ^{14}C in the glass-fibre discs and a 15% efficiency for ^3H in solution. The results were corrected to d.p.m. according to the respective efficiencies.

Results

Antitumour action of compound AHR-1911

Our first goal was to examine the effect of compound AHR-1911 on the growth of the Walker carcinosarcoma, one of the solid transplantable tumours used by the National Cancer Institute for their screening programmes (Geran *et al.*, 1973; Schepartz *et al.*, 1967). When compound AHR-1911 was given intramuscularly in two daily doses of 18 mg (200 mg/kg per day) the ratio (treated tumour wt./control tumour wt.) (T/C ratio) was 0.24, and 0.30 when the dose was 133 mg/kg per day (Table 1). No deaths occurred during treatment, although there was a considerable loss of body weight and evidence of pain in the legs, with occasional necrotic foci at the site of injections. At a lower dose (66 mg/kg per day) the antitumour effect decreased, and the same occurred with subcutaneous administration. The intravenous route was rapidly lethal, and the intraperitoneal injection caused some deaths during treatment.

Cyclophosphamide at 0.5 and 1.6 mg/kg per day induced T/C ratios of 0.35 and 0.16 respectively, which compared well with the values of 0.33 and 0.10 calculated from the tumour diameter ratios reported by Tarnowski *et al.* (1966). The administration of compound AHR-1911 reinforced the antitumour effect of cyclophosphamide, giving an inhibition 60% greater than that obtained with cyclophosphamide alone.

Inhibition of transcription and translation in liver slices by thiopseudoureas

Having established the antitumour effect of compound AHR-1911 we then faced our main objective,

Table 1. *Antitumour effect of compound AHR-1911 on the growth of the Walker 256 carcinosarcoma*

Groups of three rats (nos. 1-7) bearing Walker tumours transplanted 24h before were injected twice daily for 11 days, intramuscularly in the legs, with 0.12 ml of propylene glycol containing compound AHR-1911. Other similar groups (nos. 9 and 11) were treated with compound AHR-1911 in a single dose per day during the afternoon plus one daily intraperitoneal injection of cyclophosphamide during the morning (0.2 ml of a freshly prepared solution in 0.15 M-NaCl). The respective control groups (nos. 8 and 10) received cyclophosphamide only. As general controls 24 untreated tumour-bearing rats were used. When the rats were killed, on the twelfth day, tumour weight was 27.3 ± 5.3 g and body weight was 241 ± 16 g. This corresponds to an increase of 34 g in body weight (after subtraction of the tumour weight). All values are averages, s.e.m. values being given only for experiments with six or more animals. The treated tumour wt./control tumour wt. ratios (T/C ratio) were obtained by comparing these values with the average of the single control group. All rats (control and treated) survived until killed.

Group	No. of rats (total)	Daily dose (mg)		Animal wt. variation* (treated)	T/C ratio
		Compound AHR-1911	Cyclophosphamide		
1	3	36	—	-27	0.24
2-5	12	24	—	-25 ± 13	0.30 ± 0.09
6-7	6	12	—	-16 ± 9	0.57 ± 0.19
8	3	—	0.08	+25	0.35
9	3	12	0.08	-2	0.14
10	3	—	0.25	+40	0.16
11	3	12	0.25	-2	0.07

* Average net weight change of test group (gross weight change minus tumour weight) after 12 days of treatment.

Table 2. *Effect of compound AHR-1911 on mitochondrial and microsomal protein synthesis in liver slices*

Preincubation of liver slices was performed for 30 min in the presence or absence of cycloheximide (1.78 mM) and compound AHR-1911 (1.4 mM) as specified below. A mixture of ^{14}C -labelled amino acids was added to the samples with cycloheximide (8 μCi), or without the antibiotic (4 μCi), and the incubation proceeded for 60 min. For other details on the incubation procedure, subcellular fractionation and counting of radioactivity see the text. Values are averages of two separate experiments.

	Sp. radio-activity of control (d.p.m./mg of protein)	Inhibition by compound AHR-1911 (% compared with control)
Without cycloheximide		
Mitochondrial fraction	1150	88
Microsomal fraction	6635	96
With cycloheximide		
Mitochondrial fraction	128	44
Microsomal fraction	254	61

Table 2 shows that at a concentration of 1.4 mM there is a 96% inhibition of microsomal protein synthesis and that when the incubation was carried out in the presence of cycloheximide, to eliminate the contribution of cytoplasmic ribosomes to the synthesis of mitochondrial proteins, the inhibition of this process inside the organelle, although lower, was still significant. This experiment proves that both 55S and 80S ribosomes are sensitive to compound AHR-1911, the difference in reactivity probably being derived from a partial barrier to the entrance of the drug into the mitochondria.

At one-half the concentration originally tested, the inhibition of protein synthesis was still higher than 80%, and the incorporation of [^3H]uridine into microsomal RNA was similarly affected (Table 3). At 0.14 mM there was still a slight inhibition of both processes. When the free pools of radioactive precursors in the slices were measured and the results referred to the total radioactivity added, neither the [^{14}C]leucine (6.5%) nor the [^3H]uridine (10.9%) pools were affected by compound AHR-1911 (0.7 mM), thus showing that this compound does not inhibit the uptake of the precursors into the tissue.

When other thiopseudoureas and related compounds were tested in liver slices at 0.70 mM concentration, compound AHR-1911 was by far the most active in inhibiting not only translation but transcription as well. Fig. 1 shows a 76% decrease in microsomal protein and nucleic acid synthesis induced by the drug, whereas one of the constituents of its structure, thiourea, was only slightly inhibitory, and the other component, undecylenic acid, even

that is to define the mechanism of its action at a biochemical level. For studying protein and nucleic acid synthesis *in vitro* we preferred to use the well-defined systems obtained from rat liver instead of those from the Walker carcinosarcoma. As a first approximation we examined the effect of the drug on the incorporation of ^{14}C -labelled amino acids into protein by incubations of liver slices,

Table 3. *Effect of different concentrations of compound AHR-1911 on protein and nucleic acid synthesis by liver slices*

Slices were incubated as described in Table 2, except that three different concentrations of compound AHR-1911 were used, no cycloheximide was present, and 8 μ Ci of 14 C-labelled amino acids and 36 μ Ci of [3 H]uridine were added per flask.

Concn. added (mm) ..	Sp. radioactivity of control (d.p.m./mg)	Inhibition by compound AHR-1911 (% compared with control)		
		1.40	0.70	0.14
Protein				
Whole homogenate	5560	93	85	26
Microsomal fraction	10815	93	82	21
RNA				
Whole homogenate	28200	79	43	—
Microsomal fraction	39070	96	80	36

Fig. 1. *Effect of different thiopseudoureas and related compounds on (a) RNA and (b) protein synthesis by liver slices*

Slices were incubated as described in Table 2, except that no cycloheximide was present, and 4 μ Ci of 14 C-labelled amino acids plus 36 μ Ci of [3 H]uridine was added per flask. All drugs tested were at a concentration of 0.70 mM. Values are averages of three separate experiments, the range of variation being indicated by a bar, except for the control and compound AHR-1911, where the means are taken from five experiments and are $\pm$ S.D. Control values (in d.p.m./mg) are: 2302 $\pm$ 570 (total protein); 4354 $\pm$ 980 (microsomal protein); 47550 $\pm$ 10930 (total RNA); 50840 $\pm$ 10900 (microsomal RNA). $\square$, Whole homogenate; $\blacksquare$, microsomal fraction. TU, thiourea, UA, undecylenic acid; PPTU, *S*-(deoxypodophyllotoxin-1-yl)bisthiouronium bromide; TCFTU, *S*-(2,4,7-trichlorofluoren-9-yl)-isothiuronium bromide.

stimulated the incorporation of radioactive precursors. Two thiopseudoureas synthesized by Dr. N. Ercoli, TU-40 (cyclopentyl derivative) and TU-47

Table 4. *Effect of compound AHR-1911 on protein synthesis by liver polyribosomes*

The microsomal fraction and the C-ribosomes were prepared freshly in separate experiments as described in the text. Incubations, preparation of protein for counting and determination of radioactivity, were as indicated in the text, except for the omissions listed in the table. Compound AHR-1911 was present at a concentration of 0.42 mM, emetine at 0.18 mM and chloramphenicol at 0.16 mM. Results were calculated by subtracting zero-time values and are averages of two separate experiments for each fraction, with triplicate determinations, except for C-ribosomes with compound AHR-1911, where five experiments were considered. In this case the incorporation in the control samples was 38810 and 58035 d.p.m./mg of RNA for the microsomal fraction and the C-ribosomes respectively.

Additions to or omissions from the system	Inhibition compared with control (%)	
	Microsomal fraction	C-ribosomes
Emetine	93	79
Chloramphenicol	0	8
Minus energy source	80	83
Minus ribosomes	91	—
Minus pH 5 fraction	66	—
Zero time	95	80
Compound AHR-1911	81	78 $\pm$ 8.2

(propenyl derivative) were moderately inhibitory. As for the drugs tested by the National Cancer Institute for antitumour activity, it is noteworthy that both podophyllotoxin and trichlorofluoren thiopseudourea inhibited total protein and microsomal protein synthesis by only 25–40%, and stimulated very remarkably the incorporation of [3 H]uridine into microsomal RNA. The most potent antitumour agent, *S*-(9,10-anthrylenedimethylene)-bisthiouronium, unfortunately could not be assayed, since it is insoluble in the medium used for

incubation, even in the presence of propylene glycol (10%).

Inhibition of translation on ribosomes by thiopseudoureas

Since compound AHR-1911 affects both protein and nucleic acid synthesis we decided to approach first the mechanism of its action on translation. The subcellular test systems for protein synthesis were the microsomal fraction and the C-ribosomes, differing in that the endoplasmic-reticulum membranes were present in the former. Table 4 shows that both systems behave as expected, since the well-known inhibitor of 80S ribosome function, emetine, blocked the protein-synthesizing activity nearly completely, whereas chloramphenicol, which should not act on extramitochondrial ribosomes in eukaryotes, practically did not affect the incorporation. The omission of ribosomes or an energy system, or incubation at 37°C (zero time), blocked protein synthesis, although a considerable residual activity was left in the systems lacking pH 5 fraction, probably owing to the adsorption of soluble factors and aminoacyl-tRNA molecules on the ribosomes. Compound AHR-1911 was again a rather potent inhibitor,

since incorporation of [¹⁴C]leucine was decreased by about 80% in the microsomal fraction and the C-ribosomes. Again, its two constituents, undecylenic acid and thiourea, affected the activity of the system only slightly, and KI had virtually no effect (Table 5), ruling out the possibility that the I⁻ was responsible for the effect of compound AHR-1911. The other two thiopseudoureas, TU-40 and TU-47, were much less inhibitory on C-ribosomes than on liver slices.

The antitumour agents from the National Cancer Institute were tested in systems containing 10% propylene glycol to allow a satisfactory solution of drugs otherwise difficult to dissolve in water (particularly the anthrylene derivative). As shown in Table 5, two of them, the podophyllotoxin and trichlorofluoren derivatives, were moderately inhibitory, and the most active antileukaemic drug, the anthrylene derivative, almost completely blocked the protein-synthesizing ability of the isolated polyribosomes.

Compound AHR-1911 at 0.58mM inhibited protein synthesis by 90–95%, and at 0.18mM by about 50%, as compared with the 80–90% inhibition exerted by 0.18mM-emetine (Fig. 2). The curves relating percentage inhibition to concentration of compound AHR-1911 were parallel and nearly coincident for the microsomal fraction and the C-ribosomes. Fig. 2 also illustrates a very important feature of the action of compound AHR-1911 on ribosomes, the aggregation of polyribosomes induced by simple contact with the drug. When clarified C-ribosomes were incubated with different concentrations of compound AHR-1911 in an ice bath in the absence of pH 5 fraction and an energy system, the suspensions looked perfectly homogeneous, but on centrifugation at 2000g for 5min a substantial amount of the RNA was sedimented, in contrast with the controls where the sediment is negligible. The plot relating the percentage precipitation to the concentration of compound AHR-1911 does not follow the shape of the one for the inhibition of protein synthesis, which suggests that the two effects are not linked. This ribosomal aggregation deserves special attention, however, and a series of experiments (presented below) was performed to decide whether it has any relation to the interference with translation.

Ribosomes programmed with exogenous mRNA are also inhibited by compound AHR-1911, as is shown in Table 6. Poly(U) in two different concentrations stimulated the incorporation of phenylalanine by C-ribosomes whose endogenous mRNA was decreased by incubation under conditions of protein synthesis. Compound AHR-1911 decreased this stimulation by approx. 70%, a value comparable with the inhibition obtained in the same experiments for the ribosomes programmed with endogenous mRNA.

Table 5. *Effect of thiopseudoureas on protein synthesis by liver polyribosomes*

Only C-ribosomes were used in these experiments. Control values and other details for (a) are exactly as in Table 4. The drugs were present at a concentration of 0.42mM. In (b) the ribosomes used had been stored at -60°C and the thiopseudoureas were first dissolved in propylene glycol at 4.2mM and then added to the incubation medium. Final concentration of propylene glycol was 10% (v/v). The incorporation in the control samples was 25010 and 26530 d.p.m./mg of RNA for the samples with and without propylene glycol respectively.

	Inhibition compared with control (%)
(a) Without propylene glycol	
KI	6
Undecylenic acid	23
Thiourea	12
TU-40	18
TU-47	19
(b) With propylene glycol	
Compound AHR-1911	72
S-(9,10-Anthrylenedimethylene)- bisisothiuronium dichloride	96
S-(Deoxypodophyllotoxin-1-yl)- bisisothiuronium bromide	46
S-(2,4,7-Trichlorofluoren-9-yl)- isothiuronium bromide	46

Fig. 2. Effect of different concentrations of compound AHR-1911 on protein synthesis and ribosomal aggregation in the microsomal fraction and C-polyribosomes

Details are as given in Table 4, except that the concentrations of compound AHR-1911 were varied as indicated. The controls were those tabulated in Table 4. Separate samples of the polyribosome suspensions were incubated in triplicate at 0°C in the medium for protein synthesis, except that no pH 5 fraction or energy system was present. After 30 min the ribosomal suspensions were centrifuged at 2000g for 5 min, the sediments were suspended in medium B and the RNA concentration was measured at 260 nm. ○, Inhibition of protein synthesis by C-polyribosomes; □, inhibition of microsomal protein synthesis; ●, precipitation of RNA.

Inhibition of transpeptidation on ribosomes by compound AHR-1911

The elongation stage in translation can be studied in a simplified version through the so-called 'puromycin reaction'. It consists essentially in the simulation of the creation of a peptide bond between the carboxyl end of the growing peptide chain attached to tRNA in the P site (donor state) and an incoming molecule of puromycin bound to the A site, so that peptidyl-puromycin is formed and released from the ribosome (see, e.g., Cannon, 1968; Pestka, 1971; Vázquez, 1972; Pestka *et al.*, 1972). The reaction takes place at the peptidyl-transferase site of the larger ribosome subunit, and any drug interfering with it is assumed to inhibit some step in chain elongation that can be identified precisely by selecting the experimental conditions. Of the many versions of this reaction, some implicating short pieces of tRNA, as in the 'fragment reaction' (Vázquez, 1972; Monro, 1971), and others (Pestka *et al.*, 1972; Gottesman, 1971), we have selected a

Table 6. Effect of compound AHR-1911 on the poly(U)-directed synthesis of polyphenylalanine by C-ribosomes

C-ribosomes were preincubated to decrease the content of endogenous mRNA, and then incubated with [¹⁴C]-phenylalanine, with or without poly(U), as described in the text. Results were calculated by subtracting zero-time values (less than 2% of controls) and, for the samples with 100 µg of poly(U)/ml, are averages of two separate experiments. The stimulation factor [with poly(U)/without poly(U)] was 2.18 in the controls.

	Sp. radio-activity of control (d.p.m./mg of RNA)	Inhibition by compound AHR-1911 (% compared with control)
Expts. 1 and 2		
[100 µg of poly(U)/ml]		
With poly(U)	38 135	71
Minus poly(U)	16 950	76
Difference	21 185	67
Expt. 3		
[200 µg of poly(U)/ml]		
With poly(U)	60 840	67
Minus poly(U)	23 900	64
Difference	36 940	68

very simple one (Cannon, 1968) based on the release of growing peptide chains from ribosomes in the absence of added factors and which requires only the correct ionic environment for the function of peptidyl transferase.

Table 7 shows that when no GTP was present and only ribosomes in the donor state can react with puromycin, 27% of the labelled chains were released in the reaction. This is lower than the 35–40% released under similar conditions from *Escherichia coli* ribosomes (Cannon, 1968), but is still perfectly adequate for testing the effect of compound AHR-1911 on it. The drug does not act like puromycin, since no radioactivity was solubilized by incubating the labelled ribosomes with 0.42 mM-compound AHR-1911. However, it inhibited the puromycin reaction by an average of 49% when ribosomes were treated simultaneously with both agents. The individual inhibitions were rather variable, suggesting that there is a competition in which the results vary according to whether or not compound AHR-1911 acts before puromycin. That this might be the case is suggested by the nearly complete inhibition of the puromycin reaction when it was preceded by an incubation with compound AHR-1911. All these results indicate that compound AHR-1911 inhibits chain elongation, probably by interfering with peptidyl transfer.

To see whether translocation is affected as well, the puromycin reaction was performed in the presence of GTP and supernatant factors to induce

Table 7. *Effect of compound AHR-1911 on the puromycin reaction with C-ribosomes in the donor state*

C-ribosomes stored at -60°C were used for these experiments and pulse-labelled with [^{14}C]leucine for 5 min as described in the text. Formation of peptidyl-puromycin was performed directly (first incubation) with or without compound AHR-1911, or after (second incubation) treatment with compound AHR-1911, as described in the text. In one instance, the release of nascent chains by compound AHR-1911 alone was tested. Values are averages of 5–11 separate experiments (determinations in triplicate). The percentage of radioactivity released was calculated for each particular experiment, and the standard deviations were obtained accordingly.

Expt. no.	Inhibitors added during		Sp. radioactivity (d.p.m./mg of RNA)	Radioactivity released in soluble form (%)	Inhibition of the puromycin reaction (%)
	First incubation	Second incubation			
1–11	—	—	4404 ± 303	—	—
1–6	Puromycin	—	3220	27.0 ± 8.2	—
7–11	Compound AHR-1911	—	4420	0.0 ± 0.1	—
1–5	Puromycin + compound AHR-1911	—	3805	13.7 ± 7.8	49
7–11	Compound AHR-1911	Puromycin	4310	2.3 ± 2.8	93

Table 8. *Effect of compound AHR-1911 on the puromycin reaction with C-ribosomes in the acceptor state*

The reaction was carried out as described in Table 7, except that when indicated the incubation with puromycin was performed in the presence of 0.25 mM-GTP and 0.05 ml of pH 5 fraction (in a total volume of 0.25 ml), with another batch of pulse-labelled ribosomes. Values are averages of two separate experiments performed in triplicate.

Inhibitors added during		Sp. radioactivity (d.p.m./mg of RNA)	Radioactivity released in soluble form (%)	Inhibition of the puromycin reaction (%)
First incubation	Second incubation			
—	—	5710	—	—
—	Puromycin	4583	19.7	—
—	Puromycin + GTP + pH 5 fraction	3466	39.3	—
Compound AHR-1911	Puromycin	5490	3.8	81
Compound AHR-1911	Puromycin + GTP + pH 5 fraction	5280	7.5	81

Fig. 3. *Effect of thiopseudoureas on the polyribosome profile*

C-polyribosomes were incubated under conditions of protein synthesis (see the Materials and Methods section and Table 4) in two separate experiments except for the following: (1) no [^{14}C]leucine was present; (2) incubation was for 10 min, at either 0°C (a) or 37°C (b); (3) the samples contained compound AHR-1911 (a and b), or other thiopseudoureas (c), all at 0.42 mM. A sample of each (0.75 ml a and b, 1 ml for c) was layered on the gradient and the polyribosome profile obtained as described in the Materials and Methods section. In (a) and (b) the gradient was from 20 to 55% (w/v) sucrose. The proportion of the E_{260} recovered as pellets in the bottom of the tube, referred to the input, was 25% in the controls and 60% in the compound AHR-1911-treated samples. No similar estimation was done in (c). (a) Incubation at 0°C : —, control; ----, compound AHR-1911. (b) Incubation at 37°C : curves as in (a). (c) Incubation at 0°C : —, TU-40; ----, thiourea; ·····, TU-47. The bottom of the tube is on the right.

translocation and transform those ribosomes in the acceptor state, unable to bind puromycin, to the donor state. Table 8 shows that the proportion of radioactivity released was doubled, resembling the situation with *E. coli* ribosomes (Cannon, 1968), but again at a lower value (40% for liver compared with 65% for *E. coli*). The puromycin reaction in the donor state was strongly inhibited by compound AHR-1911, but after translocation the effect remained the same instead of increasing as should be the case if the ribosomes with compound AHR-1911 did not translocate as efficiently as control ribosomes.

Relation between ribosomal aggregation and inhibition of translocation induced by compound AHR-1911

The aggregation of polyribosomes induced by compound AHR-1911 posed, however, a fundamental question of whether the inhibition of translation was due to a physical inability of these altered ribosomes to participate in protein synthesis rather than to a more subtle effect at the level of chain elongation. Two arguments stand against this possibility. The first is the lack of correspondence between the degree of precipitation and the inhibition of protein synthesis. The second is the fact that ribosomes and subunits aggregated and even agglutinated by ethanol can perform the puromycin reaction, that is to say peptidyl transfer, very efficiently (Monro, 1971; Monro *et al.*, 1971). The best answer, however, would come from the demonstration that ribosomes aggregated by compound AHR-1911 are still able to carry out transpeptidation in the absence of excess of inhibitor. Fig. 3 shows that the aggregation effect is indeed very serious, since the polyribosome profile disappeared almost completely after incubation of polyribosomes with compound AHR-1911 in the medium used for protein synthesis (pH5 fraction and energy source) either at 0°C (Fig. 3a) or 37°C (Fig. 3b). The mono-ribosome and diribosome peaks were affected in the same way. In contrast, neither thiourea nor TU-40 nor TU-47 acted on the polyribosome distribution (Fig. 3c).

The ability of polyribosomes aggregated by compound AHR-1911 to carry on protein synthesis on removal of most of the inhibitor was demonstrated by the experiments detailed in Table 9. The ribosomes sedimented at 10000g-min after incubation with the drug at 0°C were resuspended in medium B, RNA was determined and they were tested for the incorporation of [¹⁴C]leucine into protein and for the puromycin reaction. In three different experiments the protein-synthesizing ability was restored to 73–102% of the control value, with an average of 90%. In the puromycin reaction, the antibiotic released the nascent chains from the aggregated ribosomes with the same efficiency as from the controls, indicating

Table 9. Restoration of the protein-synthesizing ability of polyribosomes treated with compound AHR-1911 on removal of most of the inhibitor

C-ribosomes stored at –60°C and thawed only once were used. The pellets obtained from incubations with 0.42 mM-compound AHR-1911 as described in the text were suspended again in 0.3 ml of fresh medium B, measured for RNA, and incubated in triplicate at an RNA concentration of 250 µg/ml under conditions of protein synthesis (see the text and Table 4). For the puromycin reaction, pulse-labelled ribosomes were incubated as described in Table 7, except that after treatment with compound AHR-1911 the ribosomes were sedimented at 2000g for 5 min, resuspended in 2 ml of fresh medium B, measured for RNA, and incubated in triplicate at an RNA concentration of 1 mg/ml with puromycin. When samples of the compound AHR-1911-treated ribosomes resuspended in medium B were centrifuged again under similar conditions, the amount of RNA precipitated was 78% of the total.

(a) Incorporation of [¹⁴C]leucine into protein

Expt. no.	Control ribosomes (d.p.m./mg of RNA)	Compound AHR-1911- treated ribosomes (% of control)
1	14680	73
2	8488	102
3	11367	97

(b) Puromycin reaction

Expt. no.	Sp. radioactivity of ribosomes (d.p.m./mg of RNA)	Radioactivity released in sol- uble form by puromycin (%)	
		No treatment	Compound AHR- 1911-treated ribosomes
4	12370	29	29

a normal performance for transpeptidation. That removal of the compound AHR-1911 in solution does not change the physical state of the treated ribosomes is shown by the fact that after a new centrifugation at 10000g-min, almost all were again sedimented. This contrasts with the homogeneous appearance of the ribosomal suspension.

Features of the aggregation effect induced by compound AHR-1911

The aggregated ribosomes, either from one or two centrifugations, had a spectrum closely resembling that of the control, except for a slight deformation in the zone at 225 nm, as referred to the peaks at 260 or 210 nm (Fig. 4a). This zone corresponds to the extinction maximum of compound AHR-1911 (strictly speaking 224 nm) (Fig. 4b), suggesting that a small amount of the drug remained bound to the sedimented ribosomes. As compound AHR-1911 does not absorb at all at 260 nm, the E_{225}/E_{260} ratio

Fig. 4. Binding of compound AHR-1911 by liver polyribosomes: spectra

C-ribosomes stored at -60°C were used for these experiments, incubated at 0°C with compound AHR-1911 and centrifuged at $10000g$ -min as described in the Materials and Methods section. The sediments were suspended in 0.3 ml of medium B. One sample (A) was centrifuged and resuspended as before. The other (B) was kept as such. Portions ($50\text{ }\mu\text{l}$) were diluted to 3 ml with medium B and the spectra recorded. A sample containing 1.66 mg of RNA/ml was used as a control after a similar dilution (C). Panel (a): —, sample A; ----, sample B; — · —, sample C. Panel (b): ----, supernatant from sample A diluted 1:10; —, compound AHR-1911 in medium B at $10\text{ }\mu\text{g/ml}$ ($28\text{ }\mu\text{M}$).

should apparently serve as an estimate of the binding. However, slight variations in the proportion of protein in the ribosomes are reflected in changes in the 210 nm peak, and thus at 225 nm . It is therefore preferable to establish an internal ratio within this peak, e.g. E_{225}/E_{210} , which should automatically compensate for the differences between batches of ribosomes. Any excess in this ratio for the treated ribosomes over the control ratio should correspond to compound AHR-1911. In this way the amounts of drug bound per mg of ribosome were calculated as ranging from 30 to $45\text{ }\mu\text{g}$ (Table 10). This represents only approximate estimates, but they are likely to be the result of binding and not of contamination with the drug remaining in solution, since after a second centrifugation the ratio did not change very significantly.

The extent of binding does not appear to depend on the compound AHR-1911 concentration, at least between 0.14 and 0.42 mM , which is understandable considering that even at the lowest

concentration the amount presumably bound is less than 30% of the total compound AHR-1911. This contrasts with the direct relation between concentration and RNA precipitation (see Fig. 2), suggesting that a fixed ratio of drug to ribosomes is necessary for precipitation, and that the proportion of ribosomes attaining this threshold will depend on the concentrations of compound AHR-1911 and RNA.

The precipitating effect of compound AHR-1911 (0.42 mM) appears to be rather specific since neither albumin (1 mg/ml), liver tRNA (0.25 mg/ml) nor liver ribosomal RNA (0.25 mg/ml), checked by polyacrylamide-gel electrophoresis, were aggregated at 0°C . A suspension of highly purified liver mitochondria ($250\text{ }\mu\text{g}$ of protein/ml) prepared by the procedure of O'Brien & Kalf (1967) was practically unaffected, either as such or after ultrasonication, as judged from the proportion of mitochondrial protein sedimented at $2000g$ for 5 min (25% in the control, 35% in the compound AHR-1911 sample). The ribosomal aggregation was not due to a change in

Table 10. *Binding of compound AHR-1911 by liver polyribosomes: quantitative ratios*

The spectrophotometric ratios were calculated from the spectra in Fig. 4 and others recorded under similar conditions. Extinction coefficients (1 mg/ml) are taken as 13.4 for ribosomes at 260 nm (Von der Decken *et al.*, 1970) and 44.7 for compound AHR-1911 at 225 nm. Values for 210 nm correspond to the maximum of the peak, irrespective of its exact position.

	No. of experiments	Spectrophotometric ratios		Calculated ratio (μg of compound AHR-1911/mg of ribosomes)
		E_{210}/E_{260}	E_{225}/E_{210}	
(1) Control polyribosomes	9	1.96 ± 0.17	0.45 ± 0.02	—
(2) Polyribosomes treated with compound AHR-1911 (150 $\mu\text{g}/\text{ml}$), centrifuged and resuspended	9	1.95 ± 0.20	0.53 ± 0.03	47
(3) As in (2), but second resuspension	2	1.75	0.52	41
(4) As in (2), but 100 μg of compound AHR-1911/ml	6	2.12 ± 0.13	0.50 ± 0.02	30
(5) As in (2), but 50 μg of compound AHR-1911/ml	5	2.11 ± 0.05	0.52 ± 0.02	41
(6) Liver tRNA	1	1.06	0.45	—
(7) Bovine serum albumin	1	26.1	0.44	—
(8) Compound AHR-1911	1	—*	0.43	—

* $E_{260} = 0$.

pH of the medium, since it remains unaltered, does not depend on the Mg^{2+} concentration (range investigated 2–20 mM), and did not increase during contact for 5–30 min at 0°C . The pH 5 fraction, however, was partially aggregated by compound AHR-1911 and 35% of its protein, as measured by the Lowry *et al.* (1951) procedure, sedimented at 10000 g-min. In contrast, the pH 5 fraction supernatant, neutralized to pH 7.4 and clarified, did not precipitate at all on incubation with compound AHR-1911, in spite of having twice as much protein as the pH 5 fraction.

Discussion

We tested the antitumour effect of compound AHR-1911 on only one system, which is less sensitive to chemotherapy than the widely used lymphoid leukaemia L-1210 (Schepartz *et al.*, 1967; Geran *et al.*, 1973). However, the drug proved to be nearly as active as *S*-(9,10-anthrylenedimethylene)-bis-isothiuronium, a thiopseudourea which considerably increases the survival time of mice bearing the L-1210 lymphoid leukaemia and other kinds of leukaemias, as well as inhibiting the growth of some solid tumours such as sarcoma 180 or P-1798 lymphosarcoma (National Cancer Institute, Drug Research and Development Chemotherapy, Screening Data Summary). According to the same source another thiopseudourea active against L-1210 lymphoid leukaemia, *S*-(deoxypodophyllotoxin-1-yl)-isothiuronium, only slightly affects the growth of the Walker carcinoma in non-lethal doses, and the third drug supplied by the National Cancer Institute for the

biochemical section of the present study, *S*-(2,4,7-trichlorofluoren-9-yl)-isothiuronium, does not seem to have been tested on our system. Of the several hundred pseudoureas that have been screened in the National Cancer Institute programme, the four that exhibited antitumour activity were those detailed above, plus *S*-[(10-methyl-9-anthryl)methyl]-isothiuronium chloride (NSC 146109), a compound that could not be supplied to us by the National Cancer Institute (H. B. Wood, Jr., personal communication). Although we did not intend to perform here a thorough study of the antitumour effect of compound AHR-1911 and we therefore used only a limited number of animals, the results are conclusive and leave no doubts in this respect.

Our main objective, however, was to study the molecular basis of the antitumour effect of compound AHR-1911, and in the present work we have demonstrated that it affects both transcription and translation in normal liver, being more active than other thiopseudoureas tested, except *S*-(9,10-anthrylenedimethylene)-bis-isothiuronium. This latter drug apparently forms interaction complexes with DNA, inhibiting the synthesis of both DNA and RNA *in vivo* in bacteria, as do ethidium bromide and proflavin (Saunders & Saunders, 1970). This may be the primary mechanism of action responsible for its growth-inhibitory activity.

It is clear that compound AHR-1911 interacts with ribosomes at two levels: one, requiring a very small amount of the drug, causes ribosomal aggregation, probably through formation of a complex with the protein constituents of the ribosomes, since ribosomal RNA is not precipitated by com-

pound AHR-1911. These aggregates are perfectly able to carry on transpeptidation and protein synthesis in the absence of excess of inhibitor. The hypothetical complexes with ribosomal proteins may result from cross-linking through bifunctional hydrogen bonds with peptide groups or interactions with non-polar amino acid chains, such as has been proposed for thiourea and several proteins, mainly plasma albumin (Gordon & Jencks, 1963; Gordon & Warren, 1968). The effect of compound AHR-1911 is, however, rather specific, since no ribosomal aggregation is caused by thiourea alone, and it is known that alkyl substitution decreases the effectiveness of the compound in denaturing plasma albumin (Gordon & Jencks, 1963). Moreover, the denaturing action of thiourea and related drugs is exerted at concentrations much higher than those used in the present work (e.g. greater than 500mM, compared with 0.4mM for compound AHR-1911). The existence of a certain target specificity is also shown by the fact that the proteins from the pH5 fraction supernatant, bovine albumin and mitochondrial proteins are not precipitated by compound AHR-1911, although it is remarkable that it aggregates about 35% of the pH 5-fraction proteins.

The other main effect of compound AHR-1911 is the inhibition of translation, exerted by higher amounts of the drug, which the puromycin experiments suggested is derived from interference with chain elongation and not translocation. The precipitation of soluble factors by compound AHR-1911 is not responsible for this inhibition, since the puromycin reaction was performed in the absence of the pH5 fraction.

The invaluable co-operation of Dr. N. Ercoli and Dr. H. Wood, Jr., in providing the pseudothiureas tested is most gratefully acknowledged. Tumour transplantation and animal treatments were efficiently performed by Miss Emilia Pérez Ayuso, and the rats were provided by the Instituto de Medicina Experimental (U.C.V.), to whom our thanks are given. This work was supported by grant DF-S1-063 from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT). F. H. Q. was a pre-doctoral fellow from the same institution.

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