trast, the LMW-kininogen behaved as a ~52,000 dalton protein in both non-dissociating and dissociating conditions.

Earlier, we reported an apparent immunologic identity between the two kininogens, using immunodiffusion [9]. Similar observations were made by Komiya *et al.* [8] with bovine kininogens. As reported in this paper, when tested functionally (kinin liberation) antibody against one kininogen can neutralize both kininogens.

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Division of Experimental Pathology, Departments of Pathology, Medicine and Medical Genetics, and Institute of Immunology, University of Toronto, Toronto, Ontario, M5S 1A8, Canada

FLAVIO M. HABAL*
BRIAN J. UNDERDOWN
HENRY Z. MOVAT†

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* Holder of an MRC-Studentship.
† MRC Research Associate.

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Binding of vinblastine *in vitro* to ribosomes of Sarcoma 180 cells*

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Among the many effects of vinblastine (VLB) on cell growth is the induction of helical arrays of ribosomes [1, 2]. The association of such ribosomal complexes with fine, granular, electron-dense material has been reported [2, 3]. On the basis of its faint linearity and the presence of occasional tubular cross sections, it has been suggested that this material consists of newly formed microtubule precursor protein organized into crystals by VLB during its synthesis on the adjacent polysomes. Other work indicates, however, that VLB also may act directly upon ribosomes in the induction of such complexes. Polyribosome arrays appear in *Escherichia coli*, which is considered to contain no microtubules [1]. VLB has been shown to precipitate 65 per cent of chick oviduct ribosomes *in vitro* when added at a high concentration (3×10^{-3} M) [4]. Furthermore, Stebbings [5] has noted a considerable reduction in the numbers of ribosomes in oocytes treated with VLB that was not attributable to inhibition of RNA synthesis. Finally, various inhibitors of protein biosynthesis failed to prevent completely the formation of ribosomal aggregates [3]. The present study finds that VLB binds ribosomes *in vitro*, and attempts to assay this binding for its effect upon ribosome function.

Sarcoma 180 (S180) cells in culture (Fisher's medium with 10% horse serum) were grown in the presence of 10 μCi [^{14}C]uridine (407 mCi/m-mole) for 24 hr to label ribosomes. These cells (100×10^6) were then collected in log phase by centrifugation, washed once in saline and resuspended in 2 ml of LS buffer [10 mM KCl, 20 mM Tris–HCl (pH 7·5), 1·5 mM $MgCl_2$, 1·5 mM β-mercaptoethanol, 10 μg/ml PVS] where they were allowed to swell for 10 min at room temperature; this cell suspension was then homogenized in a Potter homogenizer. In this and in other experiments, all further procedures were performed at 0°. Unbroken cells, nuclei and mitochondria were sedimented by centrifugation at 10,000 rev/min for 6 min, sodium deoxycholate was added (0·5%) and the postmitochondrial supernatant centrifuged at 80,000 *g* for 130 min to sediment the ribosomes as a near-transparent aggregate. The ribosome pellet was rinsed three times with LS buffer to remove traces of detergent, resuspended in buffer, centrifuged again at 80,000 *g* for 130 min and resuspended in 0·20 ml of LS buffer. Subunits were prepared from the ribosome pellet isolated as above by washing with HS buffer [880 mM KCl, 50 mM Tris–HCl (pH 7·5), 12·5 mM $MgCl_2$,

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20 mM β-mercaptoethanol, 10 μg/ml PVS], resuspension in 0·2 ml HS buffer, incubation at 37° for 5 min to decrease formation of subunit aggregates, and clarification by centrifugation at 6750 rev/min for 10 min.

Suspensions (0·2 ml) of ribosomes in LS buffer or subunits in HS buffer were incubated with 0·1 μCi [^{3}H]vinblastine (44·8 mCi/m-mole) for 30 min at 0°. The incubated suspension was gently layered onto a precooled 12·0-ml 5–20% sucrose gradient containing LS buffer with a 50% sucrose cushion, and then centrifuged in a Spinco SW-36 rotor at 24,300 rev/min for 2 hr in the case of ribosomes, and at 30,000 rev/min for 150 min in the case of subunits. Fractions (0·4 ml) were collected from the gradient with an ISCO gradient fractionator, and the radioactivity of each fraction was counted in a Packard Tri-Carb liquid scintillation counter with automatic external standardization. Counting efficiencies were close to 20 per cent for ^{3}H and 70 per cent for ^{14}C. As seen in Fig. 1, VLB (1×10^{-5} M) binds both ribosomes and their subunits with sufficient strength to survive velocity gradient centrifugation. Unbound drug rests in the top 1·6 ml of the gradient, but the [^{3}H]VLB peaks at higher sucrose concentration, well separated from the initial ^{3}H label, correspond with the [^{14}C]uridine peaks and hence represent bound drug. Furthermore, it can be seen in Fig. 2 that, although the drug binds both subunits, there is a marked specificity for the smaller subunit. The fact that VLB remains associated with ribosomal subunits suggests that the observed binding is to ribosome material as opposed to nascent polypeptide or endogenous mRNA, presumably released upon dissociation of monosomes. The data of Fig. 3 indicate that the VLB–ribosome complex is sensitive to urea. At concentrations of both 1 and 2 M, considerably less drug is associated with the ribosome peak, and correspondingly more unbound VLB appears at the top of the gradient. This is in contrast to the relative insensitivity of the microtubule interaction to urea [6].

The procedure used for measuring cell-free protein synthesis was modified from that of Zomzely-Neurath [7]. A postmitochondrial supernatant, prepared as above but without ^{14}C label and in a buffer containing 0·25 M sucrose, 25 mM KCl, 4 mM $MgCl_2$ and 0·05 M Tris HCl at pH 7·4, was centrifuged at 80,000 *g* to give large microsomes and a postmicrosomal (S100) supernatant. Ribosomes were isolated as described above but without ^{14}C label and in a different buffer (0·25 M sucrose, 25 mM KCl, 4 mM $MgCl_2$, and 0·05 M Tris–HCl, pH 7·4); where endogenous mRNA was employed as message, these ribosomes did not undergo further processing. In the polyuridylic acid (poly U)-directed amino acid incorporating system, ribosomes were dissociated into their subunits and then reconstituted in order to release endogenous mRNA. Isolation of endogenous mRNA was based on the procedure of Evans and Lingrel [8]. Polysomes were isolated from S180 cells carried in Swiss mice. Erythrocytes were first lysed by exposure to hypotonic saline (0·2%) and the cells were resuspended in Fisher's medium with 10% horse serum and grown in culture. Cells harvested in log phase were allowed to swell in LS buffer for 5 min, homogenized, and unbroken cells and nuclei were removed by centrifugation at 5000 rev/min for 5 min. After addition of deoxycholate to a final 0·5%, the supernatant was layered onto precooled 12·0-ml 5–20% sucrose gradients containing LS buffer and centrifuged in an SW 36 rotor at 24,300 rev/min for 2 hr. The polysome fractions were pooled, dissociated with 0·5% sodium dodecyl sulfate and fractionated on 5–20% sucrose gradients containing 5 mM Tris-HCl buffer (pH 7·4). The peak fractions containing the mRNA were pooled and concentrated by centrifugation. The supernatant up to the bottom 1 ml of each tube was decanted, 11 ml of 5 mM Tris-

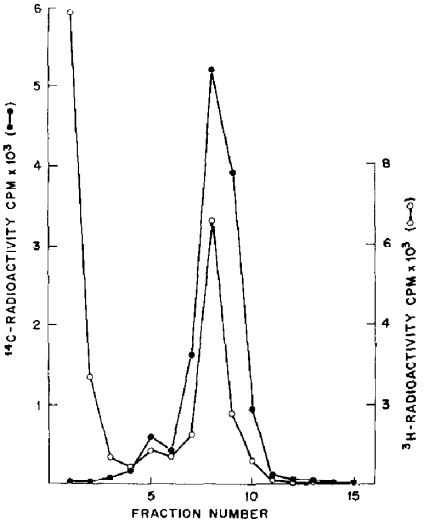

Fig. 1. Sedimentation of ^{14}C-labeled ribosomes with bound [^{3}H]VLB through a sucrose gradient. In this and all subsequent gradient profiles, the direction of sedimentation is from left to right.

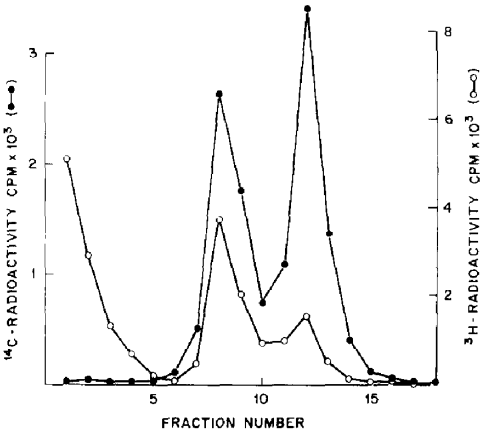

Fig. 2. Sedimentation of ^{14}C-labeled ribosomal subunits with bound [^{3}H]VLB through a sucrose gradient.

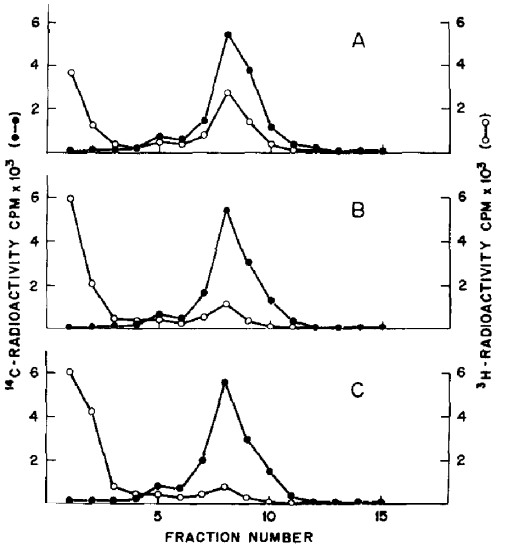

Fig. 3. Inhibition of VLB–ribosome binding by urea. A, Control; B, 1 M urea; C, 2 M urea. In B and C, ribosomes were incubated with the drug in the presence of urea for 30 min at 0° before sucrose gradient centrifugation.

HCl buffer (pH 7·4) was added to lower the sucrose concentration to less than 5%, and each tube was recentrifuged as before. This procedure was repeated twice more to further dilute the sucrose which inhibits mRNA-directed incorporation. For assay of protein synthesis, each reaction mixture contained 0·05 M Tris–HCl (pH 7·6), 2 mM phosphoenolypyruvate, 0·1 mg phosphoenolpyruvate kinase, 0·1 ml ^{12}C-amino acid mixture (19 L-amino acids minus phenylalanine at 2 mM each in 12 mM $MgCl_2$, 0·10 M KCl, 0·05 M Tris–HCl, pH 7·6), 1 μCi [U-^{14}C]phenylalanine (464 mCi/m-mole), 12 mM $MgCl_2$, 0·10 M KCl, 6·66 mM ATP, 0·20 mM GTP, and 15 μg poly U or endogenous mRNA in a final volume of 1 ml. Ribosomes (0·5 mg) were added at 0°, in some instances after incubation for 30 min at that temperature with an appropriate concentration of VLB. Then 0·2 mg of the postmicrosomal fraction (made 12 mM with respect to Mg^{2+} and 0·10 M with respect to K^+) was added and the mixture incubated at 37° for 30 min. Incorporation was stopped by adding 10 ml of cold 10% trichloroacetic acid. Each tube was heated at 90–95° for 20 min to hydrolyze amino-acyl tRNA, chilled in ice for 30 min, and the precipitates were dispersed by vigorous agitation and collected on millipore filters (0·45 μm pore size). After drying, the radioactivity on these filters was counted as above.

The results of the amino acid incorporation study with poly U appear in Table 1. The cell-free system, both with and without VLB treatment, synthesized acid-insoluble product; there was a small (16·2 per cent) but reproducible depression in the presence of VLB (10^{-6} M). When whole ribosomal preparations containing endogenous mRNA were used, VLB also lowered incorporation of amino acids into acid-insoluble material in a concentration-dependent fashion, rising from 8·2 per cent at 10^{-6} M to 20·0 per cent at 10^{-4} M and 33·0 per cent at 10^{-3} M respectively.

The biological importance of these data depends upon the concentration of drug that is attainable within the cells. In the study presented in Table 2, [^{3}H]vinblastine was added to cultures of S180 cells in log phase (1×10^5 cells/ml) at a concentration of 10^{-5} M and 1400 cpm/ml. At various times thereafter, 40-ml aliquots were removed and the cells pelleted. The supernatant was decanted, the inside walls of the centrifuge tube were wiped dry, and the cells were dissolved in 0·75 ml of 0·2 N NaOH. The radioactivity of 0·2 ml of each such solution and the growth medium was counted. To estimate cell volume, various amounts of S180 cells were pelleted in graduated Shevky-Stafford and McNaught centrifuge tubes. This procedure neglects interstitial space, and hence gives an upper limit for cell volume. There was a 9-fold concentration of the alkaloid intracellularly during the time period studied. The initial rate of uptake was 1·6 μmoles/liter of cells/min. It should be noted that the drug is also concentrated in Ehrlich ascites carcinoma cells [9], human leukocytes [10] and platelets [11].

Thus, VLB binds ribosomes and ribosomal subunits *in vitro*. The interaction shows a considerable degree of preference for the smaller subunit, and is sensitive to urea.

Table 1. Effect of VLB on poly U-directed amino acid incorporation

VLB concn (M)	[^{14}C]phenylalanine incorporated (cpm)	% Inhibition
0	22,166	
10^{-7}	21,023	5·2
10^{-6}	18,571	16·2
10^{-5}	15,387	30·6
10^{-4}	11,559	47·9
No poly U	406	
No ribosomes	70	

Table 2. Uptake of [^{3}H]VLB S180 cells

Time (min)	Intracellular VLB concn* ($\times 10^{-5}$ M)
0	1·43
10	1·90
30	6·20
45	8·09
60	7·62
90	8·15
120	7·73
150	9·22
180	8·75

* The concentration of [^{3}H]VLB in the external medium was 1×10^{-5} M. Cells were separated by centrifugation and dissolved in 0·2 N NaOH for counting.

Both these observations suggest that VLB binds directly to ribosomal material, for the association of VLB with cytoplasmic microtubule protein is unaffected by urea. This binding may well be involved in formation of the ribosome aggregates observed in VLB-treated cells. Although the inhibition of translation seen in this study required relatively high drug levels, it may be significant *in vivo*, for the drug is accumulated in a number of cell types to levels greatly in excess of its concentration in the surrounding medium. An effect on translation may aid in understanding inhibition of protein biosynthesis by VLB under conditions where incorporation of uridine into RNA is unaltered [12]. Further studies aimed at elucidating whether the subunit specificity may be reflected in specific inhibition of the synthesis of certain forms of protein are warranted. As vincristine is also known to induce such complexes [2, 13], it would be of interest to determine if vincristine is also bound by ribosomes in a fashion similar to VLB.

Departments of Pharmacology and Internal Medicine, Yale University School of Medicine, New Haven, Conn. 06510, U.S.A.

BARRY SWERDLOW
WILLIAM A. CREASEY

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