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Enhancement of cross-linking of presynaptic plasma membrane proteins by phospholipase A_2 neurotoxins

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β-Bungarotoxin (β-BuTX*) and notexin are members of a group of snake venom toxins which have phospholipase A_2 (PLA_2) enzymatic activity and whose neurotoxicity is due to a presynaptic action. These neurotoxins alter the release of acetylcholine in the peripheral nervous system and the release of many neurotransmitters (i.e. acetylcholine, γ-aminobutyric acid, norepinephrine, and serotonin) in the central nervous system (for reviews, see Refs. 1–4). The mechanism(s) by which β-BuTX and notexin alter neurotransmitter release, however, is not completely understood. PLA_2 activity alone cannot account for neurotoxicity since there is no direct relationship between the enzymatic activities and neurotoxicities of these neurotoxins and chemically related, non-neurotoxic PLA_2 enzymes (e.g.

* Abbreviations: β-BuTX, β-bungarotoxin; BS^3, bis-(sulfosuccinimidyl) suberimidate; FFA, free fatty acids; PLA_2, phospholipase A_2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and SPM, synaptic plasma membrane(s).

Naja naja atra PLA_2) [4–6]. This issue, however, is controversial, and there is an equal amount of literature in support of and against a role for PLA_2 enzymatic activity in the neurotoxicity of β-BuTX and notexin [7–12]. Since these neurotoxins have PLA_2 enzymatic activity, and PLA_2 activity has been shown to alter membrane fluidity and order [13, 14], it was postulated that these neurotoxins might exert their toxicity via a site-directed alteration in plasma membrane organization. There is evidence for a biological role of lateral motion of certain proteins in cell membranes [15]. β-BuTX and notexin could, thus, act via a site-directed phospholipid hydrolysis, to selectively alter the neighbor relationships between proteins by altering their ability to migrate laterally within the membrane. Such a role for the non-enzymatic B chain of β-BuTX in guiding the PLA_2 active A chain to the presynaptic membrane has been reviewed [16]. Alternatively, β-BuTX and notexin could act via non-enzymatic mechanisms to alter protein–protein interactions by site-directed effects on synaptic plasma membrane (SPM) proteins themselves. For example, the binding of β-BuTX to a presynaptic, voltage-sensitive K channel [17] could alter the lateral mobility of this channel protein and enhance or reduce its ability to associate with neighboring proteins. Although the protein composition of the synaptic plasma membrane has been studied [18–24], the effects of presynaptic PLA_2 neurotoxins and enzymes on the spatial relationships of proteins in this membrane have not been studied. Cross-linking reagents have been used to examine the spatial relationships of proteins in SPM [25] and synaptic vesicles [26]. The following studies were, therefore, designed to examine the effects of β-BuTX and notexin on the cross-linking of SPM proteins. We used the cross-linker bis (sulfosuccinimidyl) suberimidate (BS^3) which is an impenetrable homo-bifunctional reagent [27].

Materials and Methods

Materials. Ficoll (type 400-DL) was obtained from the Sigma Chemical Co. (St. Louis, MO). Lyophilized *Bungarus multicinctus* snake venom was purchased from the Miami Serpentarium Laboratories (Salt Lake City, UT). β_1-BuTX was isolated [28] from the snake venom of *B. multicinctus* by Dr. C-C. Yang at the Institute of Life Sciences, National Tsing-Hua University, Hsinchu, Taiwan; R.O.C. Notexin and *N. n. atra* PLA_2 were purchased from Ventoxin (Frederick, MD). The purities of the neurotoxins and enzyme were confirmed by amino acid analysis and/or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by Dr. Yang, by Ventoxin, and in our laboratory. Their estimated purities were all greater than 98%. BS^3 was purchased from the Pierce Chemical Co. (Rockford, IL). All electrophoretic reagents, including molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA). All other reagents were of analytical grade.

Preparation of synaptosomes. The cerebral cortex was dissected from the brains of decapitated male Sprague–Dawley rats (200–225 g) and homogenized in 0.32 M sucrose (containing 10 mM phosphate and 10 mM EDTA, pH 7.4) using a Ten Broeck tissue grinder. Synaptosomes were isolated from the homogenate by differential and discontinuous sucrose–Ficoll gradient centrifugations [29]. Following isolation, synaptosomes were suspended in a physiological buffer (buffer A) of the following composition (mM): 119.7 NaCl, 23.4 $NaHCO_3$, 0.36 $MgSO_4$, 3.15 KCl, 0.45 NaH_2PO_4, 2.97 urea, and 2.25 $CaCl_2$, pH 7.4 [30].

Cross-linking of synaptosomal plasma membrane proteins. Synaptosomes were suspended (approximately 10 mg protein/mL) in buffer A, and treated for 20 min at 37° with 5 and 50 nM β-BuTx and notexin and 0.5, 5, and 50 nM *N. n. atra* PLA_2. The reaction was terminated with 8 vol. of cold 10 mM EDTA in 0.32 M sucrose and 10 mM phosphate (pH 7.4) followed by cooling on ice. The resultant suspension was centrifuged (78,000 *g*, 30 min) and the synaptosomes were resuspended in 0.11 M phosphate (pH 7.4) and cross-linked with 8 mM BS^3 for 30 min at 25°. These incubation conditions were based on those established by Staros [31], and it was established in preliminary experiments that this concentration of BS^3 produced the desired degree of cross-linking where both increases and decreases in cross-linking intensity could be readily observed. The cross-linking reaction was terminated by a 10-fold dilution with excess free amino groups (25 mM Tris–HCl/150 mM NaCl, pH 7.4) and centrifugation (17,000 *g*, 15 min). SPM were isolated from treated and control synaptosomes by hypoosmotic lysis followed by differential and discontinuous sucrose gradient centrifugations [32] and stored frozen prior to SDS–PAGE. All solutions and incubation media contained 1 μM leupeptin, 0.3 μM aprotinin, and 1.3 mM EDTA (EDTA was not present during treatment with the PLA_2 neurotoxins and enzyme unless otherwise indicated). Free fatty acid (FFA) production following the various treatments with the PLA_2 neurotoxins and enzyme was assessed as previously described [6, 12]. Protein was determined by the method of Lowry *et al.* [33] as modified by Markwell *et al.* [34], using bovine serum albumin as a standard.

SDS–PAGE and analysis of results. Proteins were analyzed by SDS–PAGE (5.5% stacking and 11% resolving gels; 100 μg protein/lane) in the presence of 50 mM dithiothreitol, according to the method of Laemmli [35]. Proteins were fixed in the gels, stained with Coomassie Brilliant Blue R-250 and destained, all in methanol/acetic (40%/10%). The gels were then air dried for 24–48 hr in BioGelWrap mounted in plexiglass frames (Bio-Design Inc., Carmel, NY). The dried gels were analyzed using a soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA) driven by an IBM-compatible personal computer. Biomed SLR 2D/1D laser scanning programs were used in the quantitation of protein bands and for the estimation of molecular weight with the following standards being used (kDa): phosphorylase B (97.4), bovine serum albumin (66.2), ovalbumin (45.0), and carbonic anhydrase (31.0). The effects of cross-linking on protein band intensity and of the PLA_2 neurotoxins and enzyme on cross-linking were quantitated and compared based on the changes in protein band peak areas and peak percent distribution, which were calculated by the laser scanner. Peak area is expressed in arbitrary units and represents the total area of a given peak based on absorbance while the percent distribution is the percent of the total protein on the lane that is allotted to a given peak. The percent distribution, therefore, takes into consideration the variation in the total amount of protein which enters the gel.

Statistical comparisons. Statistical significance was determined using an analysis of variance followed by Duncan's multiple range test. The statistical analysis was run under the release 82.2B of SAS (SAS Institute Inc., Cary, NC) at The University of Connecticut. All data are presented as means ± SEM, N = 3. Correlations between two parameters were determined by calculating a correlation coefficient (r) as described by Zar [36]. A p value of < 0.05 was taken as the level of significance for all comparisons.

Results and Discussion

The effects of BS^3 on protein cross-linking, and of β-BuTX, notexin, and *N. n. atra* PLA_2 on cross-linking by BS^3 are shown in Fig. 1. BS^3 (8 mM) produced an overall decrease in protein band intensity below 200 kDa as well as the formation of cross-linked products (> 200 kDa peaks; Figs. 1 and 2, Table 1). Within the limitations of the methodology employed, there did not appear to be any selective cross-linking of any given protein band or group of bands by BS^3. β-BuTX, notexin, and *N. n. atra* PLA_2 (5 and 50 nM) produced a concentration-dependent increase in the cross-linking of proteins by BS^3 (Fig. 1, Table 1).

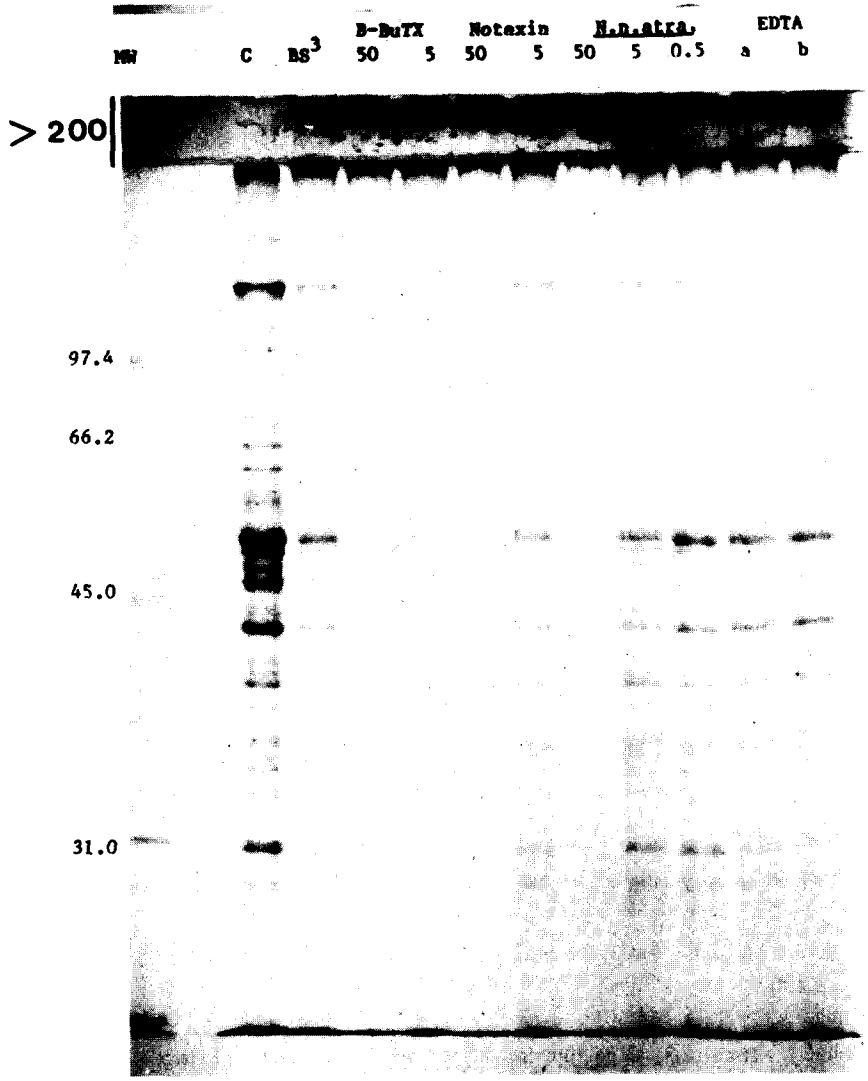

Fig. 1. SDS–PAGE of SPM proteins from BS^3-cross-linked synaptosomes pretreated with PLA_2 neurotoxins and enzyme. SPM protein (100 μg) was applied to each lane of the gel. The stacking gel was left attached to the gel and is shown at the top. Molecular weight (MW) standards are indicated to the far left. The effects of EDTA (10 mM) in the absence of Ca^{2+} are shown in the last two lanes to the right: (*a*) indicates coincubation of EDTA with 50 nM β-BuTX and (*b*) indicates coincubation of EDTA with 50 nM *N. n. atra* PLA_2. This is a representative gel from three experiments. The variation between experiments is indicated by the standard errors provided in Table 1.

N. n. atra PLA_2 (0.5 nM) had no effect on protein cross-linking while a 50 nM concentration of this enzyme produced almost 100% cross-linking of SPM proteins (Fig. 1, Table 1). As with the cross-linking by BS^3, there did not appear to be any selective alteration in the corss-linking of any particular protein band when synaptosomes were pretreated with the PLA_2 neurotoxins and enzyme (Figs. 1 and 2, Table 1). The effects of β-BuTX and *N. n. atra* PLA_2 (50 nM) on cross-linking by BS^3 were blocked by 10 mM EDTA (Fig. 1, Table 1), suggesting a role for PLA^2 enzymatic activity, since Ca^{2+} is required for enzymatic activity [37].

In an effort to quantitate the effects of the PLA_2 neurotoxins and enzyme on protein cross-linking by BS^3, the dried gels were scanned using a soft laser densitometer. An example of such a scan is shown in Fig. 2. As described above, 50 nM notexin, for example, induced a further overall decrease in protein band area (increase in cross-linking), resulting in almost complete cross-linking of all SPM proteins (Fig. 2). To further quantify these cross-linking data, four protein peaks were selected for a more detailed analysis. These peaks, > 200, 52, 45, and 36 kDa, are indicated in Fig. 2, and some of the results of this analysis are shown in Table 1. For the 52 (Table 1) and 45 kDa (results not shown) peaks, the following order of potencies in decreasing protein band area (increasing cross-linking) were observed for 5 and 50 nM treatments; *N. n. atra* PLA_2 = β-BuTX ≥ notexin > control + BS^3 > control − BS^3, while only 50 nM notexin altered the cross-linking of the 36 kDa peak by BS^3 (results not shown). Significant

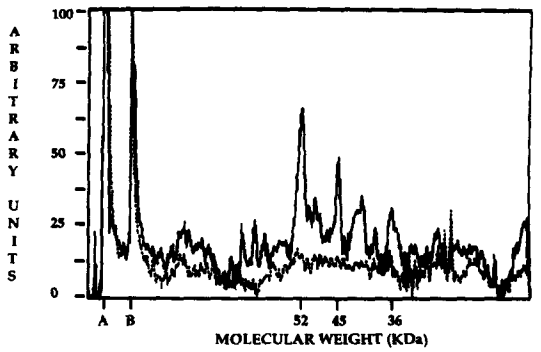

Fig. 2. Densitometric scan of SDS–PAGE of proteins from SPM prepared from BS^3-cross-linked synaptosomes treated with notexin. Synaptosomes were pretreated with 50 nM notexin and cross-linked; SPM were isolated, and proteins analyzed by SDS–PAGE. The dried gel was scanned using a soft laser densitometer and the resultant scans are shown. The variation between scans is indicated by the standard errors provided in Table 1. The stacking gel was left attached to the gel and the peaks of these proteins which did not enter the separating gel (> 200 kDa) are indicated on the left side of the scan as A and B. The 52, 45, and 36 kDa protein peaks selected for quantitation are also indicated. The solid line is the protein distribution in SPM following cross-linking of synaptosomes with BS^3 (8 mM) and the dotted line is the protein distribution in SPM following pretreatment of synaptosomes with 50 nM notexin and cross-linking with BS^3 (8 mM).

correlations between PLA_2 enzymatic activity and PLA_2 (neurotoxin- and enzyme)-induced cross-linking by BS^3 (results not shown) were found for the 52 kDa ($r = -0.93$; $P < 0.001$), 45 kDa ($r = -0.80$; $P < 0.002$) and 36 kDa ($r = -0.78$; $P < 0.005$) peaks. The extent of phospholipid hydrolysis in synaptosomes by 50 and 0.5 nM PLA_2 neurotoxins and enzymes has been reported [12]. The values for 5 nM concentrations are as follows (mean ± SEM, N = 3, 20-min incubation): control, 0.064 ± 0.008; β-BuTX, 0.149 ± 0.025; notexin, 0.137 ± 0.020; *N. n. atra* PLA_2, 0.188 ± 0.031 μmol FFA liberated/mg synaptosomal protein. The extent of hydrolysis by 50 nM β-BuTX and notexin and 5 and 50 nM *N. n. atra* PLA_2 in SPM has also been published [6].

It was expected that as the PLA_2 neurotoxins and enzyme decreased the lower molecular weight protein band intensities, they would, in turn, increase the formation of cross-linked products (> 200 kDa protein bands). Therefore, the percent of the total protein on the gel which is in the > 200 kDa bands was examined (see Materials and Methods for further explanation). The percent distribution of the > 200 kDa peaks is shown in Table 1. With this parameter the following order of potencies was observed: *N. n. atra* $PLA_2 \geq$ β-BuTX = notexin > control + BS^3 > control − BS^3. There was a significant correlation between the effects of the PLA_2 neurotoxins and enzyme on the percent distribution of the > 200 kDa peaks and PLA_2 enzymatic activity ($r = 0.76$; $P < 0.005$). A significant correlation was also found between the decreases in the 52 kDa peak areas and the increases in the percent distribution values of the > 200 kDa peaks ($r = -0.83$; $P < 0.001$).

The effects of EDTA and the correlations between FFA production and increased cross-linking suggest a role for PLA_2 enzymatic activity in the observed effects. Thus, the ability of β-BuTX, notexin, and *N. n. atra* PLA_2 to enhance protein cross-linking may be related to their ability to alter membrane order and fluidity. Studies have shown that PLA_2 activity can increase [13] or decrease ([14]; Ghassemi and Rosenberg, unpublished results) membrane fluidity; thus, the effects of PLA_2 hydrolytic products on protein cross-linking may be related to a perturbation of membrane order. These studies show a relationship between the enzymatic activities of the toxins and enzymes and their abilities to increase BS^3-induced cross-linking of proteins; therefore, the specific and potent presynaptic actions of β-

Table 1. Effects of PLA_2 neurotoxins and enzyme on cross-linking of the 52 kDa peak and the percent distribution of the > 200 kDa peaks

Treatment	Concn (nM)	BS^3	EDTA	Peak areas 52 kDa	% Distribution Peaks > 200 kDa
Control	–	–	–	37.1 ± 1.6^a	7.3 ± 1.2^a
Control	–	+	–	25.2 ± 2.6^{bc}	20.0 ± 0.8^b
β-BuTX	5	+	–	13.8 ± 0.9^d	28.1 ± 1.9^{cde}
	50	+	–	13.6 ± 2.0^d	32.4 ± 0.5^{de}
	50	+	+	29.2 ± 0.6^b	26.9 ± 1.1^{bcd}
Notexin	5	+	–	21.0 ± 3.0^c	32.9 ± 2.7^{de}
	50	+	–	8.6 ± 2.4^d	35.9 ± 5.5^e
N. n. atra	0.5	+	–	28.1 ± 1.7^b	22.3 ± 1.4^{bc}
PLA_2	5	+	–	14.0 ± 0.6^d	32.7 ± 1.7^{de}
	50	+	–	7.3 ± 0.7^d	43.9 ± 4.6^f
	50	+	+	24.6 ± 2.0^{bc}	26.4 ± 0.7^{bcd}

Synaptosomes were treated with the indicated agents for 20 min, cross-linked with 8 mM BS^3, SPM isolated, proteins analyzed by SDS–PAGE (100 μg protein/lane), and dried gels scanned by soft laser densitometry. The results (means ± SEM, N = 3) were most consistent when expressed as peak areas (arbitrary units) for the 52 kDa peak and as percent distribution (of the total protein on the gel) for the > 200 kDa peaks. The absolute values for percent of total protein entering into the gels ranged from 30 to 100 μg depending on the degree of cross-linking. Peaks > 200 kDa indicates those peaks that are products of cross-linking and represents a grouping of all proteins that were excluded from the separating gel. Some experiments were performed in the presence of EDTA (10 mM) and the absence of Ca^{2+}. Letters are used to indicate statistical significance. Within a given column (peak area or percent distribution) means with different letters are significantly different ($P < 0.05$) from each other.

BuTX and notexin (which have lower enzymatic activity than non-neurotoxic PLA_2 enzymes from other snake venoms such as *N. n. atra* PLA_2) are probably not due to alterations in the nearest neighbor relationships of SPM proteins, as measured by our cross-linking experiments. Our studies suggest that these toxins and enzyme increase cross-linking in protein bands of a wide range of molecular weights, without a specific effect on any particular protein band; however, more sensitive analytical techniques are necessary to verify these findings.

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Potentiation of the inductive effect of phenobarbital on cytochrome P450 mRNAs by cannabidiol*

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Marijuana is known to prolong the sleep induced by some barbiturates in animals. The major cannabinoid responsible for this effect is cannabidiol (CBD) [1], a component of marijuana which is devoid of psycho-pharmacological activity, but which exhibits anticonvulsant activity [2]. In animals it has been demonstrated that administration of CBD inhibited the metabolism of barbiturates and other drugs, including tetrahydrocannabinol (THC), at the level of the mixed-function oxidase system [3–5]. The mechanism by which this occurs is not clear although it has been proposed that there may be inhibition of enzymatic activity or an actual reduction of hepatic microsomal cytochrome P450 [6, 7]. In human studies, prolonged administration of CBD also was found to inhibit the metabolism of other drugs (antipyrine and barbiturates) metabolized by liver mixed-function oxidase enzymes [8].

Cloning techniques have resulted in the synthesis of DNA complementary (cDNA) to cytochrome P450 mRNAs allowing quantitation of constitutive and inducible forms of the messages [9]. Using these reagents as probes in hybridization procedures, it has been shown previously that the induction of the cytochrome P450IIB1/2 (P450b and P450e) genes by phenobarbital is mediated by increased levels of mRNA from 25- to 100-fold [10, 11]. We report here that although cannabidiol by itself produced no effect on P450IIB1/2 mRNA levels, the combination of cannabidiol and phenobarbital resulted in the superinduction of these mRNAs.

It is also known that phenobarbital treatment induces cytochrome P450IIC7 enzyme levels in immature rats [12]. We report that phenobarbital treatment induced hepatic cytochrome P450IIC7 (P450f) mRNA in immature rats and that the combination of cannabidiol and phenobarbital potentiated this effect at a suboptimal inducing dose of phenobarbital. Although many studies have been undertaken on the cannabinoids and the cytochrome P450s at the enzymatic level, this is the first which focuses on the regulation of specific cytochrome P450 mRNAs.

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Experimental Procedures

Animals and treatment. Male Sprague–Dawley rats, weighing approximately 140 g (6 weeks old), were injected intraperitoneally daily for 5 days with high (100 mg/kg) or low (30 mg/kg) dose phenobarbital. In the combined treatment the animals were co-administered, daily for 5 days, phenobarbital (high or low dose) and cannabidiol (25 mg/kg) dissolved in 50 μL ethanol, and killed approximately 20 hr after the last injection. Alternatively, animals were pre-injected with CBD (25 mg/kg) daily for 4 days and with phenobarbital (100 mg/kg) on day 5. Other animals received CBD (25 mg/kg) in ethanol daily for 5 days or just the ethanol vehicle (control) daily for 5 days.

Isolation of RNA. Livers were dissected and cut into small pieces which were immediately frozen in liquid nitrogen and subsequently ground to a fine powder, under nitrogen, in a large mortar and pestle. The powdered tissue was homogenized with a polytron in a guanidinium thiocyanate solution and total mRNA was purified by cessium chloride density gradient centrifugation using a modification [13] of the procedure of Chirgwin *et al.* [14] and Glisin *et al.* [15].

Analytical procedures. For Northern blot analysis [16], RNA was separated on 1.4% agarose gels containing formaldehyde and transferred to nitrocellulose or nylon membranes for hybridization with the cDNA probes followed by autoradiography. The recombinant plasmids containing the cDNA inserts were radiolabeled by nick-translation with a ^{32}P nucleotide (New England Nuclear or Bethesda Research Laboratories). Quantitative analysis was performed directly on the membranes using the Ambis Radioanalytical Imaging System. This system has a detector composed of 896 elements that simultaneously detect multiple beta emissions. By moving the sample beneath the detector, counts are recorded from approximately 65,000 discrete locations on the nylon membrane used for hybridization. Sample patterns are recorded in a computer file which is then used for production of a composite picture and quantitation. Dot-blot hybridization experiments were conducted with purified RNA according to the procedure of White and Bancroft [17] using a Hybridot Manifold apparatus (Bethesda Research Laboratory). A semi-quantitative assessment of mRNA was made by visual inspection of the dot-blot autoradiogram: the relative