Citrus Exocortis Disease — 1965 to 1975

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The advancement of our understanding of the causal agent of the exocortis disease of citrus during the past 10 years has been significantly influenced by the key developments of (1) the discovery of herbaceous hosts which provided both a reliable source of biologically active preparations and a rapid and accurate bioassay system and (2) the identification of a new species of low-molecular weight pathogenic RNA. These developments have permitted the formulation of a more precise definition of the exocortis disease agent, not as the presumed classical plant virus, but as one of the principal type examples of a unique class of minimal infectious molecules, the *VIROIDS*. The primary role of this review will be to trace the evolution of our knowledge of the agent of the exocortis disease from the initial observation as a transmissible principal to the present state of its molecular identity. Interaction of the viroid with the host cell will be considered in perspective to possible mechanism of synthesis or "replication" of the pathogenic molecule and to the process of pathogenesis.

NATURE OF THE EXOCORTIS AGENT AS DEDUCED FROM BIOLOGICAL PROPERTIES

The initial observations on the transmission of the exocortis disease by Benton *et al.* (1950) suggested a viral etiology. This prevailing view was sustained over several years even following the transmission of the agent to herbaceous hosts (Weathers *et al.* 1967). However, all attempts to implicate a viruslike particle as the causal agent of exocortis disease were unsuccessful. Maximum infectivity was always recovered in high speed (100,000 x *g* for 2 hours) supernatant samples from which host organelles as well as nucleoprotein particles had been sedimented. This coupled with the practical observations of the extreme thermal stability (Semancik and Weathers, 1970) of the exocortis disease agent lend credence to the unusual character of the pathogenic agent.

During the process of replication of RNA plant viruses, a phase of free-RNA molecules, either double-stranded or single-stranded, exist prior to the final assembly of the nucleoprotein particle. Furthermore, tobacco mosaic virus mutants (Siegel and Zaitlin, 1965) as well as specialized forms of virus infection, as in the case of the unstable form infection of tobacco rattle virus (Lister, 1969), have been characterized in which the structural proteins of the virus are either not functional or not synthesized. Provided with an adequate level of intramolecular interactions, these free-RNA molecules can demonstrate the inherent property of greater thermal stability than the nucleoprotein form of the virus.

Stimulated by this possibility, that a plant pathogen might exist solely as a proteinless nucleic acid species, and armed with a more workable herbaceous assay system in *Gynura aurantiaca* D.C., (Weathers and Greer, 1972) we were able to determine that preparations of the exocortis disease agent concentrated by procedures of nucleic acid extraction in phenol or alkaline-high salt were sensitive to ribonuclease (Semancik and Weathers, 1968). These techniques, coupled with

the property of thermal stability to temperature of 100° C for 10-20 minutes (table 1), still constitute valid probes for the screening of plant diseases of unknown cause in the identification of pathogenic nucleic acid.

The ability to extract a pathogenic RNA is not in itself unique, since infectious RNA can be readily obtained from the nucleoprotein particles contained in virus-infected plants. However, the indication that the CEV-RNA constituted the primary form of the infectious agent pointed to an unusual form of the principal pathogenic unit. The basic question then centered on the consideration of the molecular structure of the CEV-RNA as either (1) a double-stranded (ds) high molecular weight molecule analogous to the replicative form (RF) biosynthetic intermediate of typical virus replication, (2) a single-stranded (ss) high molecular weight viral RNA or, (3) a unique form of low molecular weight pathogenic RNA.

Since the bulk of the pathogenic RNA sedimented as a 8-16S molecule and remained soluble in a 2M LiCl medium

TABLE 1
THERMAL INACTIVATION OF CITRUS EXOCORTIS "VIRUS" PREPARATIONS

		Extracted by	
°C	Sap	HSS†	Phenol
50	5/8‡	7/8	
60	4/8	7/8	
70	4/8	5/8	
80	6/8	3/8	3/4
85			4/4
90	2/8	3/8	2/4
95			4/4
100		4/8	2/4
100§			3/4

*10 minute exposure
†HSS = ethanol concentrated high-speed supernatant
‡No. plants infected/no. plants inoculated
§20 minute exposure
Reproduced from Semancik & Weathers, 1970.

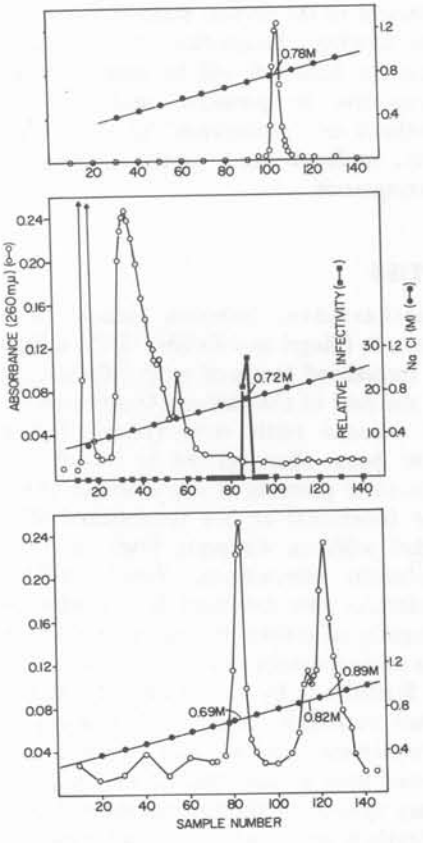

Fig. 1. Elution pattern from methylated albumin kieselguhr columns of (bottom) DNA (0.69 *M*) and ribosomal-RNA (0.82 and 0.89 *M*), (middle) 2 *M* LiCl supernatant preparation from CEV-infected *Gynura aurantiaca* indicating CEV infectivity (0.72 *M*), (upper), double-stranded RNA (0.78 *M*). Reproduced from Semancik and Weathers (1972*b*).

TABLE 2
SPECIFIC INFECTIVITY OF PHENOLIZED PREPARATIONS FROM CEV-INFECTED *GYNURA AURANTIACA* AFTER 2 M LiCl FRACTIONATION

	Relative infectivity/A_{260} = 0.5		
Days p.i.*	2 M LiCl precipitate	"DNA"	2 M LiCl supernatant
Expt. 1			
20	0	0	28
49	0	15	38
84	2	11	28
Expt. 2			
35	0	0	48
82	0	0	23
145	0	15	12

*Post inoculation
Reproduced from Semancik & Weathers, 1972*b*.

(table 2) the likelihood that CEV-RNA represented a high molecular weight ss-structure was discounted. These properties coupled with the characteristic elution patterns of the CEV-RNA from methylated albumin (fig. 1) and CF-11 cellulose further implicated a ds-like molecular structure. Nevertheless, the biological activity of the CEV-RNA could be destroyed by nucleases such as pancreatic RNase A and phosphodiesterases in high salt conditions and formaldehyde concentration to which ds-RNA is normally resistant (Semancik and Weathers, 1972*b*). This apparent paradox was explained by the suggestion that the pathogenic RNA conformed to a partially double-stranded t-RNA-like molecule. Since this highly-ordered putative structure might arise from inter- or intramolecular complementation, attempts were made to separate the single-strands of a truly ds molecule with no apparent success (Semancik *et al.*, 1973*b*).

The most compatible model which could be deduced from the properties of the pathogenic RNA was a highly-ordered single-stranded low molecular weight molecule. This model was further supported by the evidence that the "infectivity" of CEV-RNA was reduced by only 20-60 per cent by the action of diethylpyrocarbonate under conditions in which ss viral RNA was completely inactivated and the cosedimentation of the CEV-RNA with tRNA in Cs_2SO_4 equilibrium sedimentation (Semancik and Weathers, 1972*b*). This conclusion was, however, weakened by the inability to identify a distinct biologically-active RNA species in the nucleic acid extracts from diseased tissue.

IDENTIFICATION OF THE EXOCORTIS SPECIFIC LOW MOLECULAR WEIGHT PATHOGENIC RNA

Confirmation of the low molecular weight property of CEV-RNA came with the first association of pathogenic activity with a physically-discernable new RNA species from a *VIROID* infection by Semancik and Weathers (1972*a*). With the application of a tRNA-rich preparation from CEV infected Gynura to electrophoresis in 5 per cent polyacrylamide, an "infectious" RNA of about 1.0 - 1.3 X 10^5 daltons, undetected in healthy tissue, (fig. 2) was implicated as the causal agent of the exocortis disease. This discovery introduced a new threshold in the search for the molecular identify of the CEV-RNA, for even though the concentration of viroid-RNA was extremely low it was then possible to attempt to isolate the pathogenic RNA as a pure species for analysis as well as to detect the CEV-RNA in extracts from as little as 1-5 gm of infected gynura.

Utilizing the molecular properties previously described, principally involving 2M LiCl partition, chromatograph on Cf-11 cellulose and preparative polyacrylamide electrophoresis, yields of about 30 µg of purified CEV-RNA/kg

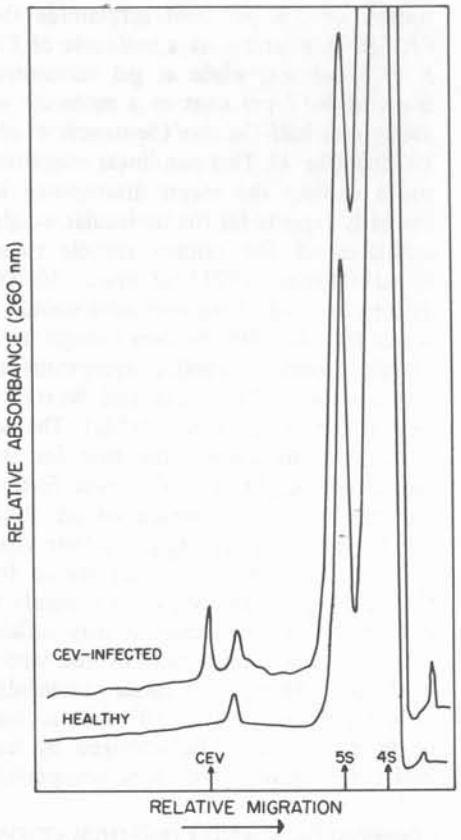

Fig. 2. Electrophoresis on 5% polyacrylamide gels of a tRNA-like fraction from healthy and exocortis-infected *Gynura aurantiaca*.

fresh weight of tip tissue were routinely obtained as shown in fig. 3 (Semancik *et al.*, 1974).

MOLECULAR WEIGHT ESTIMATION

The molecular weight of 1.0-1.25 X 10^5 daltons for the CEV-RNA detected after electrophoresis in 5 per cent acrylamide, agreed favorably with the previous estimates deduced from the bioassay of crude preparations. This estimate was further sustained by the target size estimate of 1.1 X 10^5 daltons made from preparations inactivated by ^{60}Co ionizing radiation (Semancik *et al.*, 1973*b*) and direct electron microscopy (unpublished data) of the purified pathogenic RNA.

A curious property of the CEV-RNA is the non-linear relative migration demonstrated in different concentrations of polyacrylamide gels. At a gel concentration of 2-8 per cent acrylamide the CEV-RNA migrates as a molecule of 1.0 X 10^5 daltons, while at gel concentrations of 8-17 per cent as a molecule of about one half the size (Semancik *et al.*, 1973*b*). (fig. 4). This non-linear migration could explain the major discrepancy in the early reports for the molecular weight estimates of the potato spindle tuber viroid (Diener, 1971) of about 50,000 daltons and the citrus exocortis viroid of about 100,000 daltons even though both agents produced similar symptoms in common hosts (Semancik and Weathers, 1972*a*; Semancik *et al.*, 1973*a*). The accuracy of the early estimation for the molecular weight of PSTV was further hampered by the absence of an RNA species detected by A_{260}, rather than biological activity. The exact reason for this electrophoretic migration anomaly is not understood, however, it may reflect the unusual conformation of the viroid molecule. Since the larger molecular weight estimate, 1.0 x 10^5 daltons was made in a system characterized by less molecular sieving and was compatible with the values made by the independent EM and ^{60}Co experiments, it remains the preferred value for the CEV-RNA.

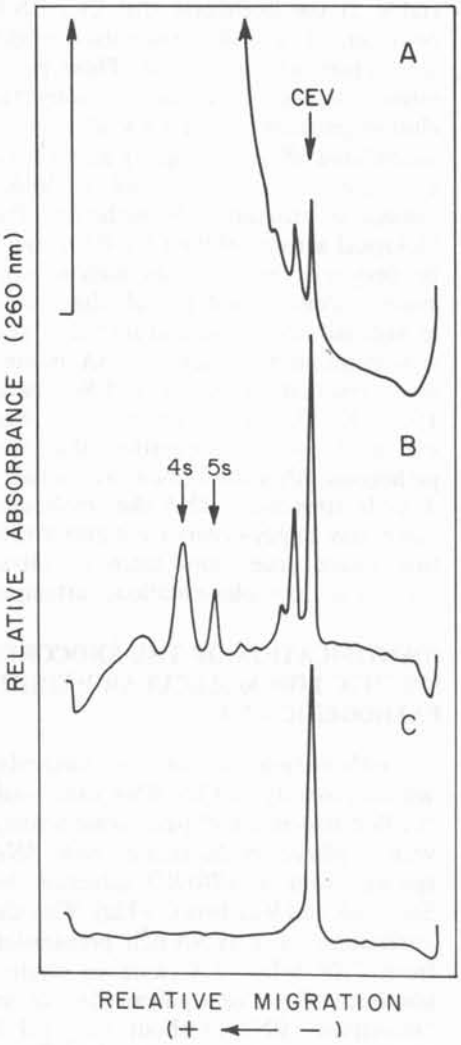

Fig. 3. Electrophoresis on 5% polyacrylamide gel in Tris-acetate-EDTA buffer, pH 7.2, with 0.2 per cent sodium lauryl sulfate at 6 mA/gel for 3 hours of a tRNA-like preparation from exocortis-infected *Gynura aurantiaca* (A); after elution from CF-11 cellulose (B); and after preparative polyacrylamide electrophoresis (C). Reproduced from Semancik *et al.* (1974).

COMPOSITION AND CONFORMATION OF THE EXOCORTIS VIROID

No significant levels of either DNA or protein could be detected in the purified CEV-RNA preparations. The stability of the molecule to thermal inactivation is understandable considering the optical properties of the viroid which indicate a

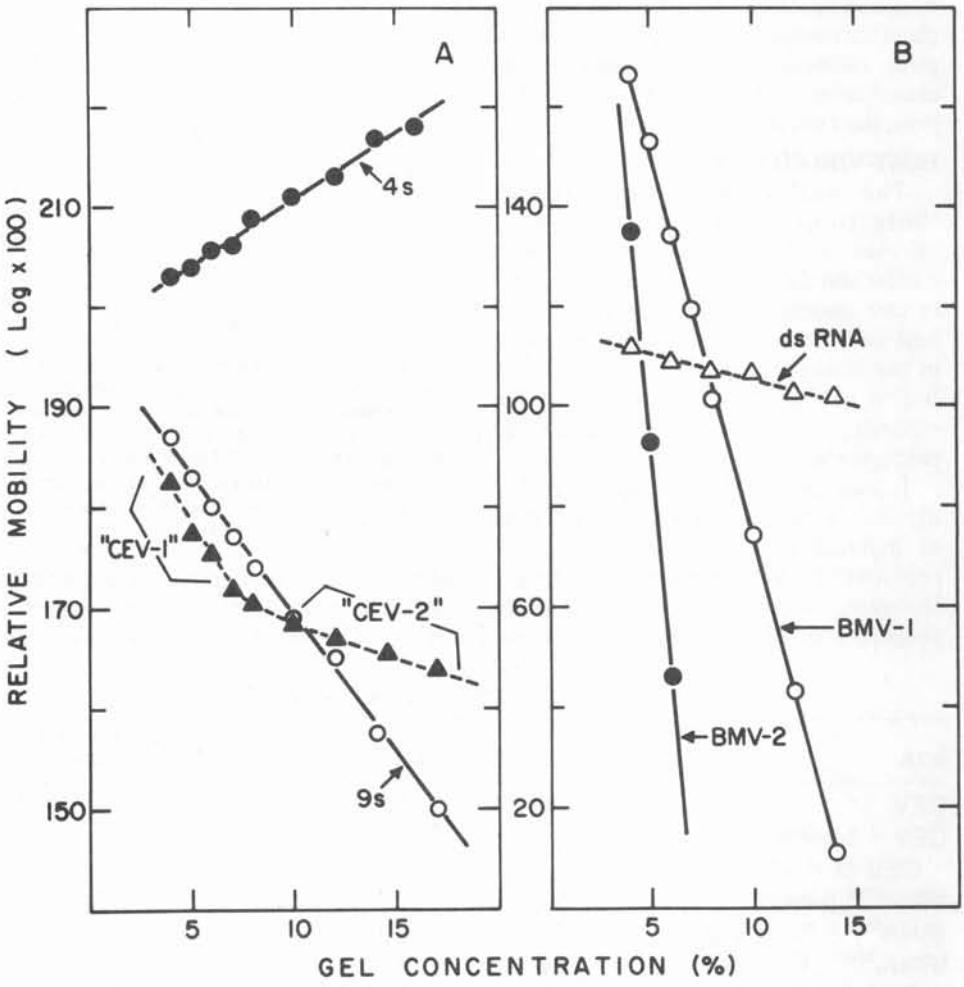

Fig. 4. Polyacrylamide gel electrophoresis at various gel concentrations in Tris-acetate-EDTA buffer, pH 7.2 with 0.2 per cent sodium lauryl sulfate at 6 mA/gel of a tRNA-like preparation (which includes 4A, 5S, 9S and CEV-RNA) from exocortis-infected *Gynura aurantiaca* (A) and the 14 and 21 S components from brome-grass mosaic virus and double-standard RNA from bacteriophage Ø6 (B). The electrophoretic mobilities are calculated relative to 5 S RNA. Reproduced from Semancik *et al.* (1973*b*).

greater degree of base pairing than even a tRNA species, but less than a pure duplex molecule (fig. 5).

The fidelity of reannealing indicates that the viroid rapidly regains molecular structure upon cooling. Yet the 24 per cent hyperchromicity and the T_m of CEV-RNA suggest only a partially double-helical configuration. The disproportionate contribution of GC base pairs in the viroid configuration is suggested by the high G + C content (58 per cent) T_m (52° in 0.1 SSC) (table 3) and low field NMR spectra (Semancik *et al.*, 1975). These properties can be taken as further indicators of the intrinsic stability of the viroid molecule to physical degradation. Nevertheless, in the absence of a completely double-helical configuration, the biological activity of the viroid remains sensitive to the action of ribonuclease.

From these data the most compatible model which emerges is a single-stranded hairpin-like RNA molecule of about 10^5 daltons with significant region of intra-

strand antiparallel GC rich sequences. The characterization of this unique pathogenic molecule has contributed to the establishment of a new class of pathogens, the viroids.

HOST-VIROID INTERACTION

The definition of these minimal "infectious" molecules as free-RNA species as characterized by physical studies should not be extrapolated to the *in vivo* association of the viroid with the host cell. For despite the advances made in the isolation and molecular characterization of the viroids, knowledge of the replication or synthesis and the process of pathogenesis remain highly speculative.

It may be tempting to assume implicitly that viroids constitute simply a class of minimal viral-RNA and thereby are replicated and function accordingly. However, a review of the viroids virus-like properties of (1) a nucleic acid composition, (2) the production of a disease condition, and (3) an increased concentration in diseased cells, might warrant caution in this extrapolation or at least the consideration of an alternative possibility. One must consider that the unique nature of the viroid-pathogenic RNA might possibly reflect or indicate an equally unique process of replication and pathogenesis by viroids. These pathogenic molecules may provide a most powerful probe for the study of the replication of any exogenous RNA which may be directly coordinated with the appearance of a pathological lesion. Therefore a basic dichotomy centers on whether the viroid RNA acts (1) as a messenger RNA in directing the *de novo* synthesis of a viroid-specific protein essential for either replication or pathology or (2) as a regulator RNA which interacts directly with the host genome thereby modifying the expression of normal metabolic systems.

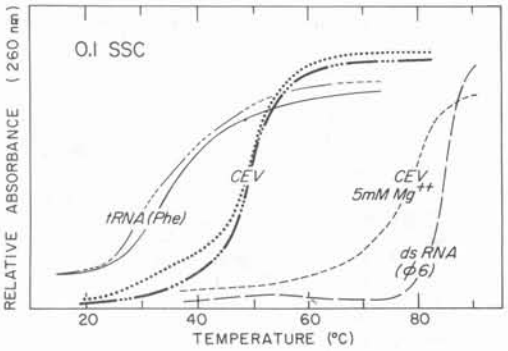

Fig. 5. Absorbance profiles of RNA species as a function of temperature. The broken line through the "tRNA (Phe)" label and the dotted line through the "CEV" label indicate the reannealing pattern for these RNA species in 0.1 SSC. Reproduced from Semancik *et al.* (1974).

TABLE 3
OPTICAL PROPERTIES OF RNA SPECIES*

RNA	Hyperchromicity per cent	T_m(°C)	G+C	GC base pairs per cent
CEV	22	52	58	—
CEV + 5mM Mg^{2+} or CEV (1 × SSC)	18	79	58	—
$tRNA^{Phe}$ (yeast)	24	37	53	57
$tRNA^{Phe}$ + 5mM Mg	21	76	53	57
$tRNA_f^{Meth}$ (*E. coli*)	23	45	66	85
dsRNA (phage Ø6)	32	85	57	57

*RNA solvent, 0.1 SSC (0.015 M sodium chloride, 0.0015 M sodium citrate, pH 7.0).
Reproduced from Semancik *et al.*, 1973*b*.

THE PROCESS OF REPLICATION OF THE EXOCORTIS VIROID

The synthesis of a virus-specific RNA polymerase translated from the viral genome constitutes a critical step in the replication of most RNA plant viruses. All attempts to demonstrate translation of viroid RNA by *in vitro* protein synthesizing systems proved unsuccessful (Hall *et al.*, 1974). Even though the pos-

sible *in vivo* translation of the CEV-RNA in host cells cannot be yet discounted, it appears that the viroid RNA does not contain significant polyadenylic acid sequences characteristic of messenger RNA (Semancik, 1974).

How, then, might a molecule with such a limited genetic potential accomplish both replication and pathogenic expression? By the direct interaction with a portion of the host genome of adequate homology, a regulatory RNA might elicit an aberrant response in a normal regulatory system. This concept has been recently reviewed by Reanny (1975). As a consequence the synthesis of a limited number of copies either during the process of cell division or amplification of a portion of the host genome might be assured. In support of this hypothetical mechanism, a CEV-like DNA has been characterized (Semancik and Geelen, 1975) on the ability of DNA from exocortis-infected gynura to hybridize with ^{125}I labelled CEV-RNA (table 4). This CEV-like DNA does not appear to result from *de novo* synthesis via a RNA-directed DNA polymerase (reverse transcriptase) nor has a viroid-specific polymerase system been detected (Geelen and Semancik, 1976). If this putative DNA complement designates critical areas of the host genome involved in regulation, a site of the viroid homology or recognition as well as an intermediate of viroid synthesis might be also so localized. It is presumed that the CEV-like DNA comprises the template critical for the synthesis of CEV-RNA. It is also conceivable that a limited number of copies of the CEV-DNA are contained in the genome of the healthy cell but are amplified with the introduction of the viroid RNA.

TABLE 4
HYBRIDIZATION OF ^{125}I-CEV-RNA TO VARIOUS DNA-RICH PREPARATIONS

DNA Source	RNase-resistant ^{125}I (c.p.m.)
Tobacco	70
Cowpea	0
Tomato	233
Gynura 5 µg	133
Gynura 10 µg	116
CEV-infected tomato	1,731
CEV-infected tomato + 1.6 µg CEV-RNA	170
CEV-infected tomato + DNase*	350
CEV-infected gynura 5 µg	1,105
CEV-infected gynura 10 µg	1,465
CEV-infected gynura + 1.6 µg CEV-RNA	153
CEV-infected gynura + DNase*	859

*RNase-resistant ^{125}I was determined after hybridization of 0.005 µg ^{125}I-CEV (50,000 c.p.m.) with 10 µg (except as indicated) of the DNA preparation.
Reproduced from Semancik and Geelen (1975).

SUBCELLULAR ASSOCIATION AND CYTOPATHOLOGY

The association of the CEV-RNA with a DNA complement is supported by the recovery of significant levels of pathogenic RNA from nuclei-rich preparations. However, up to 60 per cent of the extractable CEV-RNA was isolated from post-nuclear preparation rich in membranes (table 5). Virtually no viroid remained in a 100,000 *g* supernatant from infected tissue even though the CEV-RNA could survive in similar preparations (Semancik and Weathers, 1972*b*). These data reinforce the observation that the viroid is *not* a free-RNA *in vivo* but remains in intimate association with host constituents or membranes thus assuring its persistence as well as expression at a very high specific biological activity (Semancik *et al.*, 1976).

The dual distribution of pathogenic