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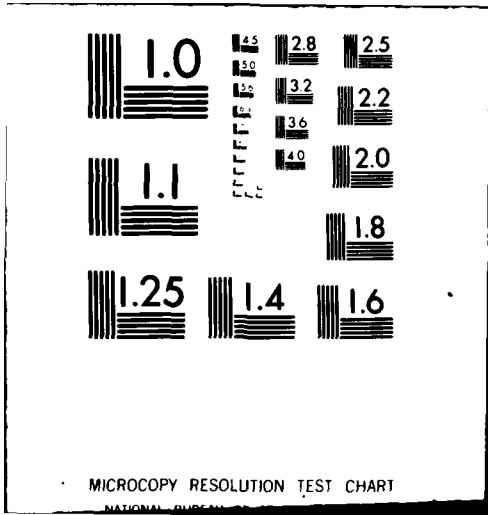
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TARGET ORIENTED DRUGS AGAINST LEISHMANIA  
(First Annual Summary Report)

URI ZEHAVI, PhD

and

JOSEPH EL-ON, PhD

supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21701

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) 1. Excreted Factor (EF) is a carbohydrate-rich protein excreted by different strains of <u>Leishmania</u> . It has antigenic properties similar to those of the parasite and plays a role in the infective process. 2. Isolation and purification of EF is necessary for: a) study of its biological function. → next page		

**BLOCK 20**

- b) the use of EF for diagnostic purposes;
  - c) the use of EF in immunization experiments;
  - d) the study of the biosynthesis of EF; *and*
  - e) the preparation of inhibitors of particular biosynthetic steps of EF.
3. Chemical analysis of partially purified EF was carried out. This indicated a high galactose content of EF. *beta*
  4. Independent reactions of EF with  $\beta$ -galactosidase and with peanut lectin (PNA) suggest the presence of  $\beta$ -galactosyl units at the non-reducing end of at least part of the carbohydrate component of EF. Interaction of promastigotes with PNA, reversed by galactose, indicates the presence of the same galactose component in cell membrane constituents of promastigotes.
  5. Purification of EF was achieved using chemical procedures including a phenol extraction method, followed by affinity chromatography.
  6. Initial experiments aimed at constructing a radioimmunoassay (RIA) for Leishmania were undertaken.
  7. Galactose residues in EF and galactose attached to the promastigote cell membranes may play an important role in the mechanism of infection by Leishmania.

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Summary

1. Excreted Factor (EF) is a carbohydrate-rich material released by different strains of Leishmania during growth. It has antigenic properties similar to those of the intact parasite and plays a role in the infective process.
2. Isolation and purification of EF is necessary for:
  - a) study of its biological function
  - b) the use of EF for diagnostic purposes
  - c) the use of EF in immunization experiments
  - d) the study of the biosynthesis of EF
  - e) the preparation of inhibitors of particular biosynthetic steps of EF.
3. Amino acid analysis of partially purified EF was undertaken and indicated a low content of aromatic amino acids and a high content of glycine, alanine, proline and hydroxyproline. Sugar analysis showed a high proportion of galactose and also the presence of glucose, mannose, xylose N-acetyl-glucosamine and N-acetylgalactosamine; there is the as yet unproven possibility of arabinose.
4. Independent reactions of EF with  $\beta$ -galactosidase and with peanut lectin (PNA) suggest the presence of  $\beta$ -galactosyl units at the non-reducing end of at least part of the carbohydrate component of EF. Interaction of promastigotes with PNA, reversed by galactose, indicates the presence of the same galactose component in cell membrane constituents of promastigotes.
5. The Leishmania parasites were grown in various media and EF was obtained from the used medium. Purification of EF was achieved using chemical procedures including phenol extraction method, followed by affinity chromatography. Affinity chromatography was based on EF-antibody interaction or on the binding of EF to insoluble PNA or  $\beta$ -galactosidase.
6. Initial experiments aimed at constructing a radioimmunoassay (RIA) for Leishmania were undertaken.
7. Galactose residues in EF and galactose attached to promastigote cell membranes must play an important role in the mechanism of infection by Leishmania.

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## 1. Introduction

Excreted Factor (EF) is a carbohydrate-rich protein excreted by different strains of *Leishmania* (1). It has immunological properties similar to those of the intact parasite and may play a role in the infective process. After it was shown to be species-specific, EF became the basis for serotyping *Leishmania* strains, and is a valuable tool in diagnostic, demographic and ecological studies of the disease (2).

## 2. Reaction between EF and Peanut Lectin (PNA)

a) Interaction of PNA with *Leishmania* promastigotes. PNA binds specifically to galactose residues and this property was utilized to test for the presence of galactose on the surface of the parasite. Peanut lectins (PNAs) were obtained from either Sigma or Makor, Jerusalem, or produced in large quantities in our laboratory by slight modification of the method of Terao *et al* (3) (Fig. 1). Commercial PNA and PNA produced in our laboratory were compared and shown to have similar agglutination and precipitin properties. Promastigotes of both *L. donovani* and *L. tropica* were examined microscopically. Both species were readily agglutinated by concentrations of PNA exceeding 10  $\mu\text{g/ml}$ . Promastigotes of *L. tropica* were somewhat more sensitive to PNA, being agglutinated by concentrations as low as 1  $\mu\text{g/ml}$ . Agglutination was completely reversed by the addition of as little as 0.01 M galactose. PNA was not toxic to the promastigotes.

b) Effect of EF on PNA-mediated agglutination. Since EF is known to contain galactose residues (4) (§5b), the ability of EF to inhibit PNA-mediated agglutination was examined. EF did not, unlike galactose, reverse agglutination. This can be attributed to the large size of EF which prevents it from penetrating the massive, tightly-packed parasites. Further experiments are planned.

c) Interaction of PNA with EF. Despite the abundance of sugars in EF (see Chemical Analysis §5b), it has been found that EF is insensitive to precipitation by a wide variety of lectins (5, 6). However PNA was found by us to precipitate the EF of *L. tropica* after TCA-treatment, using immunodiffusion methods (6). To examine further the interaction of EF with PNA, immunoelectrophoresis was performed; this was done to show the presence of soluble EF-PNA complexes. Partially purified EF from both *L. donovani* and *L. tropica* when treated with PNA (25 mg/ml) ran a shorter distance on immunoelectrophoresis than did untreated EF. This result indicates formation of a soluble EF-PNA complex.

## 3. Inhibition of $\beta$ -galactosidase by EF

Previous work showed that EF from *L. donovani* inhibited  $\beta$ -galactosidase activity prepared from C3H and C57Bl macrophages (7). This inhibition was specific against  $\beta$ -galactosidase but no inhibition was observed against three other enzymes from macrophages. In our present study, we have shown that EFs from both *L. donovani* and *L. tropica* (independent of mode of preparation) inhibit

$\beta$ -galactosidase from E. coli (Sigma). Conditions for assaying for  $\beta$ -galactosidase activity were selected to be suitable for future work with affinity chromatography.

**Table 1**  
**The effect of various EF preparations on the activity of  $\beta$ -galactosidase**

The reaction mixture for study of  $\beta$ -galactosidase inhibition (470  $\mu$ l) contained 0.11 M mercaptoethanol; 1 mM MgCl<sub>2</sub>, 5 x 10<sup>-4</sup> units of  $\beta$ -galactosidase (E. coli) and 100 mM ONPG in 0.1 M phosphate buffer pH = 7.3. For the preparation of the EFs used see Glossary § 11.

EF sample added to the reaction mixture	$\beta$ -galactosidase activity
None	100%
500 $\mu$ g EF-L32 (calf medium)*	71%
500 $\mu$ g EF-L32 (rabbit medium)*	60%
300 $\mu$ g EF-L152 (calf medium)*	72%

\* Calf or rabbit denotes the type of serum added to the original parasite culture.

#### 4. Purification of EF

EFs from both L. tropica (L137) and L. donovani (L52) were previously partially purified by physical and chemical methods including ammonium sulphate and acid precipitation, as well as chromatography on Sephadex columns.

In all the above procedures traces of substances originating from the growth medium were found to contaminate the EF preparations. This level of purification was not satisfactory for either analytical purposes nor for studying the biological function of EF. The present work includes three new approaches to this problem.

a) Phenol extraction. This method follows the previous procedure (6) through the boiling at pH = 5. After dialysis the samples are extracted twice with 90% phenol at 68°C. The aqueous phases are combined and extensively dialysed. The EF obtained by this method is immunologically identical to that previously obtained. However, it is less contaminated with medium components as shown by analysis on SDS polyacrylamide gels. This method has the advantage of being fast and producing a high yield of EF.

b) Fractionation of dissociated EF - antibody complexes. A complex of EF-antibody was produced by mixing either crude or purified EF with homologous rabbit antiserum (8). After incubation for 30 min at 37°C, and overnight at 4°C, the complex was collected by centrifugation, washed with saline and then dissociated with 2 M sodium thiocyanate in PBS (9). The dissociated complex was fractionated on a Sephadex G-100 column, using 2 M sodium

thiocyanate in PBS as eluting buffer. Two main fractions were obtained: the antibody fraction (MW = 178,000) followed by an EF fraction (MW = 33,000) (Fig. 2). After dialysis, both fractions displayed the characteristic behaviour of the original starting materials. The antibody fraction yielded only one band on immunoelectrophoresis against goat anti-rabbit antiserum. Similarly, the EF fraction showed one precipitating band which ran to the same locus as the original untreated EF. This technique, used either alone or in combination with one of the previously described methods leads to greater purification of EF as well as to the separation of monospecific anti-EF rabbit IgG. Generally, anti-leishmanial EF antibodies are produced in rabbit by the inoculation of whole promastigotes (1). Although the antibodies which are obtained by this procedure are mainly against EF they also recognize parasite somatic components. EF, which is a haptenic compound, does not elicit the production of antibodies when inoculated into rabbits or mice either alone, complexed with homologous antibodies, or complexed with methylated bovine serum albumin (8). The present technique is therefore the only available method for producing monospecific anti-EF antibodies. These antibodies are of great importance since they can be used in further studies to determine the site of origin and the localization of EF in the parasite.

c) Affinity chromatography of EF. Affinity chromatography is currently recognized as an excellent tool for isolating a specific molecular species from a mixture. We have chosen two approaches for the development of an affinity method for isolation of EF. The first is based on its reactivity with antibody. The second approach depends on the presence of galactose residues in EF and their reaction with either immobilized  $\beta$ -galactosidase or immobilized PNA.

- (i) Affinity chromatography using antibody bound to Sepharose  
Using the results obtained in § 4b, an affinity chromatography procedure was designed for the purification of EF. Rabbit anti-EF IgG was bound to activated Sepharose 4B. The EF bound to IgG was then eluted from the washed EF-antibody complex by 2 M sodium thiocyanate in PBS. This procedure was successful for both L. tropica and L. donovani EF, resulting in highly purified EF. In all cases the batch technique was used. This technique is preferable to the column method since the antibody is stable in the presence of 2 M sodium thiocyanate only for 24 h.
- (ii) Affinity chromatography using immobilized  $\beta$ -galactosidase  
Based on the inhibition study described in § 3, purification of EF was attempted by binding  $\beta$ -galactosidase to a column of AE-cellulose (10). Excess reactive groups were blocked by ethanolamine. The  $\beta$ -galactosidase-AE-cellulose column was eluted with 0.02 M phosphate buffer pH = 7.0 and EF activity was eluted only after the addition of 1 mM lactose to the eluting buffer. At least a three-fold purification with respect to protein was achieved.
- (iii) Affinity chromatography using immobilized PNA  
Based on the results described in § 2c showing the formation of an

EF-PNA complex, purification of EF was achieved by binding PNA to a column of AE-cellulose (10). The column was washed with 0.02 M phosphate buffer pH = 7.0 and the EF activity eluted immediately after the void volume (Fig, 3). Purification of EF achieved by this method was three-fold with respect to protein and four-fold with respect to carbohydrate.

We are now in the process of scaling-up these techniques in order to produce large quantities of highly-purified EF.

5. Chemical analysis of EF preparations

Typical samples of EF from L. donovani and L. tropica were assayed for protein by the Lowry method and for carbohydrate by the Dubois method. Values ranged from 17-29% for protein and 17-64% for carbohydrate. Further analysis of amino acids and monosaccharides was then performed.

a) Amino acid analysis. The various EF preparations were analyzed following acid hydrolysis using the amino acid analyzer (LKB Model 3201). The designation of EF preparations is described in the Glossary (§ 11). See Table 2 for results.

Table 2  
Amino acid analysis of EF preparations

Amino acid	% of dry weight		
	EF L137 <sup>a</sup>	EF L32	EF L32 <sup>b</sup>
Lysine	4.8 ( 6.3- 2.8)	3.7	4.4
Histidine	2.3 ( 2.8- 1.4)	1.7	2.2
Arginine	3.9 ( 4.9- 3.0)	1.4	2.6
Aspartic acid	10.7 (10.9- 6.3)	13.2	14.4
Theonine	4.0 ( 4.9- 2.6)	11.4	6.8
Serine	4.6 ( 7.0- 3.3)	8.7	8.8
Glutamic acid	10.0 (11.9- 8.8)	7.6	18.2
Proline	18.5 <sup>c</sup> (19.6-17.4)	27.8	
Glycine	29.9 (36.9-20.3)		20.4
Alanine	12.4 (15.3-11.2)	14.7	10.4
Half cystine	-	-	-
Valine	2.5 ( 2.7- 2.3)	3.6	-
Methionine	-	-	-
Isoleucine	1.6 ( 1.9- 1.4)	2.0	6.2
Leucine	2.3 ( 3.0- 1.9)	4.2	5.2
Tyrosine	0.5 ( 1.6- 0 )	-	-
Phenylalanine	0.3 ( 0.7- 0 )	-	-

- a Average of three determinations; numbers in brackets are high and low values
- b Prepared by the phenol method and further purified by  $\beta$ -galactosidase affinity column
- c Includes hydroxyproline
- d Total of both amino acids

**Conclusions:** The amino acid composition of the EFs of both strains is quite similar and seems to be independent of method of preparation. The most striking features of the analyses are:

1. complete absence of aromatic amino acids
2. high content of aspartic and glutamic acids (which may account for the negative charge of EF)
3. high content of glycine and alanine
4. low content of basic amino acids
5. absence of sulphur containing amino acids
6. remarkable presence of hydroxyproline

b) Carbohydrate analysis. Monosaccharide components of EF were determined by GLC following methanolysis and trimethylsilylation (11).

**Table 3**  
Monosaccharide composition of various EFs

sugar	EF small L137			EF L137			EF L32		
	rel. to mannose <sup>a)</sup>	nmol/mg	%	rel. to mannose <sup>a)</sup>	nmol/mg	%	rel. to mannose <sup>a)</sup>	nmol/mg	%
Arabinose <sup>b)</sup>	0.5	22	0.3	0.3	57	0.9	0.4	127	2
Xylose	1.7	78	1.2	0.5	92	1.4	1.2	410	6.2
Mannose	1	46	0.8	1	196	3.5	1	350	6.3
Galactose	7	318	5.7	4.4	860	15.5	2.8	970	17.5
Glucose	2	93	1.7	0.95	187	3.4	1.1	370	6.7
N-acetyl-glucosamine	0.8	38	0.8	0.2	40	0.9	0.3	93	2
N-acetyl-galactos-amine	-	-	-	-	-	-	0.09	30	0.7
N-acetyl-neuraminic acid <sup>c)</sup>									
total sugar			10.5			25.6			41.4
total sugar by Dubois			17			23			54

a amount relative to mannose (Man = 1.0)

b an independent determination of arabinose is still required to rule out the possibility of an artifact

c a minute amount of N-acetylneuraminic acid could be present. This, however, could not be demonstrated by neuraminidase treatment.

This sugar analysis points to very high galactose content. In addition, xylose is present in very significant amounts and there is a possibility of arabinose - normally found only as a component of plant glycoproteins.

#### 6. Radioimmunoassay (RIA) for leishmaniasis

At present the most reliable diagnosis of leishmaniasis is by directly detecting the parasite. Since the individual strains are morphologically identical further diagnostic tools are needed for full identification. The fact that EFs are immunologically active, able to bind to and precipitate homologous antibody, makes them primary objects for study and use in the immunological diagnosis of leishmaniasis. The aim of this work is to determine the usefulness of purified standardized EFs as specific RIA reagents in the differential diagnosis of leishmaniasis. RIA could be a most sensitive tool for detecting the minute amounts of EF present in body fluids.

In this study, the EF is labelled externally via the radioiodine labelling method of polyanionic macromolecules (12). The principle of this method involves the formation of a soluble, electrostatic complex of the polyanionic antigen with a radioiodinated polycation.

Application of this technique for leishmaniasis is based on two steps:

- a) formation of stable complexes of EF with suitable polycations and
- b) labelling the polycation and using this complex for RIA.

So far, we have proceeded in this preliminary work only to the formation of EF and polycations as indicated in a).

a) Normally, EFs of both L. donovani and L. tropica are highly negatively charged compounds migrating to the anode on electrophoresis. In the present work, the effect of the polycationic compounds, protamine sulphate, poly-L-lysine, histone and DEAE-dextran, on the EF charge was examined. The results indicate that complexes are indeed produced between each of these polycations and EF. These can be ordered by their electrophoretic mobility: DEAE-dextran-EF < histone-EF < protamine sulphate-EF < poly-L-lysine-EF.

#### 7. Conclusions and working hypothesis

In the vertebrate host, Leishmania are obligatory intracellular parasites of the mononuclear phagocyte, in which they grow and multiply as amastigotes even in the presence of lysosomal enzymes (13,14). The mechanism of survival of these microorganisms within these cells is still unknown. It was recently shown that EF acts as a conditioner for amastigotes in macrophages from resistant animals (15).

In our experiments we have demonstrated that different EF preparations excreted by L. tropica and L. donovani inhibit  $\beta$ -galactosidase action (§ 3) and bind to PNA (§ 2c), which like  $\beta$ -galactosidase possesses galactose-binding properties. Sugar analysis of EF preparations similarly shows high galactose content. (§ 5b). These observations suggest the presence of

numerous non-reducing terminal  $\beta$ -galactose residues in EF. Since EF reacts with anti-Leishmania antibodies we tentatively propose that cell-membrane glycoproteins and/or glycolipids possess the same antigenic determinants (of a carbohydrate nature) as does EF. This is also demonstrated by our experiments on promastigote agglutination by PNA (§ 2a). These oligosaccharide substituents, containing in particular non-reducing  $\beta$ -galactose, could be important in cell-cell recognition between the parasite and the host cell. One can speculate that the host cell-membranes might contain a galactose-binding receptor capable of binding either the parasite or EF.

This hypothesis is analogous to the well-studied system of hepatic membrane receptors - which are known to be specific to galactosyl residues (16,17). Hepatic plasma membranes bind asialoglycoproteins, where galactose is the non-reducing sugar, as a prelude to their catabolism. Similar binding is reported of asialoerythrocytes (18) and lymphocytes (19). More recently (20), it was found that Kupffer cells (macrophages) bind both asialoglycoproteins and asialo-cells (which are apparently considered as 'non-self', due to desialylation).

Leishmania might be initially attached to macrophages via galactose-binding acceptors and subsequently engulfed by them through a similar mechanism. EF could be likewise attached and could possibly cause the 'conditioning' of macrophages to infection. It is pertinent to note however, that this is no more than a working hypothesis which we would like to support with additional experimental facts.

The presence of exposed galactose residues on Leishmania could, in principle, account also for its presence in host cells and promote removal from blood circulation by the liver.

Reaction to the Leishmania appears to be primarily cell mediated and only secondarily humoral. A population of long lived, thymus-dependent lymphocytes gradually builds up, specifically sensitized to the invoking antigen and capable of multiplying in response to contact with this antigen (21). On such contact, sensitized lymphocytes release substances that induce the accumulation and activation of macrophages. The mechanism of lymphocyte-macrophage interaction and the function of  $\beta$ -galactosidase in such mechanism is not known. It is of interest to mention the work of Swain and Coons (22) who indicated that by the time of birth of mice there are T cells already committed to recognize at least one antigen -  $\beta$ -galactosidase. The cells binding  $\beta$ -galactosidase are presented with low frequency among T cells of the thymus and about equally among T and B cells of the spleen.

#### 8. Proposals for further research

We plan to pursue our research activity in the following areas:

- a) Purification of EF from L. tropica and L. donovani will be achieved through a combination of phenol extraction and affinity chromatography. This should provide us with a good yield of high-purity EF.
- b) More detailed chemical analysis and structural determination of EF will be carried out.

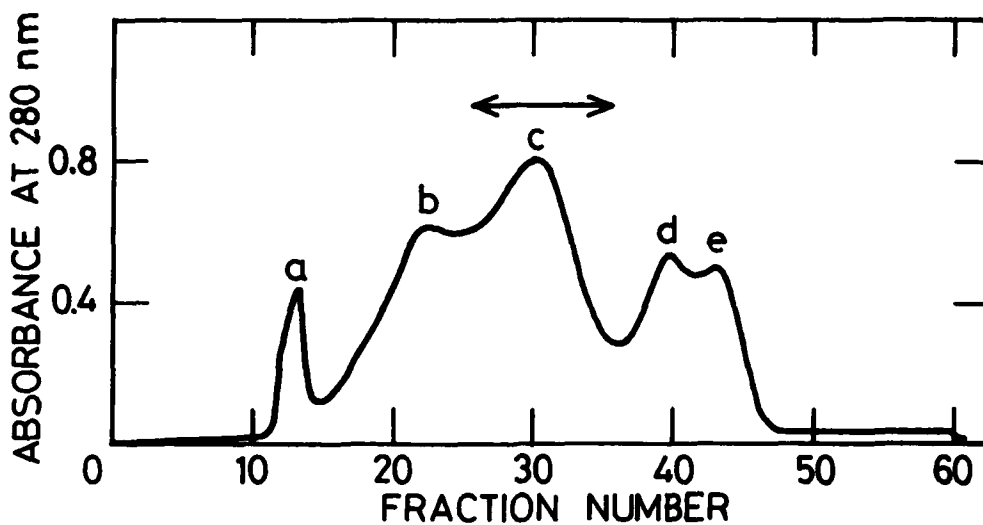
- c) Development of the RIA should provide a reliable diagnostic tool for leishmaniasis.
- d) The biological role of EF in infection will be studied by a series of haptenes, chemically and enzymatically modified EFs and modified promastigotes,
- e) We will attempt to increase the immunogenicity of EF by employing high-molecular weight carriers.
- f) Once better understanding of the structure of EF is available we will design inhibitors to the enzyme systems responsible for its biosynthesis.

9. Figures

- Fig. 1. Preparation of PNA
- Fig. 2. Separation of EF on anti-EF column
- Fig. 3. Separation of EF on a column of immobilized PNA

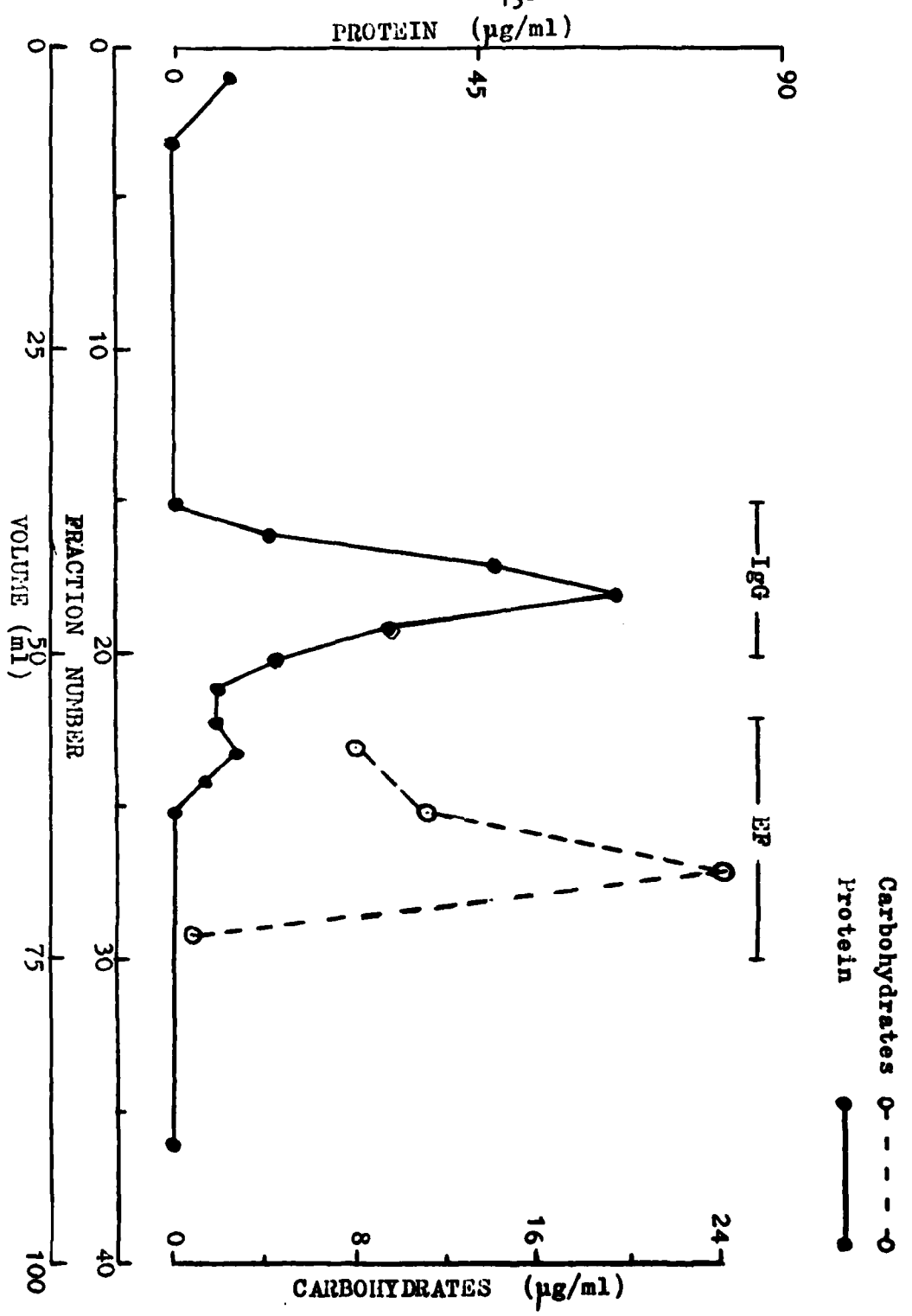


**Fig. 1.** Final Stage in Preparation of PNA-Sephrose Chromatography.

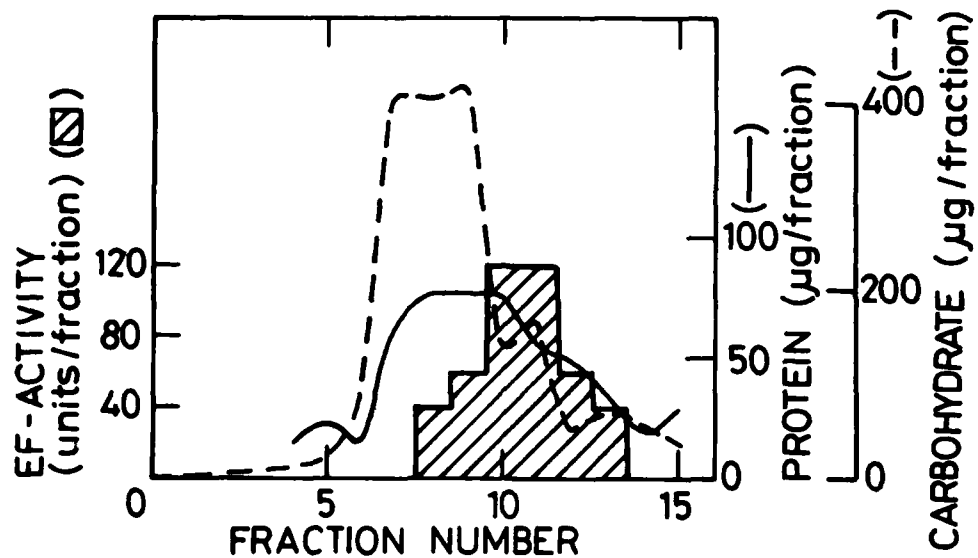


150 mg of "peanut powder" prepared as described by Terao *et al.* (1) was eluted on a Sepharose 6B column (73 cm x 2.0 cm) with 10mM tris-HCl buffer pH = 7.2. The fraction volume was 6.5 ml. The fractions indicated (peak c) showed agglutination activity with EF in immunodiffusion experiments, and were combined, dialyzed and lyophilized to produce PNA.

Fig. 2. Separation of *Leishmania donovani* EF and rabbit anti-L. donovani promastigotes IgG from dissociated complexes on Sephadex G-100 column, using 2 M sodium thiocyanate in PBS as eluting buffer.



**Fig. 3.** Affinity Chromatography of EF on a column of Immobilized PNA.



5.0 mg of EF (L-32) was chromatographed on a column of PNA- AE-cellulose (12 cm x 1.0 cm) and eluted with 0.02 M phosphate buffer pH = 7.0. The fraction volume was 1.0 ml. Fractions were assayed for EF-activity, protein and carbohydrate.

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## 11. Glossary

EF Factor excreted by Leishmania (Excreted Factor). 'Small' indicates lower molecular weight compound. EF preparations are designated with digits indicating the leishmanial source.

### Leishmanial strains

L32      Leishmania tropica LRC L32  
L137     Leishmania tropica LRC L137  
L52      Leishmania donovani LRC L52

These strains were obtained from the WHO Leishmania Reference Centre collection maintained in the Department of Protozoology in Jerusalem.

PNA      peanut lectin

RIA      radioimmunoassay

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