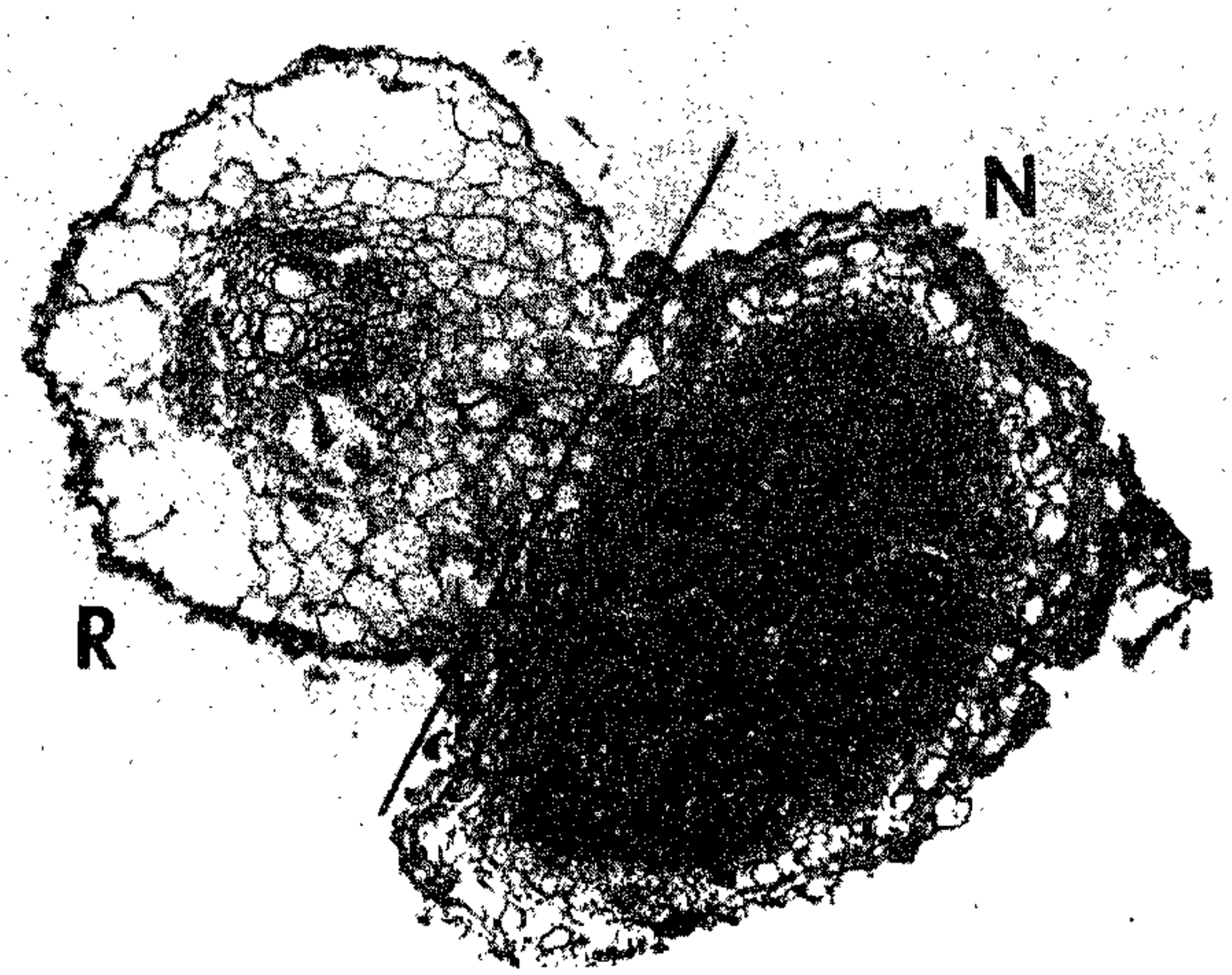

Figure 1. Micrograph of a cross-section of a nodulated root from a one-month-old soybean plant infected with *Rhizobium japonicum*. Nodule and root were separated as indicated by the solid line (N, nodule; R, root).

Table 1. Uptake and metabolism of [8-^{14}C]adenine and [8-^{14}C]-hypoxanthine exogenously supplied to the nodules and the roots of soybean plants

	[8-^{14}C]Adenine			[8-^{14}C]Hypoxanthine		
Precursor	Nodules	Roots	(N/R)	Nodules	Roots	(N/R)
Total uptake (nmol/100 mg fr. wt/4 h)	2.10	2.07	(1.0)	2.00	0.99	(2.0)
Incorporation (% of total uptake)						
RNA	21.2	14.8	(1.4)	17.6	8.5	(2.1)
DNA	1.4	2.4	(0.6)	2.2	1.7	(1.3)
Nucleotides	22.5	12.1	(1.9)	4.3	1.6	(2.7)
Nucleosides	13.6	11.1	(1.2)	3.6	7.3	(0.5)
Purine bases	10.6	30.6	(0.3)	7.3	16.6	(0.4)
Ureides	14.0	21.4	(0.7)	19.6	48.7	(0.4)
CO_2	15.2	5.7	(2.7)	43.7	14.2	(3.1)
Others	1.2	1.8	(0.7)	1.4	1.3	(1.1)

Amounts of adenine and hypoxanthine taken up by the samples are expressed as nmol per 100 mg material per 4 h. The rates of incorporation of radioactivity from [8-^{14}C]purines are expressed as the percentage of radioactivity taken up by the material. These values are averages of the results from duplicate samples. The ratio N/R, i.e. the ratio of the value in the nodules to that in the roots is also shown in parentheses for each class of compounds.

our knowledge, this is the first report of an examination of the salvage of purine bases in the nodules of leguminous plants.

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Major *Entamoeba histolytica* (200:NIH) immunogen

A. H. Khan, Sanjai Kymar, P. Kumar, M. K. Sahib* and S. R. Das
Divisions of Microbiology and *Biochemistry, Central Drug Research Institute, Lucknow 226 001, India

Antigenic analysis of axenic *Entamoeba histolytica* (200:NIH) antigen, by preparative polyacrylamide slab gel electrophoresis, reveal the presence of 13 distinct protein fractions. Major antigenicity was found in fraction 4 by indirect haemagglutination test. Fraction 4 on electrophoresis under non-denaturing conditions yielded a single band.

AMOEBIASIS is an important world wide disease resulting in an annual mortality of 10,000 to 40,000 and

considered as the second highest killing disease of humans after malaria (WHO)[1]. The invasive form of the disease is generally accompanied by the appearance of specific serum antibodies against *Entamoeba histolytica*. Detection of such antibodies is, therefore, important especially for invasive amoebiasis cases. Serological tests help in the diagnosis of infection when diagnostic stages (trophozoites or cysts) are not present or cannot be identified, specifically in the case of amoebic liver abscess and other cases of extra-intestinal amoebiasis.

Before 1968 amoebiasis was diagnosed serologically by the determination of specific serum anti-amoebic antibodies with the help of impure crude amoeba antigen. However, crude antigen was sometimes derived from contaminated *E. histolytica* cultures and therefore the results were inconsistent. In 1968 *E. histolytica* was grown axenically by Diamond and standard axenic antigen was prepared. The availability of standard antigen preparations has been helpful in the development and application of various diagnostic tests for diseases and elimination of non-amoebiasis patients.

The present study attempts to find out the major *E. histolytica* immunogen and evaluate the serological diagnosis of amoebiasis and protection.

Axenic trophozoites of *E. histolytica* (200:NIH) were grown in large scale in a modified Diamond's TP-S-1 medium (Singh *et al.*[2,3]) and amoebae from 72 h old cultures were collected and sonicated in sterile saline for 5 min at 20 Kc in a MSE ultrasonic disintegrator in an ice bath. The sonicated material was centrifuged at 105,000 *g* in an ultra centrifuge (Spine, Rotar type 50) for 60 min. The 105,000 *g* supernatant and sediment fractions were collected separately. Protein contents were estimated according to Lowry *et al.*[4]

The 105,000 *g* supernatant fraction was subjected to preparative vertical polyacrylamide slab gel electrophoresis in electrophoretic chamber (Pharmacia, Sweden) following the method of Davis[5] using 7% gel. The protein (13.2 mg) was loaded on each slab gel. Electrophoresis was carried out at a constant current of 30 mA/gel for 4 h. After electrophoresis, the gel was sliced horizontally at 0.5 cm intervals, yielding 13 strips of the gel. A vertical piece of the gel was cut and stained in Coomassie Brilliant Blue (0.1%) stain.

Each strip was minced by passing it through a 5 ml glass syringe. The gels were suspended in 2 ml of glass distilled water and allowed to stand overnight at 4°C. The eluate was separated from the gel by centrifugation at 5000 *g* for 30 min. The protein content of each was estimated according to Lowry *et al.*[4] Antigenic analysis of different fractions was carried out by gel diffusion precipitin (GDP) and indirect haemagglutination (IHA) tests following the techniques of Aurenheimer and Atchley[6], and Khan and Das[7] respectively.

Golden hamsters (wt 30–40 g) were immunized with whole amoeba antigen, 105,000 *g* supernatant and sediment fractions by three subcutaneous injections at weekly intervals with 0.1 ml, containing 500 μg antigen per animal emulsified with 0.1 ml of Complete Freund's Adjuvant (CFA). The animals were challenged with intrahepatic inoculation following laprotomy of approximately 6 to 7×10^4 trophozoites of *E. histolytica* (H-39) growing associated with bacteria. Bacterial association with *E. histolytica* is essential to produce amoebic lesions to hamster liver.

Antigenic analysis of the whole amoeba antigen, 105,000 *g* supernatant and sediment fractions against hyper-immune rabbit serum (anti-*E. histolytica* antibodies) by GDP test is presented in Table 1. Hyper immune (anti-*E. histolytica* antibodies) was produced in rabbit being immunized with *E. histolytica* antigen following the method of Khan and Das[7]. The supernatant fraction showed sharp and distinct bands. The supernatant fraction (0.5 ml of 105,000 *g*) containing 13.2 mg of protein was fractionated on preparative polyacrylamide slab gel electrophoresis and eluted in 13 different fractions, approximately 85% of the total protein was recovered. Table 2 shows the protein profiles of 13 fractions of 105,000 *g* supernatant on PAGE. Antigenic activity of different fractions obtained from preparative polyacrylamide gel electrophoresis with IHA test is shown in Table 3. Results showed that the major antigenic activity was in fraction 4 which showed single

Table 1. Antigenic analysis of whole amoeba-antigen, 105,000 *g* supernatant and sediment fractions by GDP test.

Antigen (protein concentration 2 mg/ml)	No. of precipitin bands
Whole amoeba-antigen	7
105,000 *g* supernatant	8
105,000 *g* sediment	3

Table 2. Elution profile of 105,000 *g* supernatant fraction on preparative polyacrylamide slab gel electrophoresis.

			Total protein		
Antigen		Total volume	mg	Per cent	Protein μg/ml
105,000 *g* supernatant fraction		1.0	13.2	100	13,200
Fraction	1	1.7	1.31	9.92	775
	2	0.6	0.81	6.14	1,350
	3	1.35	1.11	8.41	825
	4	1.10	1.05	7.80	950
	5	0.50	0.64	4.85	1,280
	6	1.20	1.28	9.70	1,070
	7	0.70	0.94	7.12	1,350
	8	1.00	0.90	6.82	900
	9	1.00	1.02	7.73	1,020
	10	1.20	1.02	7.73	850
	11	1.00	0.90	6.82	900
	12	0.90	0.09	0.68	100
	13	1.30	0.18	1.36	140
Recovery			11.25	85.10	

Table 3. Antigenic analysis of different fractions by indirect haemagglutination (IHA) test against hyperimmune rabbit serum.

Protein concentration (μg/ml)	IHA titres obtained with												
	105,000 g Supernatant	Fraction											
		1	2	3	4	5	6	7	8	9	10	11	12	13
40	2048	16	128	4093	131072	4096	128	64	64	512	512	128	16	128
20	512	16	32	1024	32768	1024	16	16	16	128	128	16	16	32
10	32	16	16	128	4096	128	16	16	16	16	16	16	16	16
5	16	16	16	32	2048	32	16	16	16	16	16	16	16	16
1	16	16	16	16	16	16	16	16	16	16	16	16	16	16

band on SDS-polyacrylamide gel electrophoresis (figures 1 and 2). The method used is the conventional SDS–PAGE. The molecular weight of major antigenic fractions (single band protein) was determined by extrapolating mobility plot of relative migration in SDS–PAGE against log molecular weight of standard protein markers.

The relative electrophorectic mobility (R_f) of the single band protein was 0.34 which corresponds to the molecular weight of 90,000 daltons.

Protective effect of the whole amoeba antigen, 105,000 g supernatant and sediment fractions is presented in Table 4. Complete protection was reported in the experimental animals which were immunized with 105,000 g supernatant fractions.

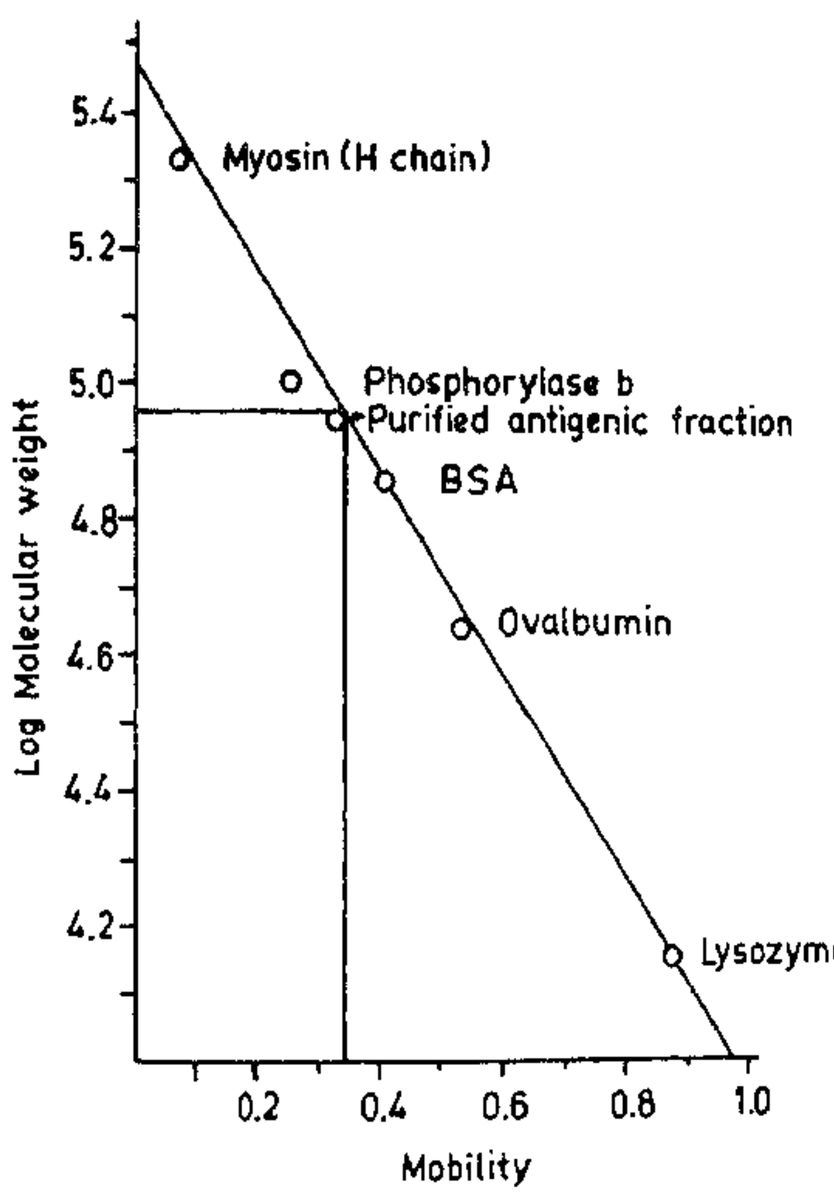

Figure 2. Molecular weight determination of purified antigenic fraction of *E. hystolytica* using myosin (H chain) 200,000; phosphorylase b (97,400); BSA (68,000); ovalbumin (43,000); lysozyme (14,300) as standards.

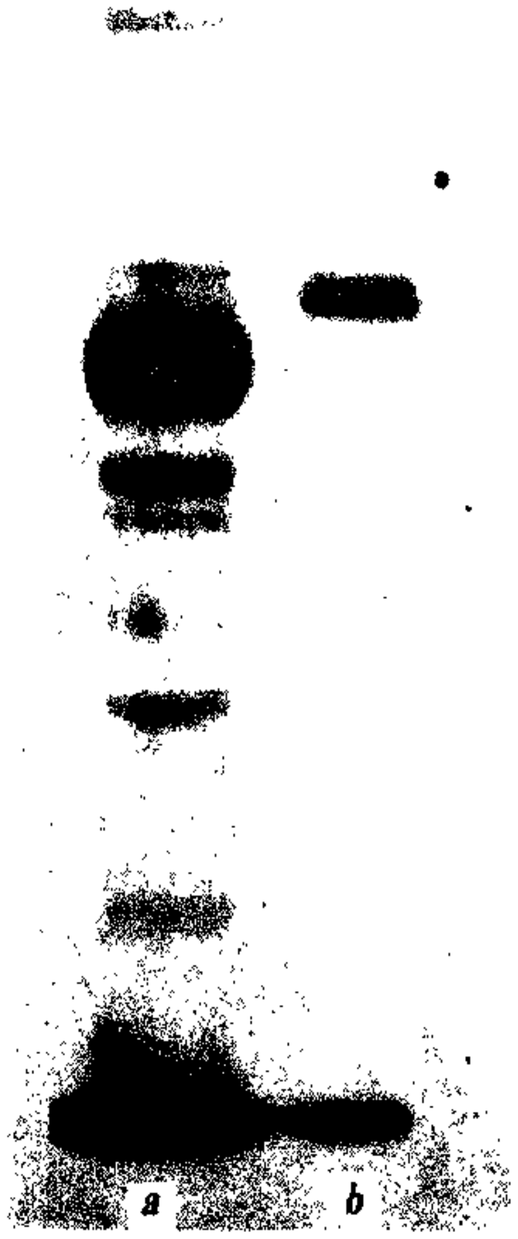

Figure 1. SDS-PAGE profiles of purified single band antigen fraction 4 of *E. hystolytica* and standard protein markers ***a***, standard protein makers. ***b***, Purified fraction 4.

Table 4. Protective effect of whole amoeba-antigen, 105,000 g supernatant and sediment fractions of axenic *E. histolytica*.

Group	No. of hamsters free of liver abscess/number of inoculated	% Protection *P value
Whole amoeba-antigen + CFA	5/8	62.5 ($P<0.05$)
105,000 g sediment fraction + CFA	1/8	12.5 (NS)
105,000 g supernatant fraction + CFA	8/8	100.0 ($P<0.01$)
Isotonic saline + CFA	0/8	Nil
Control (No. immunization)	0/8	Nil

CFA, Complete Fruend's Adjuvant. NS, Not significant.
*Student's test.

Krupp[8] and Sawhney *et al.*[9] purified axenic *E. histolytica* (strain NIH:200) antigen on sephadex G-200 column and obtained three (F_1, F_2 and F_3) fractions. The molecular weights of these fractions ranged from 650,000 to 1,450 and found that the major antigenic activity were associated in fraction F_1.

The axenic *E. histolytica* antigen was purified by using the 105,000 *g* supernatant fraction of whole amoeba extract. This fraction was purified on preparative polyacrylamide slab gel electrophoresis. A comparative antigenic activity obtained with GDP and IHA tests clearly shows maximum precipitin bands and haemagglutinating activity in the 105,000 *g* supernatant fraction. Among the 13 fractions isolated the highest haemagglutinating and precipitin activity was in fraction 4. Fraction 4 gave maximum antigenic response in GDP and IHA tests. This fraction at the protein concentration of 5 μg/ml gave the titre in IHA test similar to 105,000 *g* supernatant fraction at protein concentration of 40 μg/ml.

Fraction 4 gave single protein band on analytical polyacrylamide slab gel electrophoresis. This shows that the isolated antigen fraction, which showed highest immunogenicity, is a pure protein (Table 3).

Ganguly *et al.*[10] purified axenic *E. histolytica* antigen on SDS-polyacrylamide gel electrophoresis and isolated 9 fractions they have reported that fraction 5 showed a significant macrophage migration inhibition of 100 μg/ml concentration in amoebic liver abscess.

Results on protective immunity show 100% protection for 105,000 *g* supernatant fraction against experimental hepatic amoebiasis of hamsters whereas partial protection was obtained with whole amoeba-antigen (62.5%). The 105,000 *g* sediment fraction gave only 12.5% protection (Ghadarian *et al*[11], experimental hepatic amoebiasis in hamsters.)

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31 October 1988; revised 21 February 1989

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