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Application of New Techniques in Biochemistry and Molecular
Biology for Studies on Parasite/Vector Relationships in Leishmaniasis

Charles L. Greenblatt Yosef Schlein
Joseph Shlonai Lionel F. Schnur

Hebrew University-Hadassah Medical School, Jerusalem

In the interim report which we submitted in April of 1985 we dealt with three aspects of the work, leishmania-sandfly interaction, parasite heterogeneity, and kinetoplast DNA. In this report, we shall utilize these same frameworks and give in more detail the results of the second half of the year. All areas of the work have advanced, with one exception- the sandfly colony has suffered infections of both viruses and yeast which have prevented the studies of the development of the leishmanial clones in the vector.

LEISHMANIA-SANDBLY INTERACTION

The last report concentrated on the selective effect of the vector digestive enzymes on two different species of Leishmania, one which is successfully transmitted and one which is not. It was found that there was a significant inhibition of proteolysis by the transmitted parasite and by its extracellular products. In this report, we shall emphasize the diet of the sandfly on the success of transmission.

Transmission of leishmania by the bite of laboratory reared sandflies is difficult to achieve and unpredictable for reasons which can, in part, be attributed to the lack of specific nutritional factors (1). Sandfly diets in nature contain sugars probably of plant origin (2,3). Under experimental conditions, laboratory-reared Phlebotomus papatasi obtain sugars from plants, by probing leaves and stems, as well as honeydew secreted by plant-sucking hemipteran insects (1). Apart from sugars, phloem sap and honeydew also contain significant amounts of amino acids which may facilitate the transmission of Leishmania.

We assessed the effects of some artificial post blood-meal diets of infected Phlebotomus papatasi females on the transmission of Leishmania major. Forced-feeding of sandflies by micro-capillaries simulated normal blood-feeding and enabled the individual testing of a large number of infected flies. Microscopical observation of the mouth parts of flies, made during forced feeding, provided us with information relevant to the possible mechanism of transmission by bite.

Materials and Methods

Forced-feeding was by micro-capillaries that were prepared according to the method described by Hertig and McConnel (4). A capillary, prefilled with a mixture of saline and rabbit blood (9:1), was slipped over the mouth parts forcing them to assume the blood-feeding mode (Fig. 1). Flies were allowed to feed for 10 mins, after which they were dissected and examined under a phase-contrast microscope. Infections in the different parts of the alimentary canal were scored, and parasites egested into capillaries were counted after the fluid was extracted onto microscopical slides. The results were analysed using the X2 test. Flies egesting parasites into capillaries are hereafter referred to as transmitting flies.

Results

An average infection rate of 73% was scored in the 241 flies which were dissected between 10-12 days after the infective meal. Almost all the infected flies (97%) had parasites in the anterior midgut and the stomodeal valve, and in 54% of them free promastigotes were observed in the head region of the gut. The percentage of transmitting flies was significantly higher ($p < 0.01$) in the group fed on a mixture of sucrose and albumin than any of the other groups (table 1).

Many of the flies that did not gorge, did regurgitate fluid containing parasites into capillaries (table 2).

Abdominal midgut infections were heavy, in all the groups, consisting mainly of elongate, thin nectomonads (15-25 μm x 1 μm thick). The thoracic midgut and stomodeal valve were crammed with parasites of different morphological forms including short (4-5 μm), highly active unattached promastigotes which were found in the esophagus and in fluid from capillaries. No attached parasites were found anterior to the esophagus, and head infections consisted of free, highly active forms which may have been flushed forward during the feeding. Most of the transmitting flies (75.6%) egested between several and 100 parasites, some (19.5%) egested 100-1000 parasites and two flies (4.9%) egested over 1000 parasites each. No difference was observed in the development of leishmania parasites between the groups fed on different diets and between transmitting and non-transmitting flies. Since there were transmitting flies in all groups, it appears that there is no single essential nutritional requirement for transmission of leishmania. Nevertheless, the group fed on a mixture of protein and sugar yielded the highest proportion of infective flies. This diet was meant to simulate plant sap and honeydew, since it contains sugar and protein upon which *P.papatasi* feed (5).

Although unattached parasites were observed in the head region of the guts of all the transmitting flies, they were also seen in many non-transmitting ones (Table 2). Therefore their presence should not be considered indicative of potential transmission by bite.

A high percentage of the flies that did not ingest fluid were shown to be transmitters (Table 2). No parasites were observed attached to the cibarium in any of the flies, but the masses of attached parasites were in the anterior thoracic midgut.

These attached forms in the pocket between the gut wall and the stomodeal valve may constrict the lumen of the valve by compressing its surrounding lobes and thus prevent or reduce the passage of blood into the midgut (2). These observations support the "blocked fly" theory of transmission by bite originally suggested by Shortt and Swaminath (6). Infective stages of *L.major* have been experimentally demonstrated to be present in the midguts of infected *P.papatasi* (7). Our results and observations demonstrate that large numbers of these infective promastigotes from the stomodeal valve and the esophagus can be egested during blood feeding of flies which have no permanent infection in the head.

Summary

There are 3 main observations in this study: 1. infected flies which took in small quantities or no fluids, regurgitated and ejected parasites into the capillaries, 2. Transmission of parasites occurred in flies which had no infection of the head region of the gut (3). More transmitting flies were recorded in the group that had been kept on diet of sucrose and protein than in the groups that had just one of the components.

It is postulated that natural sugar meals, when identified will promote transmission beyond that of the crude imitation used in the study.

HETEROGENEITY OF PARASITE POPULATION

We have continued utilizing the techniques described in the first report and have now almost completely characterized 45 clones separated from 21 isolates from patients attending our leishmaniasis clinic. These we refer to as mother strains. Eight mother strains from the Jordan Valley region gave 10 clones, 6 mother strains from the Western Negev-Eastern Sinai region gave 20 clones and 3 mother strains from the Arava region gave 15 clones (see map for regions). A picture is emerging of the microheterogeneity of the leishmanial populations in nature.

Virulence - All the clones have been uniformly virulent in outbred Sabra mice injected at the base of the tail with a dose of 10,000 parasites. Usually all three mice in an experimental group injected with a given clone were positive, producing a lesion in which parasites could be found. In a few cases only two or even just one mouse were shown to be positive.

Morphology - All the clones showed considerable polymorphism giving variation in size and shape. No special morphological features were noticed as being associated with any particular strain or clone.

Serotyping - By using excreted factor (EF) serotyping, to characterize Israeli strains over many years, we have shown that most strains from the regions considered here typed as EF subserotype A1 organisms. A few strains isolated from cases from the Arava some thirty years ago were of mixed serotype, EF subserotype A1B, where a B determinant was also shown to be present in their EFs, but where it was not possible to designate a subserotype. A single strain from Ein Gedi by the Dead Sea, also isolated many years ago was shown to be EF subserotype A4.

Serotyping of the recently isolated mother strains and their clones demonstrated further serotypic variation, with subserotypes falling into regional clusters. The 8 mother strains from the Jordan Valley and the 10 clones derived from them were all EF subserotype A1. The 3 mother strains from the Arava and the 15 clones derived from them were all EF subserotype A1B, confirming the serologically mixed nature of strains from this region. The 6 mother strains from the Western Negev - Eastern Sinai region, proved to be alike, but somewhat different from strains described so far of the subserotypes A1, A4 and A1B and they probably constitute a new subtype of the A serotype. This needs further investigation, since, of 20 clones

derived from these 6 mother strains, 11 were of the parental subserotype, whereas 9 were EF subserotype A1.

Enzyme Variant Typing- This was carried out by cellulose acetate electrophoresis, comparing the mobility of 11 different enzymes: MDH, 6PGD, SOD, ALAT, ASAT, PK, PGM, NH, PEPD, MPI and GPI against those of three Israeli reference strains, each one representing one of the three geographical regions; since 3 different variants of 6PGD have been found, each one apparently associated with a different region (see Fig.2) (8).

Generally this geographical separation of 6PGD variants was upheld, but not entirely. The 8 mother strains from the Jordan Valley region and all but two of their clones were like the Jordan Valley region reference strain. The two exceptional clones were like the Western Negev-Eastern Sinai region reference strain in its 6PGD. The six mother strains from the Western Negev-Eastern Sinai region and all 20 clones derived from them were identical with regard to their 6PGD and like the reference strain for this region in this respect. However, one variant was seen with regard to the enzyme NH in the case of one clone from this region.

Concerning the mother strains from the Arava region and their clones the situation was more complicated and interesting. One mother strain and the clones from it were all like the reference strain for the region, with regard to their 6PGD. Another mother strain from this region and the clones from it were all like the reference strain for the Jordan Valley region in this respect. While the third mother strain and three of the clones from it were like the Jordan Valley region marker and two clones from it were like the Western Negev-Eastern Sinai region marker in terms of their 6PGD. In all other respects concerning enzymes profiles all the strains and clones were alike.

Kinetoplast DNA analysis by restriction endonuclease analysis- Kinetoplast DNA (kDNA) from a number of clones and uncloned populations was isolated by the rapid method of Morel (9) and digested with several restriction enzymes. The digests were electrophoresed on agarose and acrylamide gels, using standard buffers and electrophoretic conditions. The enzymes which gave the clearest results were Hae III, Eco RI, Taq I, and Hpa II.

1% and 2% agarose minigels provided insufficient resolution of the bands, so that the final results were obtained from 5% acrylamide gel electrophoresis. Clones from each of the geographical regions fell into schizotypes (schizodeme) specific for the area. In the area the grouping could be subdivided further into two subschizotypes. Strain LRC-L 137's clone 121 showed a very simple pattern which was clearly like that of all the L. majors, but very distinctive in its lack of multiple banding. Whether this loss of the usual heterogeneity is due to the nature of cloning or to laboratory passage is unknown, since LRC-137 has been maintained in our laboratory since 1967.

The length of the L. major linearized minicircle in the agarose gels was 710 bp and appeared as a conserved band in all the digests. On acrylamide gels this band migrates more slowly at approximately 800 bp. In acrylamide, this band expands to a large number of bands, indicating abnormal migration of different minicircle sequence classes. Supercoiled and open minicircles released from the network run in front of and behind the linearized

minicircles respectively.

Fig. 3 and table 3 summarize the results for eleven clones and three uncloned populations, the latter are the marker strains for the three zymodemes. The minicircles have a conserved and a variable region. If restriction sites are in the conserved region, as with Hae III and Taq I, the network is completely disrupted because all the minicircles are cut. The wells will contain only high molecular weight oligomers. When the site is in the variable region, as with Hpa II and Eco I, a substantial number of minicircles do not contain sites and undigested network kDNA remains as a very bright band in the wells, with fainter bands in the cut region.

The kDNA of each clone were distinguishable. However, clones from the same geographical area were more similar to each other than to those from other areas, implying a closer genetic relationship. This is supported by similar enzymological and serological evidence (see table 3).

STUDIES ON KINETOPLAST DNA

Introduction

The project deals with two aspects of the kinetoplast DNA (kDNA) study: (A) The control of kDNA topological state; (B) kDNA minicircles coding capacity. Since the previous report we have made progress on both aspects of this project. Crithidial kDNA minicircle sequences which were expressed in *E. coli* are now analyzed, and analysis of rRNA transcripts expressed in Crithidia-infected sandflies guts to detect possible kDNA minicircle mRNA transcripts was initiated. However, in this report we would concentrate on the progress made in the study of the control of kDNA topological state.

As was mentioned in the proposal and the interim report, Crithidia DNA topoisomerase was suggested by us as the central enzymatic activity functioning in the "Release and Reattachment" system in the course of kDNA minicircle replication. This is in concert with at least two other proteins: a DNA aggregating protein factor, and a unique DNA nicking enzyme. Englund's model, which describes the replication of kDNA minicircles as free individual DNA rings, assumes the existence in the cell of an enzymatic system which can catalyze the topological interconversions of monomeric DNA circles and catenanes, discriminating between newly replicated progeny DNA minicircles and mature parental ones. Such a discriminatory capacity is required in order to insure the release, and thereby the replication, of each of the minicircles only once in every generation. Since newly replicated DNA minicircles, either free or reattached to the DNA network, are nicked, while mature parental ones are covalently sealed, it has been previously suggested that nicking might provide the signal for discrimination between replicated and unreplicated DNA minicircles. The mechanism by which this nicking is carried out is not yet clear. It has been previously suggested that nicks either remain in the replicated minicircles due to inefficient ligation, or alternatively, that they are introduced post replication by a special nicking enzyme. Here, we report on the purification to homogeneity and the characterization of crithidial nicking enzyme, and discuss its unique effects on the topological interconversions of monomeric DNA minicircles and DNA networks.

Results

Purification of Crithidia Nicking Enzyme- Purification of Crithidia nicking enzyme was based upon both the nicking of duplex circular DNA, and inhibition of the decatenation of kinetoplast DNA networks catalyzed by Crithidia DNA topoisomerase. Using these assays, Crithidia nicking enzyme was purified approximately 3000 fold over the cell cleared lysate (fraction i), to near homogeneity, with an overall yield of about 15% (Table 4). The procedure yielded about 1 mg of pure nicking enzyme from about 80 g of Crithidia fasciculata wet cell paste (about 2×10^{12} cells).

Lysis conditions were design here, for the gentle disruption of the Crithidia cell membrane, using low concentrations of nonionic detergent (0.02% of Brij-58) in hypotonic solution. The cleared lysate (Fraction i) contains about 24,000 units of nicking activity per each gram of wet cell paste. One has to consider other endonucleolytic activities, as well as inhibitors of the assay in the evaluation of these figures.

Upon ammonium sulfate fractionation of the cleared cell lysate Crithidia nicking activity was precipitated (about 70%, Tables 4) at the 20-40 percents of saturation (Fraction ii), where a fraction of about 28% of the total, cleared extract proteins is recovered. Binding of crude enzyme preparations (fraction ii) to DEAE-cellulose (fraction iii) is probably due to the enzyme interactions with other tightly bound proteins or with bound nucleic acids, since this capacity to bind to the resin is lost (Fraction v) following its chromatography on hydroxyapatite (Fraction iv) (Fig. 4A). Hydroxyapatite chromatography separates the nicking enzyme eluted at a low phosphate concentrations (about 60 mM), from a DNA topoisomerase activity eluted at a rather high phosphate concentration (300-400 mM). The DEAE-cellulose flow-through chromatography (Fraction v) step is essential, since it enables the subsequent step of chromatography on phosphocellulose (Fraction vi) (Fig. 4B), probably due to the removal of the remaining nucleic acids from the enzyme preparation. The final affinity chromatography step on single stranded DNA cellulose (Fraction vii) can be followed, if necessary, by a gel filtration step on Bio-Gel P-300.

Stability of Crithidia Nicking Enzyme- Purified enzyme preparations were found to be unstable at 0-4 C. The enzyme lost about 50% of its initial activity within 10 min. at 36.5 C (Fig. 5). Glycerol increases the heat stability of pure enzyme preparations (Fig. 5, insert). No decrease in nicking activity for 30 min at 36.5 C in the presence of 25% (v/v) glycerol. The enzyme is stable for at least 6 months at -20 C in the presence of 50% glycerol.

Physical Properties- Molecular weight and subunit structure: Crithidia nicking enzyme migrates in SDS- polyacrylamide gel electrophoresis, under denaturing and reducing conditions, as a single polypeptide band of 60,000 daltons (Fig 6,7) On the final purification step by affinity chromatography on single stranded DNA cellulose (fraction VII), this 60,000 daltons band corresponds well with the DNA nicking activity profile and it is separated from an accompanying 53,000, 83,000, and 89,000 dalton polypeptide bands (Fig. 6). The apparent molecular weight of the native Crithidia nicking enzyme, determined by P-300 gel filtration (Fig. 8) according to the method of Andrew is about 129,000. Gel filtration data give a stokes radius of 40.4A as calculated by the method of Siegel and Monty (Fig. 9). A sedimentation coefficient of 6.8 S was estimated upon sedimentation through a 20-40% (v/v) glycerol gradient (Fig. 7), according to the method of Martin and Ames. The apparent native molecular weight of the enzyme calculated from the experimental sedimentation coefficient and Stokes radius, and assuming a partial specific volume (\bar{v}) of 0.725 ml/g, is estimated as 114,000. The frictional coefficient calculated from the equation.

$$f/f_0 = \frac{a}{(3\bar{v}M/4\pi N)^{1/2}}$$

is 1.241 (where a, Stokes radius; M, molecular weight; \bar{v} , partial specific volume (assuming a value of 0.725 ml/g); and N, Avogadro's number).

Based on the data obtained from the SDS-polyacrylamide gel electrophoresis analysis and the estimation based on sedimentation and gel filtration analyses it is suggested that the native Crithidia fasciculata nicking enzyme is a dimer protein with a protomer of 60,000 daltons. Based on the enzyme activity in cleared cell extracts, its molecular weight and the specific activity of the apparently homogeneous enzyme it is estimated that there are approximately 15,000 molecules of this enzyme per trypanosomatid cell.

Catalytic Properties of the Nicking Reaction-

Requirements of reaction and its inhibition: Nicking of duplex DNA by Crithidia nicking enzyme requires Mg^{2+} , optimal levels of Mg^{2+} were at the range of 5-15 mM. Monovalent ion salts at concentrations higher than 10 mM are inhibitory; inhibition was 50% at the concentrations of about 60 mM NaCl or 100 mM KCl. Glycerol concentrations beyond 10% were inhibitory; inhibition was 25% and 50% of the optimum in the presence of 15% (v/v) and 30% (v/v) of glycerol, respectively.

The pH optimum is in a narrow range: of 7.50-8.25 (in Tris, phosphate and glycine buffers). At pH 7.20 (phosphate) and 8.40 (borate) activity was 75% of the optimum and it dropped down to 50% at pH 6.6 (phosphate) and 8.75 (glycine), and to about 15% of the optimum at pH 5.2 (acetate) and 9.85 (borate). (Not shown).

Products of the enzyme reaction are nicks in duplex DNA circles: Treatment of covalently closed duplex DNA circles with the purified enzyme, results in the breakage of phosphodiester bonds in the molecules. Treated pBR322

duplex DNA circles migrated as nicked circular molecules upon electrophoresis in ethidium bromide containing agarose gels (Fig. 11A). The interruptions introduced by the enzyme in the circular DNA molecules were defined as single stranded DNA nicks containing 3' hydroxyl and 5' phosphoryl groups. This is by virtue of their capacity to be covalently resealed by the action of DNA ligase, as indicated by their supertwisting in the presence of ethidium bromide (Fig. 11B). The minor fraction of non ligated pBR322 molecules was also observed in the preparation prior to the treatment with the nicking enzyme.

Nicking of kDNA minicircles affects their reversible decatenation by Crithidia DNA topoisomerase: It has been previously reported that newly replicated kDNA minicircles, either free or reattached to the network, are nicked, while unreplicated ones are covalently sealed. It was suggested that nicking might provide the signal for discrimination between replicated and unreplicated minicircles to insure the replication of each minicircle only once in every generation. We have studied the hypothesis that such a discrimination of nicked versus covalently sealed kDNA minicircles is executed by a DNA topoisomerase upon the prereplication release of kDNA minicircles from the network and their post replication reattachment to it. Our previous observations, using crude enzyme preparations, have suggested that Crithidia type II DNA topoisomerase has the capacity to catalyze the catenation, but not the decatenation of nicked DNA circles.

Nicking of free monomeric kDNA minicircles, using the apparently homogenous nicking enzyme, had no significant effects of either the rate or the extent of the catenation reaction catalyzed by purified Crithidia topoisomerase (Fig. 12). The slightly increased rates of the catenation reaction measured using nicked monomeric circles was reproducible. While nicking had no significant effects on the catenation of free monomeric circles, it had dramatically affected both the rates and the extents of the reversible decatenation reaction catalyzed by Crithidia topoisomerase (Fig. 13).

Reversibility of the topological reaction is specifically affected by Crithidia nicking enzyme; Crithidia nicking enzyme introduces a single nick into the negatively supercoiled duplex DNA circles (Fig. 14) used as substrates. We have previously reported that inhibition of the topological reaction does not result from the relaxation per se of interlocked kDNA minicircles due to their nicking. The results presented in Fig. 14 strongly suggest that the effect on the topological reaction is neither the result of a random nicking of the interlocked DNA circles. Whereas nicking of duplex DNA minicircles, interlocked into a kDNA network, results in significantly reduced rates (<4% of the rate measured using untreated networks) of decatenation (Fig. 15), no inhibition could be measured when kDNA networks nicked by DNase I (using limited DNase I digestion in the presence of ethidium bromide) were used (Fig. 4). Rates and extents of decatenation reactions using DNase I nicked minicircles, (notice that nicked monomeric minicircles are the sole products of such a reaction, Insert B vs. A in Fig. 15), were either equal to, or slightly higher than the values measured using untreated networks (Fig. 15). Further studies on the site specificity of Crithidia nicking enzyme, and the mechanism by which it specifically affects the topological reaction are now in progress.

Conclusions and Discussion

An enzyme purified to apparent homogeneity from Crithidia fasciculata cell extracts introduces nicks into duplex DNA circles and thereby interferes with trypanosomatid DNA topoisomerase. It has been previously observed that E. coli and M. luteus DNA gyrases as well as T4 DNA topoisomerase unlink nicked catenanes and knobs less effectively than they unlink the covalently sealed forms. However, as was found in the present study, introduction of nicks into random sites in either free or topologically interlocked kDNA duplex minicircles has no effect upon the reversible catenation-decatenation reaction catalyzed by either at random, using limited digestion by DNase I (Fig. 15), or nicked at their unique Xho I restriction endonuclease site (cleaved in the presence of ethidium bromide, not shown).

The observations suggest that nicking of the DNA substrate per se does not affect the reversibility of the topological reaction, using Crithidia DNA topoisomerase. Since nicking of the interlocked duplex DNA circles by the Crithidia nicking enzyme does interfere with the reversibility of this reaction (Fig. 13), one could argue that this enzyme action upon the DNA substrate results in a change of the DNA molecule which specifically affects its decatenation. The product of the enzyme reaction was identified as a proper single stranded DNA nick in the duplex DNA molecule, possessing a 3'-hydroxyl and a 5'-phosphoryl groups, which could be efficiently ligated by DNA ligase (Fig. 11), or used as a template-primer for nick translation (but not for gap-filling) by E. coli DNA polymerase I (data not shown). In as much as one could consider the nicking as the sole product of the enzyme reaction, this might imply that the targets for such nicking are some specific sequences or secondary structures which are crucial for the topoisomerase reaction. Such a model to describe the effect of nicking on the topological reaction has to explain the differential effects of the change in the interlocked molecules on the two mechanistically identical reactions of catenation versus decatenation. Possible differences in the sites of the topoisomerase action upon the DNA substrate under catenation versus decatenation conditions might provide the answer for these questions. We are currently studying both the Crithidia DNA topoisomerase and the nicking enzyme site specificities.

Crithidia nicking enzyme is located within a cell which contains a unique and most complex topological structure - the kinetoplast DNA network. Replication of the monomeric units of this network as free individual DNA minicircles implies the requirement for an enzymatic activity which catalyzes their release from the network prior to replication and their subsequent reattachment to it once their replication is terminated.

It has been previously suggested that nicking could provide the signal for discriminating between replicated (nicked) and unreplicated (covalently sealed) minicircles. If nicking bears such a special significance in this replication system, by conferring the capability of the crithidial topoisomerase to distinguish between replicated and unreplicated DNA minicircles, then it could not be the result of a random inefficient ligation during replication. Instead, our results are in accord with the

notion that such a nicking is carried out through an active nicking event, carried out at a special target site, in the DNA molecule, by a unique nicking enzyme.

In the next year on this aspect of the project, we intend to study the mechanism by which the introduction of a single nick at a unique site could affect the reversibility of the topological reaction. Experiments will be carried out to detect the precise location of the critical topoisomerase target sites under both catenation and decatenation conditions. These sites will be studied in comparison with the nicking enzyme target site, to elucidate the mechanism for the differential effect of the specific nicking on the catenation versus decatenation reaction. We believe that these studies will shed some light on the mechanism of discrimination of replicated versus unreplicated kDNA minicircles in the course of kDNA network replication.

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Table 1

Effect of post blood-meal nutrition of P. papatasi on the transmission of L. major (10-12 days after infection).

	1	2	3	4
	10% Sucrose	10% Trehalose	10% Albumin	2% Albumin 10% Sucrose
Total number of infected flies	55	20	35	66
Number of transmitting flies	11	3	4	23
% transmitting flies	20.0	15.0	11.4	34.8

Table 2

Correlation of transmission with feeding and presence of parasites in the head.

	NORMAL ENGORGEMENT	NO ENGORGEMENT	PARASITES IN HEAD
Total number of flies	176	14	95
Number of transmitting flies	31	10	41
% transmitting flies	17.6	71.4	43.2

Table 3

Comparison of biochemical (kDNA and enzyme) characters of strains and clones of Leishmania major from different regions where cutaneous leishmaniasis is endemic

Region	Jordan Valley	Western Negev- Eastern Sinai	Arava
kDNA schizotype	α	β, γ	β
6 PGD Variant	<u>a</u>	<u>b</u>	<u>c</u>
EF serotype	A ₁	A ₁ , A ₄ and new subtype	A ₁ B _x
Reservoir-host	<u>Psammomys obesus</u> subspecies I	<u>Psammomys obesus</u> subspecies II <u>Meriones crassus</u>	<u>Psammomys obesus</u> subspecies II <u>Meriones crassus</u>
Vector	<u>Phlebotomus papatasi</u>	<u>Phlebotomus papatasi</u>	<u>Phlebotomus papatasi</u>

TABLE 4

Crithidia fasciculata cells were grown as described under "Materials and Methods". 2×10^{12} cells were harvested by centrifugation and washed twice using TS buffer (50 mM Tris-HCl pH 7.4, 10% (w/v) sucrose). The washed wet cell paste (80 g) was frozen in liquid nitrogen and stored at -70°C . All subsequent operations were carried out at $2-4^{\circ}\text{C}$. Thawed cells were kept on ice and lysed using the following "gentle lysis" procedure: Cells were diluted in five volumes of sterile distilled water and kept in the hypotonic solution for 1 min at 0°C . Brij-58 (Sigma) was added with gentle mixing to final concentration of 0.02% and the suspension incubated for 1 min at 0°C . The suspension was made up to 5 mM EDTA, 20 mM spermidine- Cl_3 , 5% (of saturation at 0°C) ammonium sulfate and 10 mM DTT. Lysis was confirmed by light microscopic observation. The cell lysate was centrifuged in a Sorvall SS-34 rotor at 17Krpm for 2 h, to yield the cleared cell lysate supernatant (Fraction I, 425 ml/80 g of cell paste).

Ammonium sulfate (0.079 g/ml) was added over a 30 min period to Fraction I; stirring was continued for another 30 min. The precipitate was collected by centrifugation in the Sorvall SS-34 at 15 Krpm, for 30 min. Ammonium sulfate (0.113 g/ml) was added to the supernatant as above. The second ammonium sulfate precipitate (20-40% of saturation at 0°C ammonium sulfate) dissolved in 50 ml Tris-HCl pH 7.5, 25% glycerol, 1 mM EDTA and 5 mM DTT, was Fraction II (50 ml).

Fraction II was stored for several months at -70°C without significant loss of enzymatic activity. Fraction II was diluted 22 fold with Buffer A

(50 mM Tris-HCl pH 7.5, 50% glycerol, 1 mM EDTA, 5 mM DTT) to a conductivity equivalent to Buffer A plus 50 mM KCl, and applied to a 225 ml DEAE-cellulose column (6x8 cm) equilibrated with Buffer A plus 50 mM KCl. The column was washed with 450 ml of Buffer A containing 50 mM KCl, followed by 450 ml of Buffer A containing 100 mM KCl, and eluted with 450 ml of Buffer A containing 300 mM KCl to yield Fraction III. This fraction (450 ml) was diluted with 300 ml of Buffer B (50 mM Imidazole Cl pH 6.95, 50% (v/v) glycerol and 5 mM DTT) to a conductivity of Buffer B plus 150 mM KCl and applied to a 60 ml hydroxyapatite column (3.5x6.5 cm) equilibrated with Buffer B containing 150 mM KCl. The column was washed with 120 ml of the equilibration buffer and eluted by 600 ml of a linear 0-100 mM potassium phosphate (pH 6.95) gradient in Buffer B plus 150 mM KCl. Active fractions were pooled to yield Fraction IV (125 ml).

Fraction IV was diluted with 420 ml of Buffer A containing only 10 mM Tris-HCl pH 7.5 to a conductivity equivalent to Buffer A plus 50 mM KCl and was applied to a 5 ml DEAE-cellulose column (2.0x1.6 cm) equilibrated with Buffer B containing 50 mM KCl. The fraction unbound to the column under these conditions (Flow Through fraction), combined with the subsequent 10 ml wash in equilibration Buffer is Fraction V (555 ml). This fraction was applied immediately without further dilution to a 2.0 ml phosphocellulose column (1.6x1.0 cm), equilibrated with Buffer A containing 50 mM KCl. The column was washed with 5 ml of the equilibration buffer and eluted with 20 ml linear 50-100 mM KCl gradient in Buffer A. Active fractions were pooled to yield Fraction VI (4.5 ml). 0.75 ml of Fraction VI was diluted

with 3.25 ml of Buffer A to give a conductivity equivalent to Buffer A plus 50 mM KCl and applied to a 1.0 ml single stranded DNA-cellulose column (0.64x0.8 cm) equilibrated with Buffer A containing 50 mM KCl, and subsequently with 20 ml of Buffer A containing 100 mM and 250 mM KCl. The active fraction eluted with 250 mM KCl is Fraction VII (2.0 ml).

Pure enzyme preparations (Fraction VII) were stored at -20°C for at least 6 months without significant loss of activity.

TABLE 4 Purification of *Crithidia fasciculata* nicking enzyme

Fraction	Activity (units x 10 ⁻⁴)	Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification
I. Cleared lysate	24.0	21,600	11	(100.0)	1
II. (NH ₄) ₂ SO ₄ precipitate	16.0	5,000	32	66.7	3
III. DEAE-cellulose	18.0	580	310	75.0	28
IV. Hydroxyapatite	18.0	109	1,650	75.0	149
V. DEAE-cellulose	11.0	34	3,230	45.8	291
VI. Phosphocellulose	6.0	2.7	22,200	25.0	2,000
VII. SS-DNA-cellulose ^a	3.7	1.1	34,600	15.4	3,030

^a The values given for Fraction VII are corrected for the fact that only 0.75 ml (16.7%) of Fraction VI was used; SS-DNA is single stranded DNA.

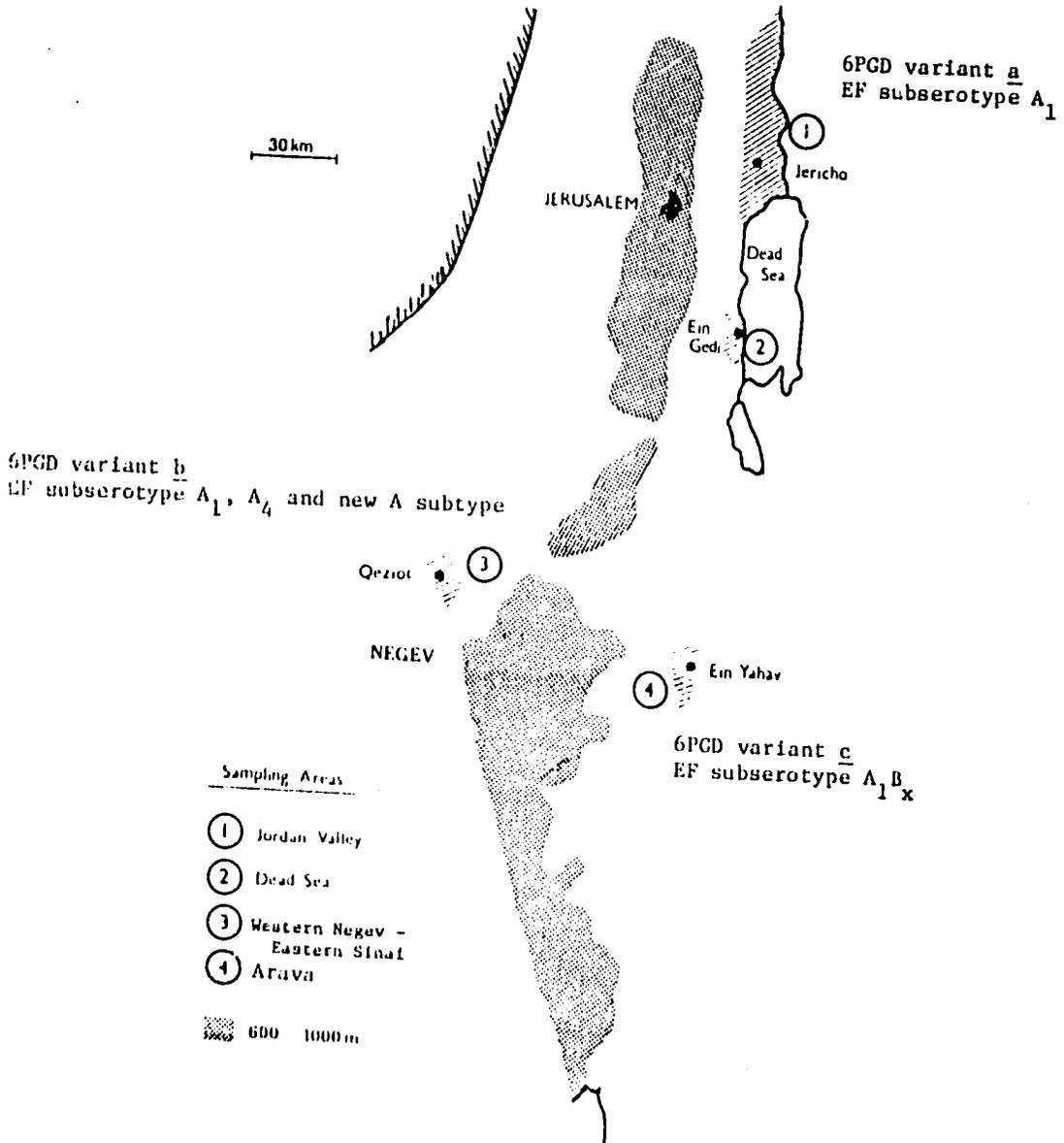


Figure 1

Forced-feeding of P. papatasi. The constricted end of a microcapillary (CP) is slipped over the mouth parts folding back the labium (LM). Sucking is evidenced by flexing of the clypeus and the fluid passes to the midgut.

Figure 2

Map of the principal regions where Leishmania major is endemic.



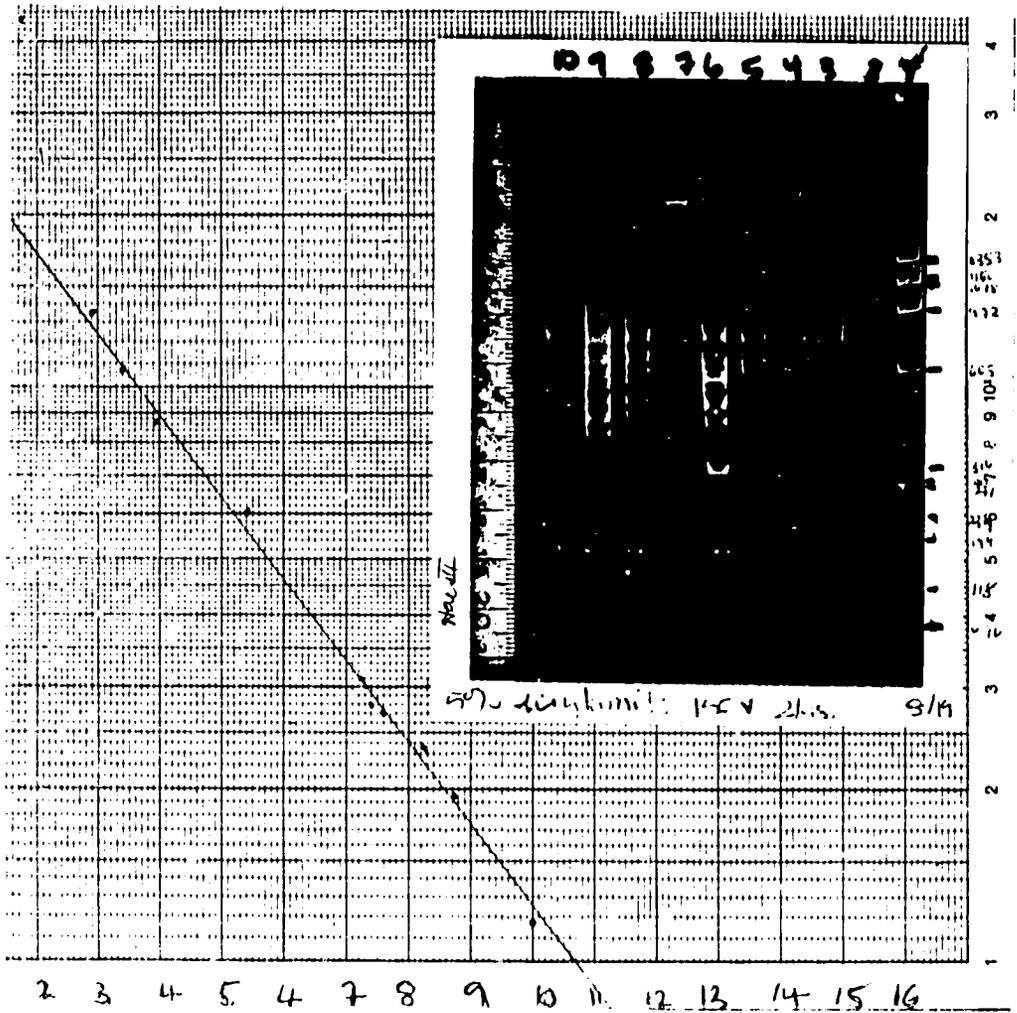


Figure 3

A 5% acrylamide gel of Hae III digests. Track one contains the markers, tracks 2, 3 and 4 are digests of Qeziot clones representing schizotype γ , track 6 is of a Jordan Valley strain, schizotype α and tracks 8, 9 and 10 are clones from the Arava which are schizotype β . The graph is a standard plot of the markers to determine the number of base pairs per fragment.

Figure 4

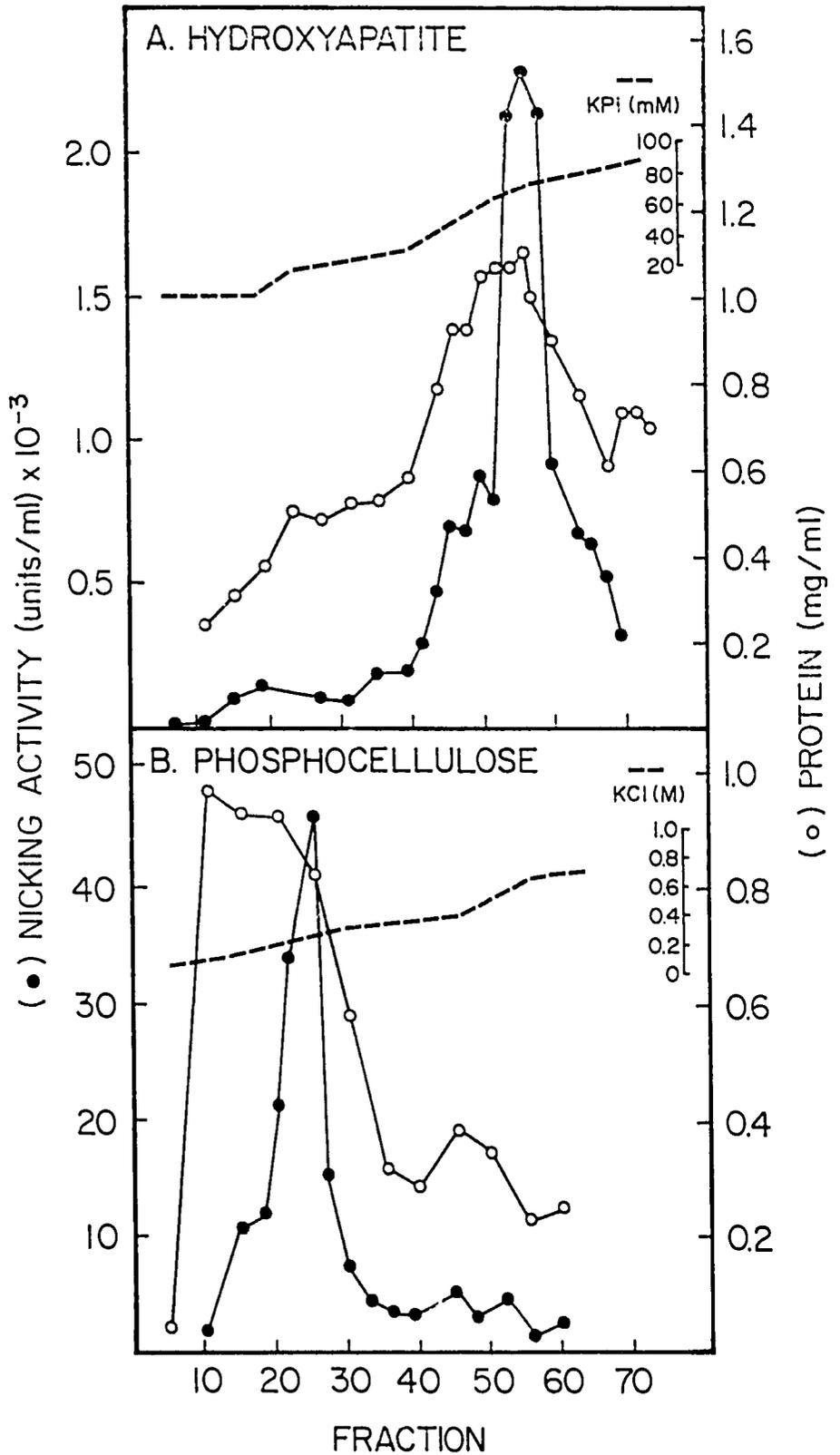


Figure 5

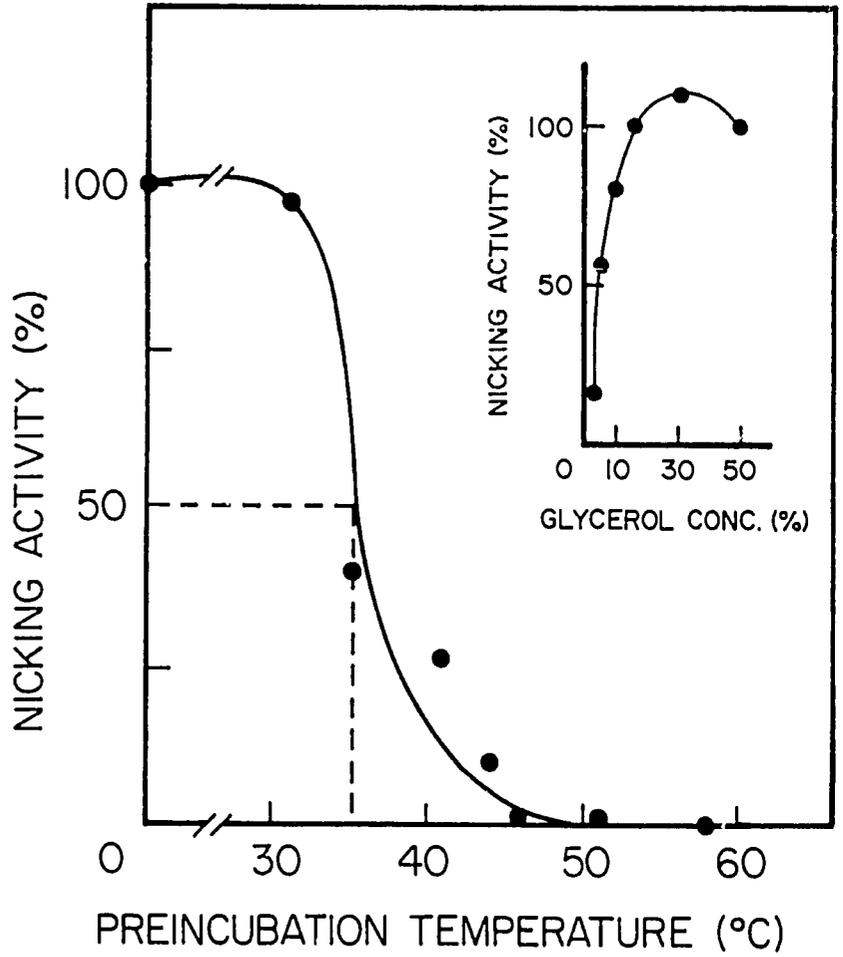


Figure 6

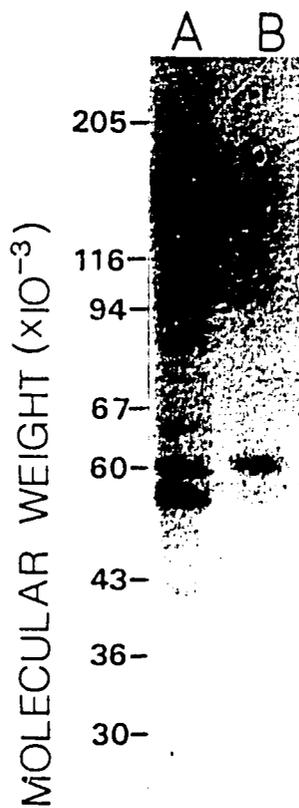


Figure 7

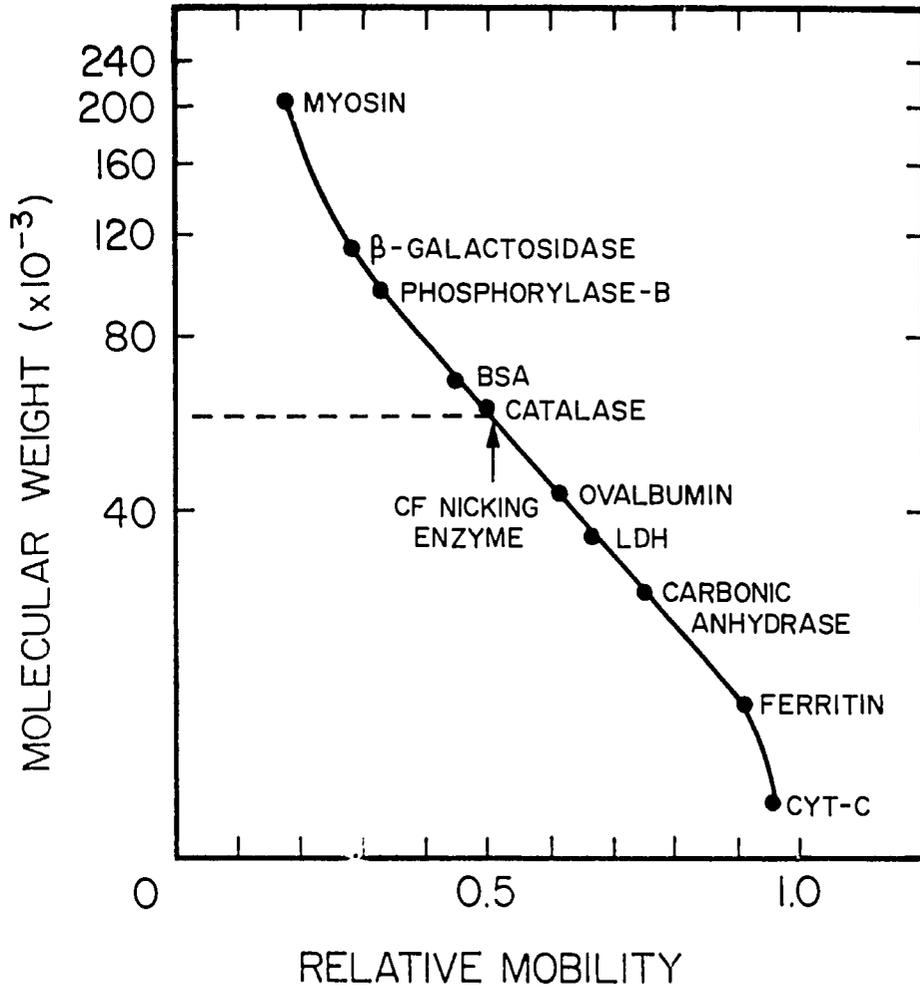


Figure 8

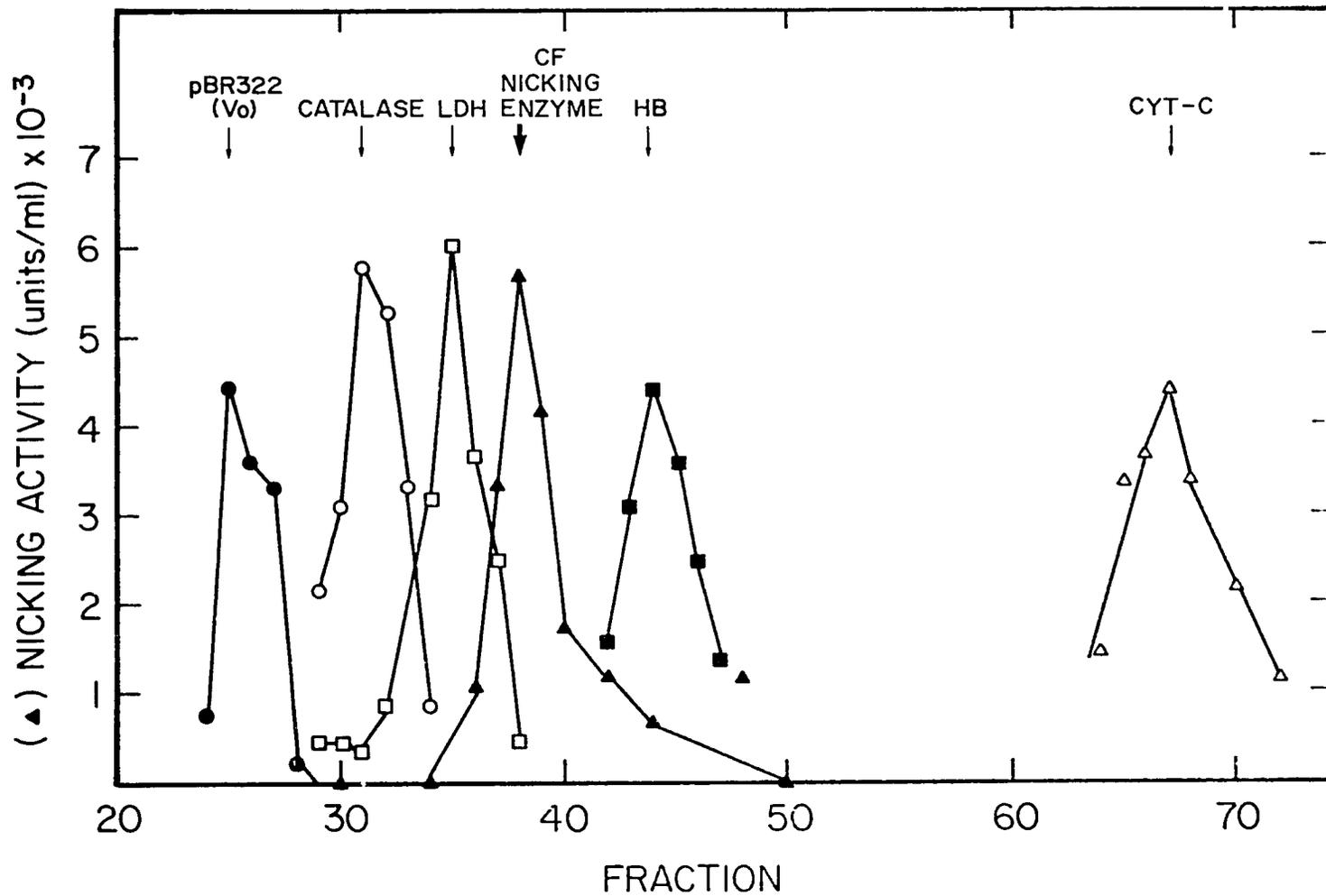
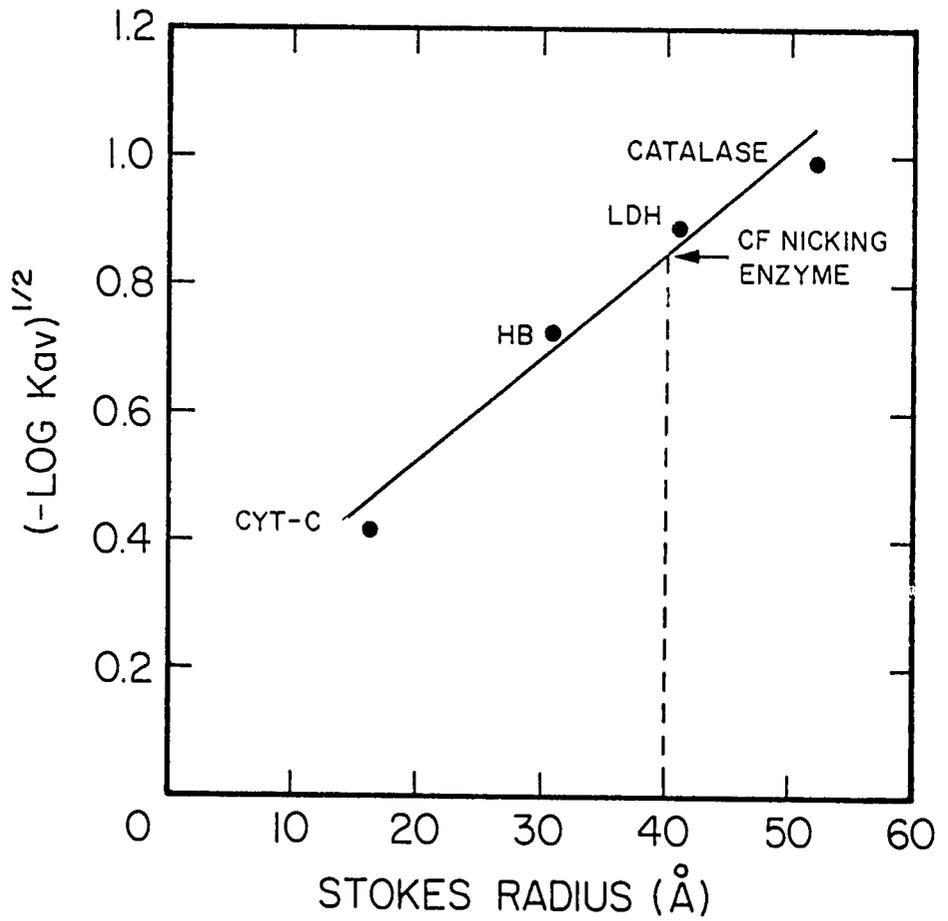


Figure 9



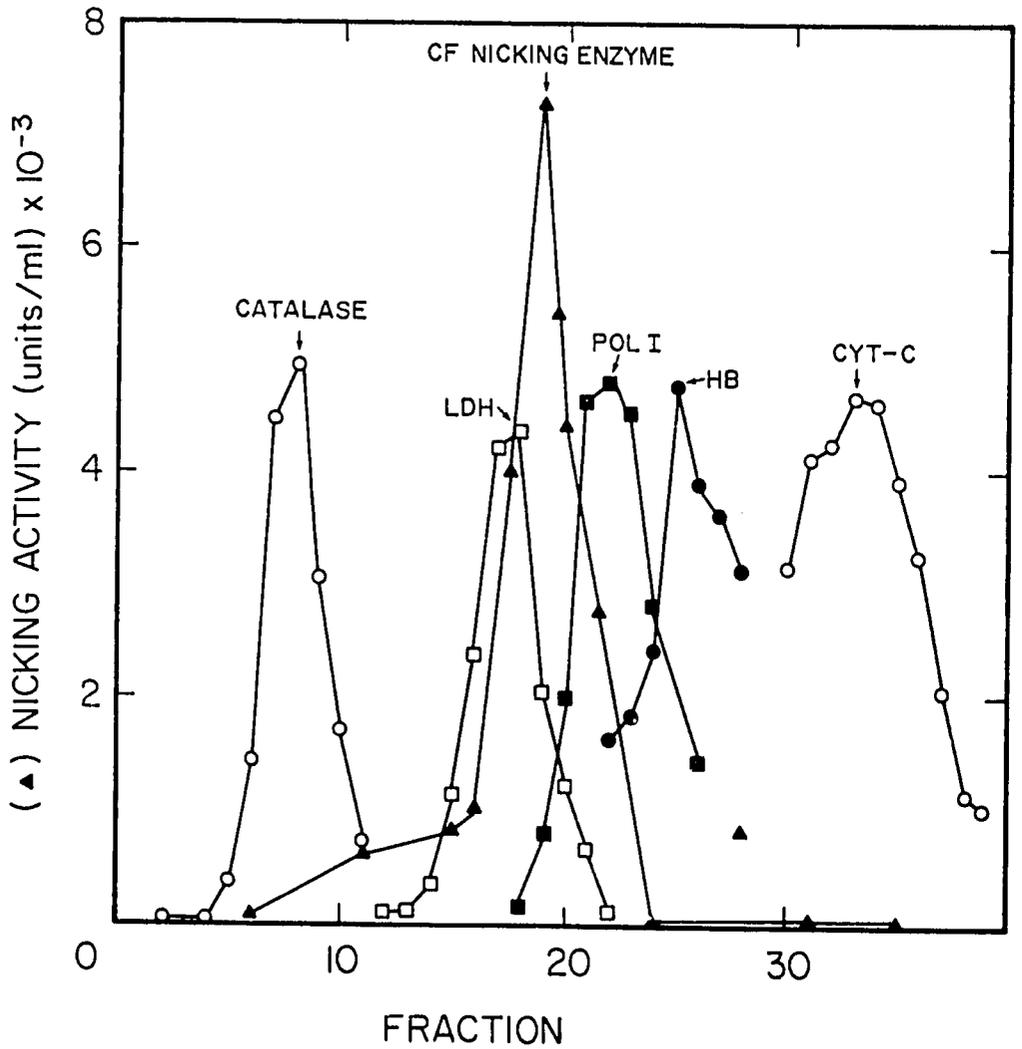
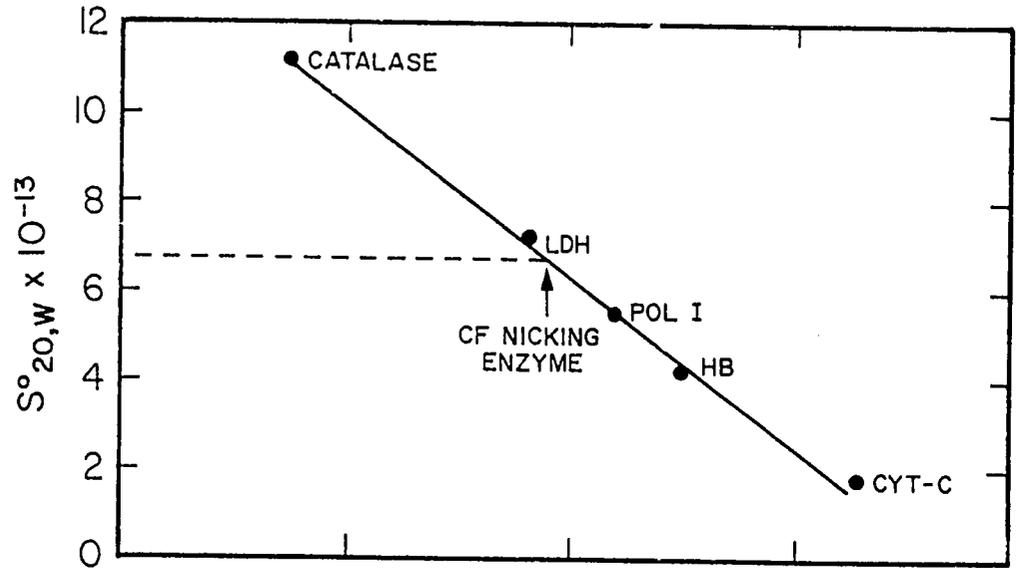


Figure 11

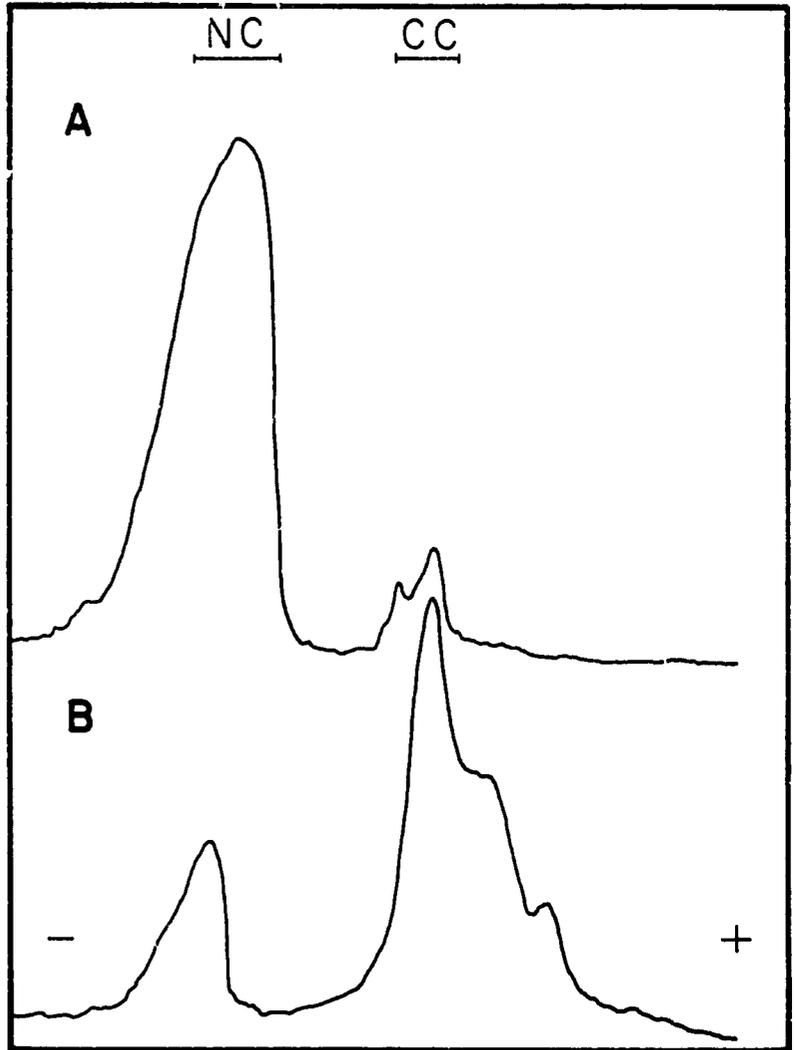


Figure 12

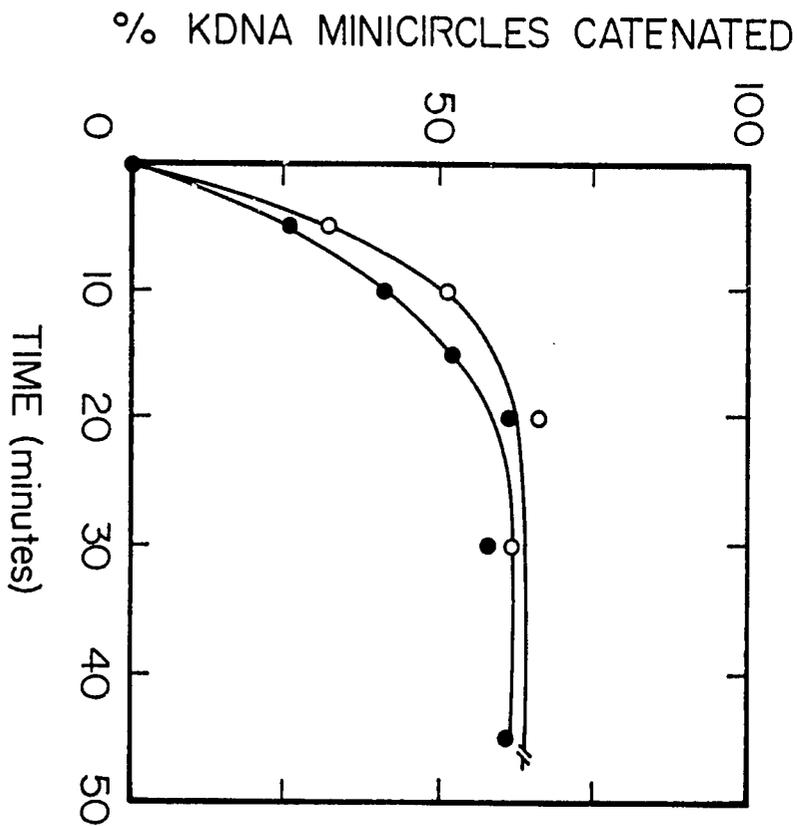


Figure 13

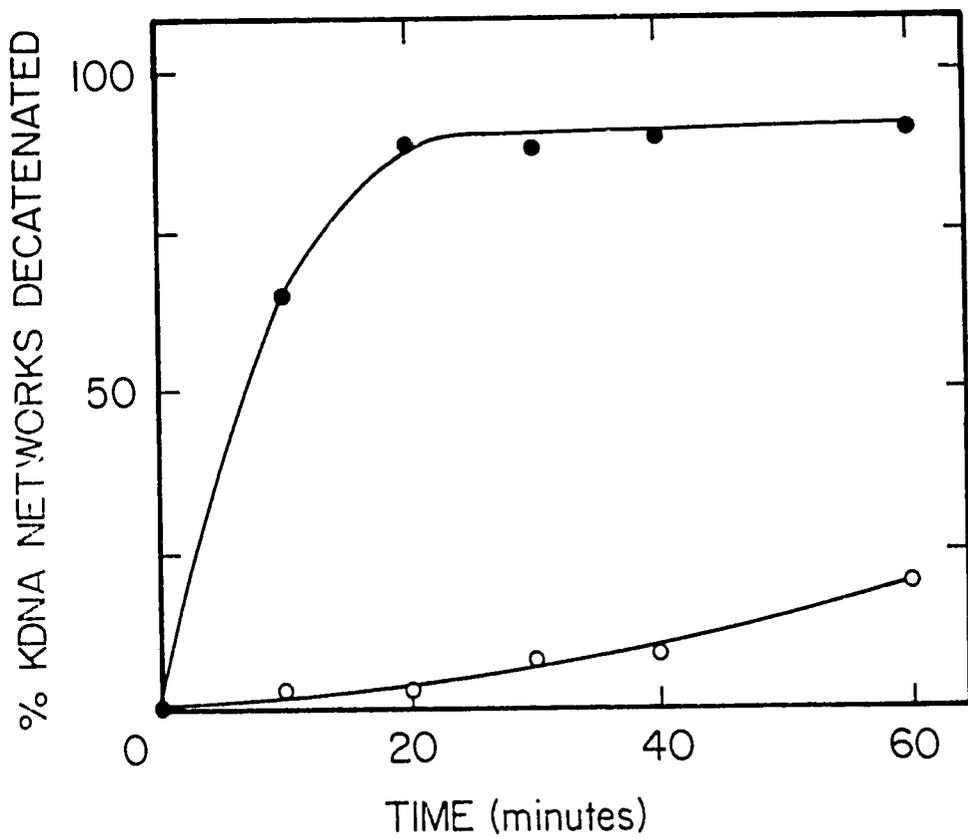


Figure 14

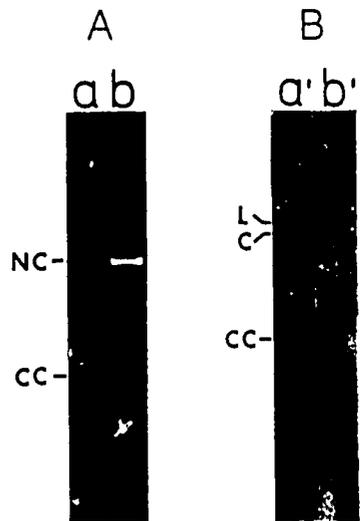
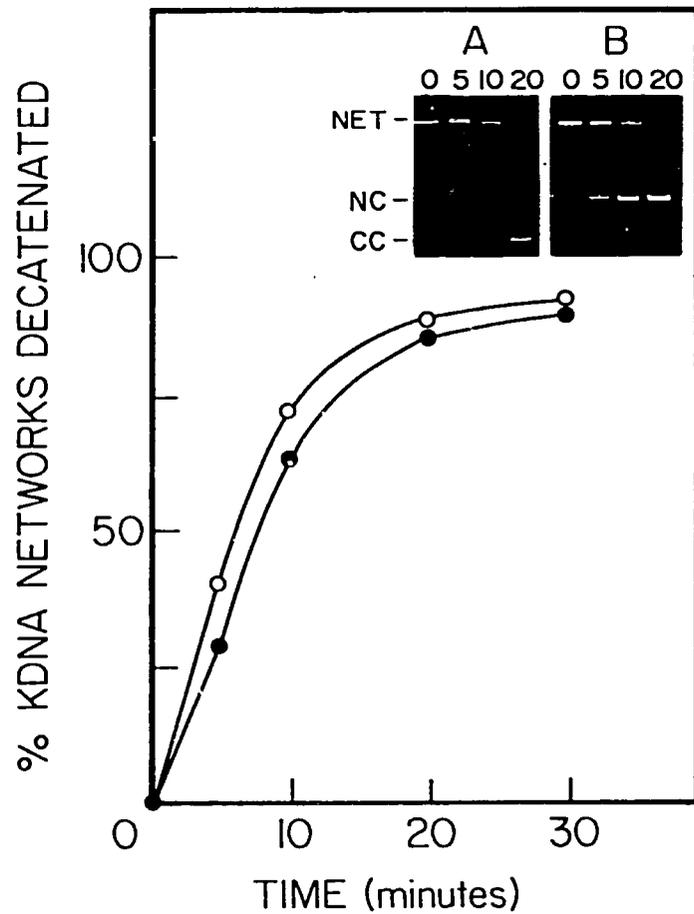


Figure 15



LEGENDS TO FIGURES

Figure 4

Purification of *Crithidia* nicking enzyme.

- A: Hydroxylapatite is the second chromatographic step. *Crithidia* nicking enzyme is eluted in the range of 60-80 mM of the linear potassium phosphate gradient. 75 fractions were collected and assayed for nicking activity as described under "Materials and Methods". Fraction numbers 52-64 were pooled and stored (-20°C) for further purification. An ATP-dependent DNA topoisomerase is eluted by a subsequent 100-400 potassium phosphate gradient.
- B: Phosphocellulose is the fourth chromatographic step. *Crithidia* nicking enzyme is eluted in the range of 250-400 mM of the linear KCl gradient. 80 fractions were collected and assayed for nicking activity. Fraction numbers 20-30 were pooled and stored (-20°C) for further purification (see the legend to Table I and text for details).

KPi is potassium phosphate.

Figure 5

Heat stability of *Crithidia* nicking enzyme.

Samples of *Crithidia* nicking enzyme (10 units, Fraction VII) in buffer A (see legend to Table I) containing 2.5% (v/v) glycerol were incubated for 10 min at the indicated temperatures followed by incubation for 10 min at 0°C. The treated enzyme samples were assayed for residual nicking activity as described under "Materials and Methods". In the insert, samples of nicking enzyme (10 units, Fraction VII) were incubated for 10 min at 36.5°C at various glycerol concentrations (percentage, as v/v), followed by 0°C incubation and nicking assay as above. Percentage of nicking activity is calculated measuring the rates of nicking reaction relative to the rate measured with the unheated enzyme in the presence of 2.5% (v/v) glycerol.

Figure 6

Molecular weight determination of *Crithidia* nicking enzyme by SDS-polyacrylamide gel electrophoresis.

Mobilities are calculated relative to the mobility of bromophenol blue. Molecular weight standards were: myosin (205 K), β -galactosidase (116 K), phosphorylase-B (94 K), bovine serum albumin (BSA) (67 K), catalase (61.5 K), ovalbumin (43 K), L-lactate dehydrogenase (LDH) (35 K), carbonic anhydrase (29 K), ferritin (small sub unit) (13.5 K) and cytochrome-c (cyt-c) (12.3 K). 4 μ g of Fraction VII enzyme were analyzed along with the above markers in a 5-15% (v/v) polyacrylamide gradient gel as described under "Materials and Methods".

Figure 7

SDS-polyacrylamide gel electrophoresis analysis of *Crithidia* nicking enzyme.
Samples of 10 µg protein of Fraction VI (A), and 5 µg protein of Fraction VII (B), were analyzed in a 5-15% (w/v) polyacrylamide gradient gel as described under "Materials and Methods", along with molecular weight protein markers as described in the legend to Figure 3.

Figure 8

Bio-Gel P-300 gel filtration.

A Bio-Gel P-300 column was prepared and calibrated using protein markers as described under "Materials and Methods". Assays and units were as follows: (o) catalase (units/ml $\times 4 \times 10^{-3}$) by decomposition of hydrogen peroxide followed by decrease in A_{240} ; (□) L-lactate dehydrogenase (LDH) (units/ml $\times 8 \times 10^{-5}$), by decrease in A_{340} from oxidation of NADH; (■), hemoglobin, (HB) A_{430} ($\times 5$); (Δ) cytochrome c (CYT-C), at A_{410} ($\times 10$). The V_0 was determined using pBR322 DNA (in µg/ml) (o) followed by fluorescence at 250 nm in the presence of 1 µg/ml ethidium bromide. *Crithidia fasciculata* nicking enzyme (CF NICKING ENZYME, (▲), 2.6×10^3 , Fraction VI), assayed as described under "Material and Methods" was filtered separately under identical conditions.

Figure 9.

Stokes radius determination of *Crithidia* nicking enzyme.

The Stokes radius was estimated from a plot of the Stokes radii of protein markers (as described under "Material and Methods") versus $(-\log K_{av})^{\frac{1}{2}}$ as shown (21). *Crithidia* nicking enzyme (CF NICKING ENZYME) was as in Figure 5.

Figure 10

Glycerol gradient sedimentation.

Crithidia nicking enzyme (2.8×10^3 units, Fraction VI) was sedimented on a 20-45% (v/v) linear glycerol gradient as described under "Materials and Methods". Assays and units are as follows: (o) catalase (units/ml $\times 10^{-3}$); (□) L-lactate dehydrogenase (LDH) (units/ml $\times 8 \times 10^{-5}$); (●), hemoglobin (HB) ($A_{430} \times 10$); and (◊) cytochrom-c (cyt-c) ($A_{400} \times 10$). These markers were assayed as described in Figure 5. DNA polymerase I (POL I, (■), units/ml $\times 20$) was assayed according to the method of Richardson et al. (20). *Crithidia* nicking enzyme (CF NICKING ENZYME, ▲) was assayed as described under "Materials and Methods". The sedimentation coefficient ($S_{20,w}^{\circ}$) was estimated using protein markers (see "Materials and Methods") according to the method of Martin and Ames (22).

Figure 11

Covalent closure of *Crithidia* nicking enzyme-treated duplex DNA circles by DNA ligase.

2.5 µg of supercoiled pBR322 DNA was treated using 2.5 units of *Crithidia* nicking enzyme (Fraction VII, 100 ng) under standard assay conditions (see "Materials and Methods") (A). The reaction was stopped by addition of 20 mM EDTA pH 8.0. DNA was extracted using phenol and treated with T4-DNA ligase (0.3 units) for 30 min at 37°C (B). Products of the reactions were electrophoresed in agarose gel containing ethidium bromide and analyzed by microdesitometry as described under "Materials and Methods". NC (nicked circles) and CC (closed circles) are the nicked and covalently sealed forms of the double stranded circular DNA, respectively. The direction of electrophoresis is indicated by the polarity (- to +).

Figure 12

Effect of nicking on the catenation reaction.

kDNA monomeric minicircles were prepared by decatenation of native kDNA networks using *Crithidia* DNA topoisomerase, extracted from the reaction mixture using phenol and ethanol precipitated. These kDNA minicircles were used as substrate in a catenation reaction catalyzed by *Crithidia* DNA topoisomerase either with (o) or without (●) prior treatment with *Crithidia* nicking enzyme. When treated with *Crithidia* nicking enzyme, kDNA minicircles were reextracted from the nicking reaction mixture using phenol and chloroform, before the topoisomerase assay. Samples withdrawn at the indicated time intervals were submitted to agarose gel electrophoresis followed by microdensitometry, as described under "Materials and Methods". *Crithidia* topoisomerase (1.2 units) and nicking enzyme (2.5 units, Fraction VII) assays were carried out on 2.5 µg samples of kDNA minicircles as described under "Materials and Methods". Percentage of catenation is calculated from the total DNA monomers used as substrates.

Figure 13

Effect of nicking by *Crithidia* nicking enzyme on the decatenation of kDNA networks.

5 μ g of native kDNA networks were nicked using *Crithidia* nicking enzyme (5 units, Fraction VI). Nicked DNA networks were phenol extracted and used as a substrate in a decatenation reaction catalyzed by *Crithidia* DNA topoisomerase (2.4 units). (●), Native untreated kDNA networks; (○), nicked kDNA networks. Samples withdrawn from the topoisomerase reaction at the time intervals indicated were submitted to agarose gel electrophoresis followed by microdensitometry, as described under "Materials and Methods". Percentage of decatenation is calculated from the total kDNA networks used as substrate.

Figure 14

Products of the *Crithidia* nicking enzyme using duplex DNA circles as a substrate.

1 μ g pBR322 DNA (85% negatively supercoiled, 15% containing naturally occurring nicks and gaps) were treated using *Crithidia* nicking enzyme (2 units, Fraction VI). Reaction was stopped by addition of 20 mM EDTA (pH 8.0), and the DNA products were extracted with phenol and ethanol precipitated. In A, a sample of 0.5 μ g of the DNA precipitate was dissolved in neutral stopping buffer and loaded onto a 1% neutral agarose gel. In B, a sample of 0.5 μ g of the precipitate was dissolved in alkaline stopping buffer and loaded onto a 1% alkaline agarose gel.

a, a' - untreated pBR322; b, b' - *Crithidia* nicking enzyme treated pBR322.

Nicking enzyme assay and the neutral and alkaline gel electrophoresis protocols were as described under "Materials and Methods".

NC (nicked circles), and CC (closed circles), are the nicked and covalently sealed forms of the circular duplex DNA, respectively. L (linear), and C (circle), are the linear and circular forms of single stranded DNA.

Figure 15Effect of nicking of DNA networks at random sites on their decatenation.

5 μg of native kDNA networks were nicked by limited DNase I digestion in the presence of saturating ethidium bromide, as described by Barzilai (24), introducing approximately one nick in each DNA circle (25). 5 μg of kDNA in a volume of 100 μl containing 10 mM Tris-HCl pH 8.0, 2 mM MgCl_2 , 1 mM EDTA pH 8.0, 100 $\mu\text{g}/\text{ml}$ bovine serum albumine and 0.12 mg/ml of ethidium bromide, and 2 $\mu\text{g}/\text{ml}$ DNase I (40 ng of DNase per μg DNA). Incubation was at 30°C for 15 min, followed by extensive phenol extractions of the DNA prior to its further use. Nicked DNA networks were used as substrate in a decatenation assay catalyzed by *Crithidia* DNA topoisomerase. (●), and insert B, native untreated networks; (○), and insert B, nicked kDNA networks. Samples withdrawn from the topoisomerase reaction at the time intervals indicated were submitted to agarose gel electrophoresis (inserts A and B) followed by microdensitometry, as described under "Materials and Methods". Percentage of decatenation is from the total kDNA networks used initially as substrate. NET (networks), NC (nicked circles), and CC (closed circles) are the catenane network, nicked minicircle, and covalently sealed minicircle forms of kinetoplast DNA, respectively.

APPENDIX

TRAINING ASPECTS

Foreign trainees visiting the Kuving Centre have received instruction within the context of studies undertaken during the period of the tenure of this grant.

Dr. Sylvia Le Blancq, previously of the London School of Hygiene and Tropical Medicine, joined us in February 1984 for a two-year postdoctoral study period. She will be with us until April 1986. She has done enzyme analysis, being instrumental in increasing our enzyme typing capability and has extended the range of enzymes systems being used. She has also participated in work on sandflies and is currently learning technique for studying DNA.

Mr. John Githure from the Kenyan Medical Research Institute spent from March 1984 to April 1985 with us learning enzyme typing and other techniques.

Mr. John Kaddu from the International Centre for Insect Physiology and Ecology (I.C.I.P.E.), Nairobi, Kenya, spent three weeks with us in November 1985. His special interests were sandflies and leishmanial parasites.

In December 1985, Dr. N. Massamba of the Free University of Brussels joined us for two weeks to learn enzyme typing techniques. He is due to join the group working on leishmanial parasites and sandflies at I.C.I.P.E.

Ms. Robyn Juster of the University of California, School of Medicine at Davis, spent the summer months of 1985 with us. Her work was an invaluable contribution to the analysis of kDNA variation.