A COMPARATIVE STUDY OF THE DNA OF

ACANTHAMOEBA

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S U M M A R Y

1. The literature on DNA base composition of protozoa was reviewed to illustrate the relationship between the taxonomic position of protozoa and their DNA base ratios.

2. The method of Adam, Blewett and Flamm (1969) for the extraction of DNA from Acanthamoeba was modified by substituting chemical purification procedures in place of repeated cycles on preparative CsCl density gradients.

3. A method was developed for growing Acanthamoeba in the presence of high concentrationsof P32 orthophosphate and for extracting and purifying high specific activity DNA from as little as 0.1ml of packed radio-active amoebae.

4. The minor component in the DNA of Acanthamoeba was shown to be of mitochondrial origin by i) extracting enhanced amounts of the minor DNA from isolated mitochondria and ii) by demonstrating the presence of circular DNA molecules in samples of minor DNA collected from preparative CsCl density gradients.

5. Electronmicrographs of spreads of mitochondrial DNA from the Neff, Singh, H and W1 strains of A.castellanii and from A.polyphaga A.palestinensis and A.astronyxis showed a low proportion of circular molecules with a contour length of approximately 15 μ.

6. The DNA base composition was determined of the Singh, W1 and P27 strains of A.castellanii and of isolates A1, A30 and A35. The DNA of isolate A1 had a base composition of 56% GC for the major component and 37% GC for the minor component; the other isolates and strains of Acanthamoeba which were tested had base ratios of 61% GC for the major component and 34% GC for the minor component.

7. The renaturation of A.castellanii DNA was studied using the spectrophotometric technique of Wetmur and Davidson (1968). Under the conditions used in these studies (30ug/ml DNA in 0.1M NaCl) there was little evidence of renaturation apart from a small rapidly renaturing fraction comprising 7 - 10 of the total DNA.

8. A method was developed for the denaturation and renaturation of small volumes of concentrated DNA solution. This method was designated the capillary tube renaturation technique.

9. The capillary tube renaturation technique was used to study the renaturation of the Neff, H, Singh and W1 strains of A.castellanii and of A.polyphaga and A.palestinensis. The DNAs of all the amoebae examined renatured slowly with values for Cot $\frac{1}{2}$ in the range 50 - 100. The final levels of renaturation achieved exceeded 60%.

10. The capillary tube renaturation technique was used to prepare DNA-DNA hybrids between radio-active DNA from the Neff strain of A.castellanii and DNA from the H, Singh and W1 strains of A.castellanii and from A.palestinensis, A.polyphaga, A.astronyxis and T. pyriformis. The quality of the hybrid DNAs was tested by comparing the thermal stability of hybrid DNA with that of renatured homologous DNA.

11. Hybrids formed between DNA from the Singh, W1 and H strains of A.castellanii and Neff strain DNA showed a depression of Tm of 2 °C, indicating that 4% of the base sequences in the hybrid DNAs were mismatched.

12. Hybrids formed between the three species of Acanthamoeba showed much greater depression of Tm. The Neff-polyphaga and Neff-palestinensis hybrids showed Tm depressions of 5 °C and 6 °C respectively, corresponding to 7.5% and 9%

mismatching of base sequences. There was no detectable base sequence homology between the DNAs of A.astronyxis and T.pyriformis and the DNA of A.castellanii Neff strain.

13. The methods developed were criticised and the results of the hybridisation experiments were discussed in relation to the taxonomy of Acanthamoeba.

C O N T E N T S

INTRODUCTION

Amoeboid cells are found throughout the Animal Kingdom. They occur in the body fluids of vertebrates and invertebrates; they may be apparently simple, undifferentiated cells as in the sponges, or highly specialised cells as in the amoeboid spermatozoa of the nematodes. There are also a large number of species of protozoa in which the predominant phase is amoeboid. These species are grouped together to form a major sub-division of the Phylum Protozoa; the Superclass Sarcodina (Corliss 1967). The organisms which make up the Sarcodina may be conveniently grouped into three broad categories, the naked amoebae, the testate amoebae and the communal amoebae.

The communal amoebae are distinguished by the ability of the trophozoites to aggregate to form cell masses, or to grow into plasmodia and differentiate to produce spores or cysts. These anoebae are assigned to the Subclass Mycetozoia, the slime moulds. The testate amoebae are divided between 2 Classes. Species in the Class Actinopodea are typically spherical cells with radiose pseudopodia and either an internal capsule, (Subclass Radiolaria) or a peripheral skeleton of discrete spicules (Subclass Heliozoia). The second group of testate amoebae are included in the Class Rhizopodea and form the Order Foraminiferida, which possess an often complex calcareous test, and the Order Arcellinida in which the cell is enclosed in a simpler shell-like test.

The third category, the naked amoebae, are also members of the Class Rhizopodea and form a single Order, the Amoebida. The common characteristic of the species in this Order is that they are all amoeboid. In view of the widespread occurrence of amoeboid cells, the possession of the amoeboid form

would not appear to be a sound criterion on which to base phylogenetic relationships. It is not surprising therefore that the Order Amoebida is generally acknowledged to be a heterogenous group.

One of the most striking features of the naked amoebae is the lack of distinguishing features upon which a taxonomic system might be based. While these organisms may be readily grouped into sub-divisions within the Order, using characteristics such as size, form in locomotion, life cycle and number of nuclei (Jepps 1956), it is extremely difficult to use these characters to determine phylogenetic relationships between genera or to distinguish between species and strains of amoebae. This problem is not peculiar to the amoebae or to the protozoa. It can be argued that the concept of species is not applicable to asexual organisms. Nevertheless the relationships between protozoa and the identity of isolates are of great interest, and in the case of pathogenic protozoa, of considerable practical importance.

Any attempt to examine more closely the phylogeny of protozoa, and especially amoebae, must make use of the biochemical as well as the physical properties of the organisms. There are several techniques which have been widely and successfully used to compare the proteins of protozoa, and have proved useful in exploring relationships between amoebae.

Adam (1964) used the immobilising action of specific antisera to distinguish between several strains and species of hartmannellid amoebae (Acanthamoeba) and Entamoeba invadens and Naegleria gruberi. These results were confirmed and extended by Siddiqui and Balamuth (1965) who used the immuno-diffusion and fluorescent antibody techniques to explore the relationship between three species of Entamoeba and two species of hartmannellid amoebae.

They showed that there was no reaction between antiserum to Entamoeba and the hartmannellid amoebae and, using the fluorescent antibody test, demonstrated a serological relationship between E.invadens antiserum and, in descending order of similarity, E.invadens, E.histolytica and E.moshkovskii antigens.

Serological and electrophoretic techniques were used by Kates and Goldstein (1964) to compare amoebal proteins. They were unable to distinguish between Amoeba proteus and Amoeba discoides, but found that the proteins of both these amoebae were different from those of the giant amoeba Chaos chaos. Montalvo and Reeves (1968) made a comparison of the enzyme glucose phosphate isomerase of 18 different strains of Entamoeba. Five atypical strains of E.histolytica (recognised by the smaller size and ability to grow in culture at 25°C) and two strains of E.moshkovski were characterised by a fast moving enzyme band on electrophoresis gels. Two strains of E.invadens and E.terrapinae gave rise to a slow moving enzyme band, and the 8 typical strains of E.histolytica (recognised by their inability to grow in culture at 25°C) showed an enzyme band of intermediate mobility. In a more detailed study Reeves and Bischof (1968) examined the electrophoretic mobilities of five different enzymes from 21 strains and species of Entamoeba. They found six enzyme bands which were common to all 15 strains of E.histolytica which were tested, 5 atypical strains were distinguished from 10 typical strains by the different mobility of the isomerase band and by the lack of a 2nd glucokinase band. Two strains of E.invadens and of E.terrapinae shared 7 enzyme bands, but there was only 1 enzyme band common to these reptillian species and to E.histolytica and E.moshkovski.

Friz (1968) measured the free amino acid levels in Pelomyxa carolinensis, Amoeba proteus and Amoeba dubia, and subsequently (Friz 1970) determined the amino acid composition of the bulk proteins of P.carolinensis, A.proteus, A.dubia and A.discoides. In common with the other comparative biochemical techniques, amino acid analysis showed the expected differences between the species of amoebae which were tested.

The chemical composition of a cell results from an interaction between the genetic information within that cell and its environment. Many protozoa, especially parasitic species, undergo marked changes in form, metabolic activity and antigenic structure in response to changes in their environment. Clearly, any techniques which permit direct comparisons of the genotype of different organisms will provide an invaluable taxonomic tool. The recognition of deoxyribonucleic acid as the genetic material stems from the observation of Avery, McLeod and McCarty (1944) that an unencapsulated strain of pneumococcus could acquire the ability to produce a capsule when exposed to DNA extracted from an encapsulated strain. It was not until Watson and Crick (1953) described the structure of the DNA molecule that it became possible to consider the probable mechanisms by which the genetic material was able to replicate itself and by which the genetic information was encoded. According to the Watson Crick model the DNA molecule is composed of two sugar phosphate chains with the purine and pyrimidine bases adenine, guanine cytosine and thymine arranged in pairs between them, the whole double stranded structure being coiled into a double helix.

The molecular structure of the bases is such that they will only form stable pairs which will fit within the helix when adenine pairs with thymine and guanine pairs with cytosine. This provides the specificity of replication since the sequence of bases along one strand dictates the sequence along its partner. Because of its great length the DNA molecule can carry linear arrays of the four bases in any of an almost infinite number of different sequences. A structure of this type could efficiently code for a large amount of information.

Meselson and Stahl (1958) showed that DNA replicates as predicted from the Watson Crick model; by strand separation followed by the synthesis of a new chain on to each of the existing strands. To prove that the genetic code was carried in the sequence of bases along the molecule was more difficult. It could be shown that, theoretically, at least three bases would be required for each code word, this would provide 64 words to code for ~~each~~ of the 20 naturally occurring amino acids. Groups of 2 or 4 bases would provide too few or far too many code words.

The observation by Nirenberg and Mathei (1961) that the addition of the synthetic polynucleotide poly-~~uracil~~ uridylic acid to a cell free protein synthetic system stimulated the incorporation of phenylalanine into acid insoluble protein, led to an experimental system for the direct investigation of the genetic code. Using this and similar systems it was eventually possible to assign triplets of bases to code for each of the 20 naturally occurring amino acids (Ochoa 1966). Although most of the work on the genetic code was performed on microbial systems, it is generally acknowledged that the genetic code is universal (Crick 1966).

The DNA molecule has three basic physical properties, its molecular weight, its base sequence and its base composition (the molar proportions of A-T and G-C base pairs). *In vivo* there is a fourth property, the amount of DNA per cell. Comparisons of the genetic material of different organisms must therefore involve one or more of these properties.

The DNA content of a cell can be measured by microspectrophotometry (Davies and Walker 1953) or by its chemical extraction and estimation (Schneider 1945). The base composition of DNA can be readily determined either by measurement of the buoynat density on a CsCl gradient (Schildkraut Marmur and Doty 1962) or by measuring its thermal denaturation temperature (Marmur and Doty 1962). The molecular weight of DNA *in vitro* is totally dependent on the extraction procedure used. With the exception of those organisms or organelles which contain DNA in the form of covalently closed circles, it is not possible to determine the molecular weight of DNA *in vivo*.

The number of base pairs in DNA varies from 10^6 in bacteria up to 10^9 in vertebrates, and it is not practical to attempt to determine the complete base sequence of even a simple DNA. There are therefore only two measureable properties of DNA which might be useful in taxonomic studies, they are the amount of DNA per cell and its base composition.

An examination of the DNA from a wide variety of organisms has revealed a general evolutionary trend towards an increased amount of DNA per cell and a corresponding trend towards uniformity in base composition (McCarthy 1969), see Table I.1

Table I.1

Group	% G-C max	% G-C min	% G-C range	Group	Mol.wt. DNA (daltons)
viruses	28	74	46	viruses	10^6–10^8
bacteria	24	75	51	bacteria	10^8–10^9
blue-green algae	35	71	36		
fungi	38	63	23		
protozoa	22	66	44	invertebrates	10^{10}–10^{12}
algae	36	66	30		
sponges	35	60	25		
molluscs	31	47	16		
arthropods	32	45	13		
echinoderms	35	45	10		
chordates	38	47	9	vertebrates	10^{12}–10^{14}

The mechanisms responsible for these trends are not fully understood, and while it is understandable that the DNA content of cells should increase as organisms become more complex, the trend towards uniformity in base composition is less easy to explain. It is possible that micro-organisms are subjected to selective pressures in favour of divergence in base composition or, more simply, it may be that their very long evolutionary history has allowed sufficient time for the slowest changes in base composition to become apparent. The universality of the genetic code implies strong selective pressures for itsmmaintenance, variations in base composition are therefore unlikely to be transitory but rather to be reflective of evolutionary descent.

The DNA has been extracted and characterised from more than 150 species and strains of protozoa. The reported values for the base composition (%GC) of protozoan DNA's are listed in Table I.2 and shown in Figure I.1.

Table I.2 The base composition of protozoan DNA.

Ref.	Organism	DNA base composition nuclear DNA	m DNA
CILLIATES			
1	Tetrahymena patula LFF1	22	-
1	T.pyriformis W	30	-
2	T.pyriformis	33(t)	-
3	T.pyriformis	29	-
4	T.pyriformis	29	22
7	T.rostrata	24	-
2	Paramecium caudatum	39(t)	-
2	P.aurelia	34(t)	-
2	P.aurelia stock 540 syngen 1	32(t)	-
2	P.aurelia stock 513 syngen 1	33(t)	-
2.	P.bursaria	38(t)	-
4	P.aurelia (51)	30	43
1	P.aurelia 517s	29	-
1	Colpidium C. carolina	32	-
1	Colpidium C. burbank	33	-
1	C.truncatum	35	-
1	C.camphylum	32	-

Ref.	Organism		DNA base composition nuclear DNA	m DNA	c DNA
2	C.carolina		32(t)	-	
2	Glaucoma chattoni		34(t)	-	
2	Dileptus anser		32(t)	-	
2	Didinium nasutum		36(t)	-	
2	Stentor polymorphus		45(t)	-	
3	S.coerruleus		33	-	
6	T.pyriformis strains -	EU 6002	24	-	
		1 - A	26	-	
		1-WH-52	26	-	
		1-1L-12	26	-	
		2-1	26	-	
		5-1	26	-	
		8-2	26	-	
		6-1	26	-	
		HSM	26	-	
7	T.pyriformis strains	3-1	28	-	
		7-1	28	-	
		9-1	28	-	
		GL	30	-	
		E	32	-	
PHYTOFLAGELLATES					
1	Euglena gracilis		46	-	-
9	E.gracilis (bacillaris)		48	-	25
10	E.gracilis W3		48	-	26
11	E.gracilis (bacillaris)		48	-	26
14	E.gracilis BUL		48	-	-

Ref.	Organism	DNA base composition nuclear DNA	m DNA	
27	E.gracilis Z	48	-	-
1	Isochrysis galbana	61	-	-
1	Prymnesium parvum	58	-	-
12	Chlamydomonas reinhardi	64	56	33
27	C.eugametos	56	-	-
27	C.moewusii	60	-	-
27	C.reinhardi	66	-	-
27	C.angulosa	68	-	-
27	Ochromonas danica	48	-	-
27	Polytoma obtusum	66	-	-
27	Astasia longa	56	-	-
17	P.obtusum	51	-	23(?)
17	P.uvella	51	-	21(?)
17	Polytomella caeca	37	-	-
17	Polytomella agilis	42	-	-
17	A.longa	47	-	21
AMOEBAE				
20	Acanthamoeba castellanii Neff	61	34	
20	A.castellanii H	61	34	
20	A.palestinensis	62	37	
20	A.polyphaga	57	43	
20	A.astronyxis	50	30	
27	Naegleria gruberi	34	24(?)	43(?)
27	Entamoeba invadens	24	-	
27	Amoeba proteus	66	-	

Ref.	Organism	DNA base composition nuclear DNA	m DNA
19	Entamoeba histolytica strains-		
	NRS(M)	27	-
	HK9(A)	27	-
	JS(M)	28	-
	N(M)	28	-
	JH(M)	28	-
	200,200;NIH(M4A3)	29	-
	BH(M)	29	-
	DKB(M8)	29	-
	K9(M)	29	-
	F22(M)	30	-
	JA(M)	28	-
	AG(M)	29	-
	403(M)	30	-
	Huff(MA)	30	-
	Laredo(A)	30	-
26	E.histolytica strains-		
	HK9	22(t)	-
	F22	22(t)	-
	HB301 NIH	27(t)	-
	200 NIH	27(t)	-
	Laredo	38(t)	-
	Huff	26(t)	-
26	E.moshkovskii(FI6)	32(t)	-
26	E.invadens IP	26(t)	-
26	E.invadens PZ	33(t)	-

Ref.	Organism	DNA base composition nuclear DNA	m DNA
26	E.invadens 165	33(t)	-
2	Actinosphaerium nucleophilum	43(t)	-
27	Dictyostelium discoideum NC4	22	-
27	D.purpureum VI	22	-
27	D.mucoroides S2	22	-
27	D.purpureum	26	-
27	Polysphondilium violaceum SR2	28	-
27	P.pallidum	28	-
27	Protostelium irregularis	34	-
1	Dictyostelium discoideum	22	-
27	Acrasis rosea	36	-
27	Actyostelium leptosmum	36	-
28	Physarum polycephalum	41	-
30	D.purpureum 2	24	36
30	D.rosarium F11-8	24	36
30	D.discoideum Hi 66-128	22	31
30	D.mucoroides G39	22	30
30	D.mucoroides Hi-1	22	-
30	Polyshondilium pallidum Pal-4	31	25
30	P.pallidum W2	33	26
30	P.violaceum V-9	30	-
30	Acrasis rosea NC-18	37	-
30	Protostelium irregularis Mex-81	35	-
30	Actyostelium leptosomum	37	-

Ref.	Organism	DNA base composition nuclear DNA	m DNA
PLASMODIUM			
16	Plasmodium knowlesi (nuri)	37	19(?)
16	P.falciparum (Camp)	37	19(?)
16	P.berghei berghei (N30)	24	-
16	P.berghei berghei (WLTM)	24	-
16	P.vinckei (52)	24	-
16	P.gallinaceum (8B)	18	-
16	P.lophurae	20	-
TRICHOMONAS			
15	Trichomonas gallinae	34	-
15	T.vaginalis	29	-

TRYPANOSOMES			k DNA	s DNA
1	Crithidia lucillae	57	-	-
1	C.fasiculata	58	-	-
1	C.oncopelti	54	-	-
29	C.oncopelti	50	40	32
27	C.fasiculata Anopheles	54	-	-
27	Blastocrithidia culicis	56	-	-
32	C.acanthocephali	58		
32	C.fasiculata	58		
32	C.fasiculata (Culex pipiens)	58		
32	C.lucillae	58		
32	C.rileyi (Arilus)	58		
32	Crithidia sp. (E.davisi)	58		
32	Crithidia sp. (Syrphid)	58		
1	Leishmania tarentolae	54	-	-

Ref.	Organism	DNA base composition nuclear DNA	k DNA	s DNA	
21	L.enriettii	62	43	-	
22	L.enriettii	61	43	-	
22	L.mexicana	58	42	-	
22	L.donovani	59	45	-	
22	L.tropica	61	47	-	
22	L.tropica major	59	42	-	
1	Trypanosoma lewisi	59	-	-	
23	T.theileri (Ayr)	41	36	48	
23	T.theileri (Bogota)	40	38	47	
23	T.melophagium	42	35	-	
23	T.avium	32	36	46	
23	T.lewisi	46	38	-	
23	T.musculi	46	38	-	
23	T.cruzi	49	38	-	
23	T.cruzi (culture)	49	38	-	
23	T.dionisii	52	40	57	
23	T.vespertilionis	58	38	-	
24	T.vivax	34	36	-	
24	T.congolense	48	37	33	
24	T.brucei	47	31	42	
24	T.rhodesiense	47	30	41	
24	T.gambiense	47	30	42	60
24	T.evansi NS	47	29	42	
24	T.evansi SAK	49	-	42	
24	T.equiperdum	46	33	41	

Ref.	Organism	DNA base composition nuclear DNA	k DNA	s DNA
25	T.gambiense	48	31	42
25	T.cruzi	51	40	-
19	T.brucei	47	32	42
19	T.evansi SAK	47	32	42
19	T.equinum	47	32	42
33	T.mega	44	34	-

Footnote.

The values for the base composition of protozoan DNA are expressed to the nearest whole number. When authors reported only the buoyant density of the DNA their results have been converted into %GC using the formula -

$$\%GC \frac{\text{buoyant density} - 1.660}{0.00098}$$ (Schildkraut, Marmur and Doty 1962)

Minor DNA components have been reported for many different protozoa. Where the sources of these minor DNA's have been identified, their base ratios are listed under appropriate headings, e.g. m(mitochondrial)DNA; c(chloroplast)DNA and k(kinetoplast)DNA. Minor components of nuclear DNA are listed as s(satellite)DNA; minor DNA's of unknown origin are marked (?) Values of base composition calculated from thermal denaturation temperature determinations are marked (t).

References.

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8. Behme 1963.

9. Ray and Hanawalt 1964.

10. Edelman, Cowan, Epstein and Schiff 1964.

11. Ray and Hanawalt 1965.

12. Chiang and Sueoka 1967.

14. Leff, Mandel, Epstein and Schiff 1963.

15. Mandel and Honigberg 1964.

16. Gutteridge, Trigg and Williamson 1969.

17. Kieras and Chiang 1971.

18. Reeves, Lushbuagh and Montalvo 1971.

19. Gutteridge, Stuart, Williamson and Vickerman 1970.

20. Adam, Blewett and Flamm 1969.

21. DuBuy, Mattern and Riley 1966.

22. Chance 1972.

23. Newton 1971.

24. Newton and Burnett 1972.

25. Riou and Pautrizel 1969.

26. Gelderman, Keister, Bartgis and Diamond 1971a.

27. Mandel 1967.

28. Holt and Gurney ~~1961~~ 1969

29. Marmur, Cahoon, Shimura and Vogel 1963.

30. Dutta and Mandel 1972.

31. Mandel and Muzyka ~~1972~~.1971

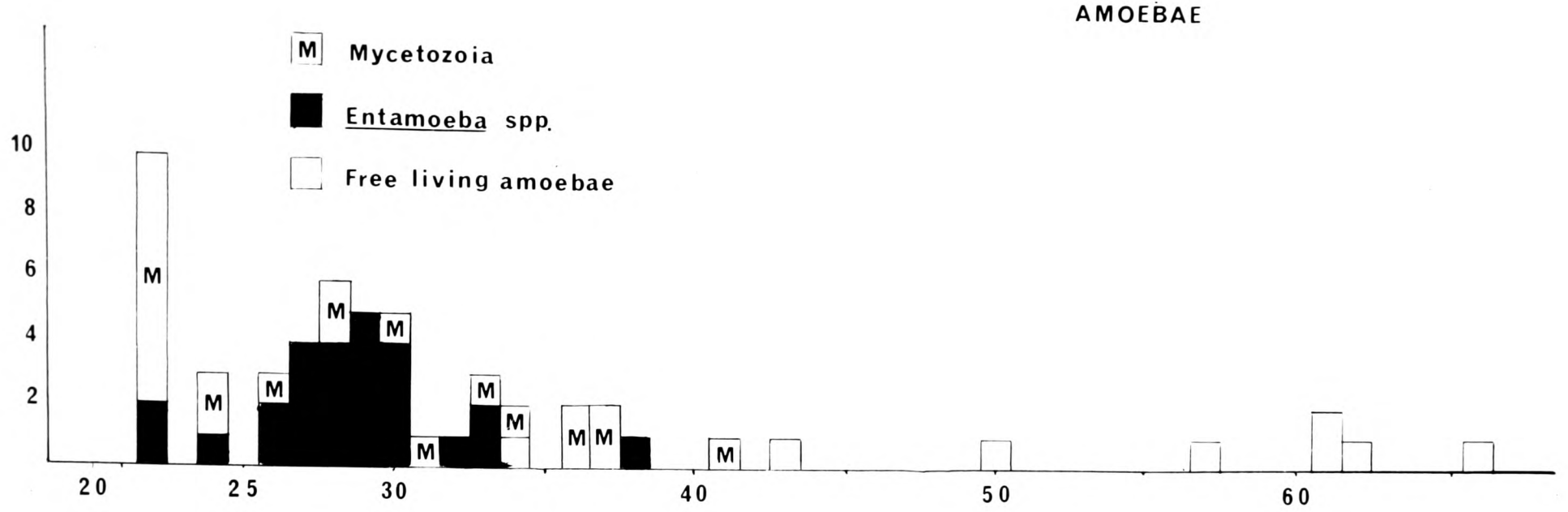
32. Renger and Wolstenholme 1972.

33. Steinert and Van Assel 1967.

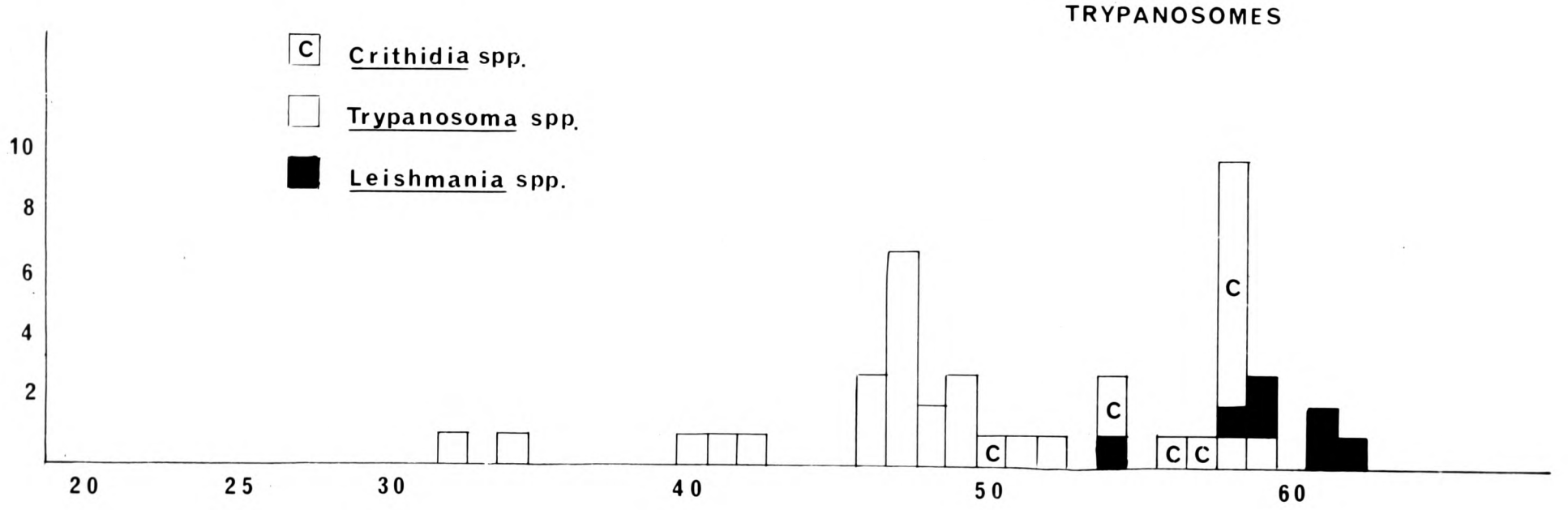
Fig. I.1.

The relationship between DNA base composition and the taxonomy of four major groups of protozoa. The frequency distribution of % G-C in the amoebae, the Kinetoplastida, the ciliates and the phytoflagellates. Compiled from the data in Table I.2.

AMOEBAE

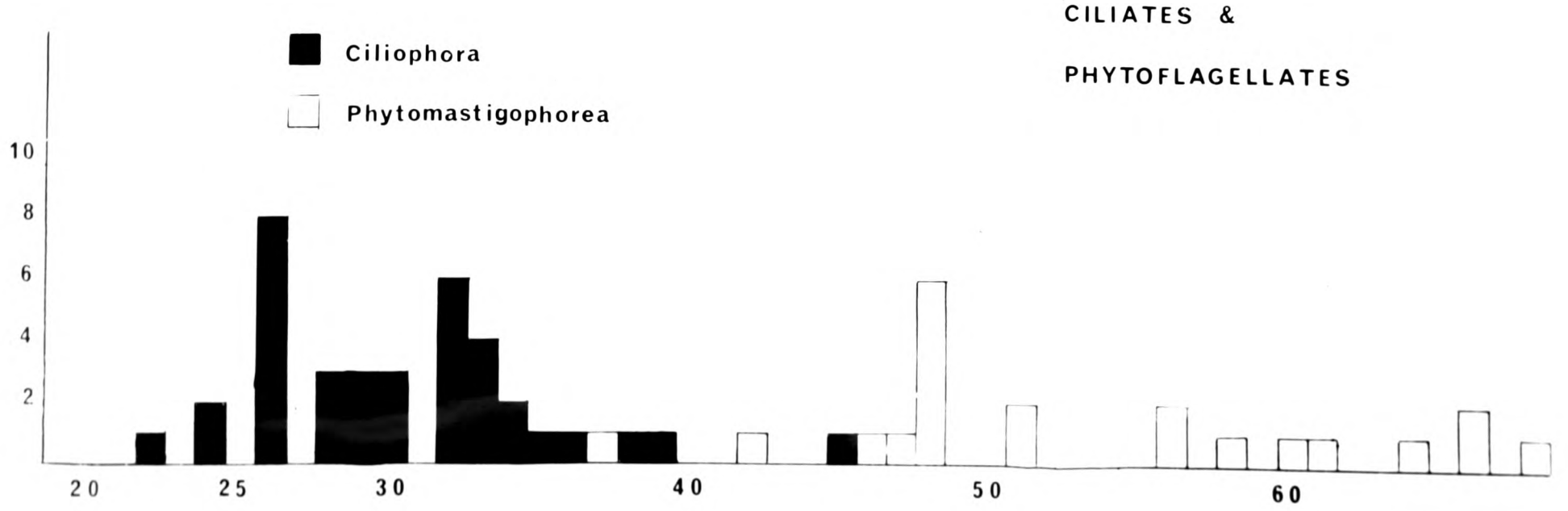


TRYPANOSOMES



CILIATES &

PHYTOFLAGELLATES



It should be noted that although the DNA from a large number of protozoa has been characterised, these organisms represent only a small fraction of the estimated 30,000 species in the Phylum. Moreover, the species which have been examined were not selected at random but were either readily cultured laboratory strains (such as Tetrahymena, Euglena, Chlamydomonas) or were important pathogens (such as Trypanosoma, Entamoeba, Plasmodium).

Amongst bacteria there is a relationship between taxonomic position and DNA base composition, (Silvestri and Hill 1965), Bohacek, Kocur and Martinec 1971, Auletta and Kennedy 1966, Hill 1966). Unlike bacteria protozoa are eucaryotes and their DNA often contains minor components which are recognised by different buoyant densities on caesium chloride gradients. The minor DNA may originate from organelles such as mitochondria (Suyama and Preer 1965, Edelman, Epstein and Schiff 1966), chloroplasts (Leff, Mandel, Epstein and Schiff 1963) or kinetoplasts (Marmur, Cahoon, Shimura and Vogel 1963, DuBuy, Mattern and Riley 1965), or more rarely it may be a fraction of the nuclear DNA (Williamson et al 1971). Minor components have been discovered in the DNA of many species of protozoa, but since minor DNAs may be lost during DNA extraction (Skinner and Triplet 1967, Rolfe 1963, Newton and Burnett 1972, Britten, Pavich and Smith 1969) their occurrence in the protozoa is possibly more widespread than the present data suggest. Brunk and Hanawalt (1969) showed that in 4 strains of T.pyriformis (6III, HOM, ST and GL strains) the mitochondrial DNA did not differ in buoyant density from the nuclear DNA.

Although the nuclear DNA of protozoa may range in base composition from 18%GC - 68%GC, the organisms in the major taxonomic groups exhibit more restricted ranges. The ciliates (Subphylum Ciliophora) are characterised by DNA of low GC content, while the phytoflagellates (Class Phytomastigophorea) tend to possess DNA of high GC content. Within the Class Phytomastigophorea there

is an indication that the DNA of the euglenoid flagellates (Order Euglenida) has a lower GC content than the DNA of the organisms in the Order Chloromonadida (Chlamydomonas and Polytoma). Representatives of the Order Kinetoplastida have GC rich DNA, and as in the Phytomastigophorea there is evidence that well defined groups within the Order have distinctive DNA base ratios. The trypanosomes have DNA of lower GC content than species of Crithidia or Leishmania. It is interesting that the amoebae (Superclass Sarcodina) range in base composition from 22 - 66% GC, a range almost as great as that of the whole Phylum. The slime moulds (Subclass Mycetozoia) are characterised by DNA of low GC content, the naked amoebae (Order Amoebida) may be divided, on their base ratios, into two groups, Entamoeba which has DNA with low GC content and Acanthamoeba and Amoeba proteus which have GC rich DNA.

In the well defined higher taxa of the Phylum the organisms tend to resemble each other in base composition, but within small well defined taxonomic groups differences in base composition have been used to distinguish between closely related organisms. Measurements of the base composition of the nuclear and minor DNA have been used to demonstrate differences between strains and species of Leishmania (Chance 1972) and of Acanthamoeba (Adam, Blewett and Flamm 1969). Similarly, characteristic base ratios have been recorded for species of Chlamydomonas (Mandel 1967), Plasmodium (Gutteridge, Trigg and Williamson 1969), Paramecium (Gibson 1966), Trypanosoma (Newton and Burnett 1972) and Polytoma (Kieras and Chiang 1971).

There appears also to be a relationship between the GC content of minor DNA and taxonomic position. The kinetoplast DNA of trypanosomes and the mitochondrial DNA of Acanthamoeba and Leishmania are as restricted in base composition as the nuclear DNA of these organisms. Similarly, the base composition

of the mitochondrial and chloroplast DNA of the phytoflagellates falls within a restricted range. The minor components of protozoan DNA usually have a lower GC content than the corresponding nuclear DNA, but there is insufficient data to assess either the extent or the significance of this trend.

There is usually good agreement between the results obtained by different workers using the same strain or species of protozoan e.g. Dictyostelium sp. (Schildkraut et al 1962, Mandel 1967, Dutta and Mandel 1972), Leishmania enriettii (DuBuy, Mattern and Riley 1966, Chance 1972) and Trypanosoma cruzi (Newton 1971, Riou and Pautrizel 1969). There are some conflicting results, e.g. Astasia longa (Kieras and Chiang 1971, Mandel 1967) and Trypanosoma lewisi (Schildkraut et al 1962, Newton 1971).

The base composition of Entamoeba DNA was determined by the Buoyant density techniques (Reeves et al 1971) and by the thermal denaturation technique (Gelderman et al 1971). Four strains of E.histolytica were examined by both authors and the values of base composition obtained by the two techniques used differed by 2 - 8% GC. These discrepancies in base composition measurements were attributed by both authors to the possible presence of unusual bases in the DNA of Entamoeba. Analogues of the four common bases are known to occur in some DNAs; if a sufficiently high proportion of an unusual base is substituted into a DNA molecule then the relationship between base composition and buoyant density no longer holds (Schildkraut et al 1962), although the thermal denaturation temperature may not be affected (DeLey 1970).

Other workers have reported differences in the values of DNA base ratios obtained by the thermal denaturation and buoyant density techniques. DuBuy, Mattern and Riley (1966) obtained values of 62%GC and 57%GC (Tm) for the nuclear

DNA of *L.enriettii* and of 43%GC and 33%GC(Tm) for the kinetoplast DNA. Edelman, Cowan, Epstein and Schiff (1964) characterised the nuclear and chloroplast DNA of *E.gracilis* using the thermal denaturation and buoyant density techniques and found that the base composition of the nuclear DNA was 48%GC or 55%GC(Tm) and of the chloroplast DNA 26%GC or 30%GC(Tm). They attributed these differences to the presence of 2.3% 5-methyl cytosine in the DNA (Brawerman, Hufnagel and Chargaff 1962). On the other hand, the buoyant density and thermal denaturation techniques have provided consistent results when applied to the DNA from *A.castellanii* (Blewett 1969), *T.cruzi* (Riou and Paoletti 1967), *T.gambiense* and *T.equiperdum* (Riou and Pautrizel 1969) and *C.lucillae*, *T.patula* *T.pyriformis* and *D.discoideum* (Schildkraut et al 1962). Gibson (1966) characterised the DNA from *C.carolina* and *P.aurelia* using the thermal denaturation technique and obtained values for DNA base composition which were in close agreement with results of buoyant density determinations by Schildkraut et al (1962) for *C.carolina* and by Suyama and Preer (1965) for *P.aurelia*.

The significance of variations in base composition is not fully understood, such variations are possible because the genotype of the organism may change and because the genetic code is degenerate and the same information can be coded using A-T or G-C rich sequences. It is assumed that changes in base composition are an evolutionary process, arising from a gradual accumulation of base substitutions (Kimura 1968). This is not necessarily true of procaryotes; mutations resulting in very large changes in base composition have been reported for several species of bacteria (Gause, Loshkareva, Zbarsky and Gause 1964). Nevertheless, analysis of the DNA base composition of micro-organisms can be a valuable guide to their taxonomy and is especially useful in

differentiating between organisms within a well defined taxonomic group.

The usefulness of base composition analysis in the taxonomy of protozoa is limited by the difficulties in assessing the significance of small variations in base ratios. The nuclear and mitochondrial DNA of A.castellanii and A.palsetinensis differ by only 1% and 3% GC respectively (Adam et al 1969) and the nuclear DNA of L.mexicana and L.tropica major differ by only 1% GC (Chance 1972). The measurement of the DNA base composition of salivarian trypanosomes has revealed only small differences in the nuclear DNA (Newton and Burnett 1972). The work on Entamoeba has shown (Reeves et al 1971, Gelderman et al 1971a) that small variations in base composition may not necessarily reflect strain differences, yet these differences may be expressed in the pathogenicity of the organism.

Closely related protozoa frequently have identical or near identical DNA base composition. A more sensitive method of comparing DNA in such cases is the study of DNA base sequence homology. Direct determination of base sequence is impracticable, but it is possible to compare DNA base sequences. When DNA is exposed to extremes of pH or temperature the molecule is denatured (Doty 1957). This reaction is reversible, (Marmur and Doty 1961) and DNA which has been denatured at high temperature will renature if incubated at a lower temperature. The extent to which the DNA renatures depends on its complexity; virus DNA renatures more readily than bacterial DNA under conditions in which calf thymus DNA does not renature at all. Renaturation occurs most readily at temperatures 20 - 30°C below the thermal denaturation temperature, it is dependent on the ionic strength of the medium and on the DNA concentration. (Marmur Rownd and Schildkraut 1963). The mechanism of renaturation appears to be the joining of a short length of matching sequence followed by a reversible zippering action along the reacting strands.

Britten and Kohne (1966) examined the renaturation rate of the DNA from higher organisms and demonstrated a relationship between renaturation rate and the complexity of the DNA. This examination of the renaturation rates of the DNA of higher organisms showed that while the bulk of the DNA renatured very slowly, a proportion of the DNA (usually about 10 - 20% but in some organisms up to 70%) renatured much more rapidly. Wetmur and Davidson (1968) described a relationship between kinetic complexity (calculated from the rate constant of the renaturation reaction) and the analytical complexity (molecular weight) of the DNA. They also showed that the genome of higher organisms contained DNA fractions with different renaturation rates.

The rapidly renaturing fraction consists of repeated sequences, that is, sequences which are repeated from a few hundred to more than a million times throughout the genome (Waring and Britten 1966, Britten and Kohne 1966). The slowly renaturing fraction contains sequences that are unique, or are at the most repeated a few times only.

The DNA strands which reassociate during renaturation need not originate from the same organism. Schildkraut, Marmur and Doty (1961) renatured together normal and heavy (N15 labelled) bacterial DNA and were able to recognise a hybrid DNA by its intermediate density on a CsCl density gradient. The amount of DNA in the intermediate band gave an estimate of the sequence homology between the different DNAs in the renaturation mixture. With this system it was found that the yield of hybrid DNA was greatest between homologous DNA or DNAs with the same base composition.

Bolton and McCarthy (1962) isolated RNA complementary to DNA by physically immobilising the DNA and collecting the RNA which reacted with it. To do this they trapped denatured high molecular weight DNA in agar gel and allowed radio-active RNA to diffuse into the gel and blind to the DNA. The agar was then subjected to washing procedures to remove unbound RNA. The RNA which had hybridised with complementary sequences in the DNA was then recovered and estimated. This technique is also suitable for preparing DNA-DNA hybrids. Gillespie and Speigleman (1965) described a technique for producing RNA-DNA hybrids in which the denatured DNA was immobilised by adsorption on to cellulose nitrate filters, which were then heat dried to fix the DNA. The filters could then be incubated in solutions of radio-active RNA. The amount of hybrid formed was determined by measuring the radio-activity on the filters after non-specifically bound RNA had been removed by washing and RNAase treatments. Denhardt (1966) modified Gillespie and Speigleman's technique by treating the heat dried filters so that they no longer adsorbed single stranded DNA, thus making it possible to use the filter technique for the production of DNA-DNA hybrids. Laird, McConaughy and McCarthy (1969) used hydroxyapatite fractionation (Miyazawa and Thomas 1965) to isolate DNA-DNA hybrids formed by the reassociation of heterologous DNA solution.

Hybridisation techniques have been used to study DNA base sequence homologies among related bacteria. Kingsbury (1967) used the filter technique of Denhadt (1966) to compare base sequence homologies between species of Neisseria. Heberlein, DeLey and Tijtgat (1967) compared the sequence homologies between three genera in the Family Rhizobiaceae using the agar technique. Hybridisation studies appeared to provide good estimates of the degree of sequence homology between the DNA of higher organisms (Hoyer, McCarthy and Bolton 1964). But hybridisation is essentially the renaturation of

heterologous DNA; the work of Britten and Kohne (1966) and Wetmur and Davidson (1968) required a more cautious interpretation of the results of hybridisation studies. The greater part of the genome of eucaryotic organisms does not renature under the conditions normally used in hybridisation techniques. Estimates of base sequence homology obtained by these techniques were therefore based on comparisons of only part of the genome. Also, the renaturation rate of heterologous DNAs is lower than that of the homologous reaction (Sutton and McCallum 1971), so that comparisons of the final levels of renaturation reached in homologous and heterologous reactions may not give a true measure of sequence homology.

Comparisons of DNA base composition and base sequence have been useful in determining relationships between bacteria. These techniques are of little practical importance in the classification of the metazoa, but DNA base composition analysis has proved a useful aid to the taxonomy of protozoa. Among protozoa differences in DNA base composition can be used to justify species separation, similarity in base composition, even between closely related organisms, does not imply synonymity. Comparisons of base sequence, using hybridisation techniques, may be useful in resolving the relationships between apparently identical organisms.

The hybridisation technique is probably sufficiently sensitive to detect the small differences in base sequence that might occur between very closely related protozoa. The sensitivity of the technique can be increased by measuring the quality of the hybrid formed rather than its quantity. Laird, McConaughy and McCarthy (1969) calculated that for reassociated DNA or hybrid DNA the thermal denaturation temperature was depressed by 1.0°C for every 1.5% of base pairs mismatched. If the base sequences of related protozoa are compared by hybridising as much of the DNAs as possible and then measuring the

thermal stability of the hybrid it should be possible to obtain reliable estimates of the degree of sequence homology.

The application of hybridisation techniques to the taxonomy of protozoa requires techniques for the extraction and purification of comparatively large amounts of DNA, and for the renaturation and hybridisation of the DNA. A variety of techniques have been used to extract DNA from protozoa. Many of these techniques are modifications of the method of Marmur (1961) (e.g. DuBuy et al 1966, Sager and Ishida 1963, Mandel and Honiqberg 1964, Riou and Paoletti 1967 and Kieras and Chiang 1971). The pronase technique of Berns and Thomas (1965) was used by Gibson (1966) to extract DNA from several species of ciliate, and Chiang and Sueoka (1967) used a modification of this technique to prepare DNA from Chlamydomonas. Reeves Lushburgh and Montalvo (1971) used a modification of the method of Britten, Pavich and Smith (1969) to extract DNA from Entamoeba. The extraction of DNA from some species of protozoa appears to be difficult. Gelderman, Keister, Bartigs and Diamond (1971) reported that the methods of Marmur (1961), Kirby (1957) and Mandel and Honigberg (1964) were unsuitable for the preparation of DNA from Entamoeba, and Kirtikar, Jensen and Myers (1967) and Mandel (1967) experienced difficulty in extracting DNA from Acanthamoeba.

The amount of DNA which can be obtained from protozoa depends largely on the ease with which the organism can be cultured. Species which grow well in axenic or monaxenic culture can usually be grown in sufficient quantity to provide the amount of DNA required for hybridisation studies.

Comparatively little is known of the renaturation rates of protozoan DNA, DuBuy, Mattern and Riley (1966) found that L.enriettii DNA would renature to the extent of 35% if incubated overnight in the spectrophotometer. Bastia,

Siersma, Swift and Chiang (1970) showed that the nuclear DNA of Chlamydomonas renatured as a single kinetic species, and did not contain repeated sequences Britten and Kohne (1966) reported the presence of a small rapidly reassociating fraction in the DNA of Euglena. Apart from the work of Gibson (1966) there is no information of base sequence homologies between protozoa.

The object of the experiments described in this thesis was to compare the DNA of amoebae in the genus Acanthamoeba. Members of this genus are ubiquitous in soil and freshwater where they probably feed on bacteria. The genus is well defined (Page 1967); but there is considerable confusion as to the identity of the large number of laboratory strains of Acanthamoeba which have been isolated.

Acanthamoeba grows readily on peptone or on simple defined media (Adam 1959) and the nutritional requirements of several strains have been determined (Adam 1964, Band 1962, and Adam and Blewett 1967). The ultrastructure of both the trophozoites (Bowers and Korn 1968) and the cysts (Bowers and Korn 1969, Pasternak, Thompson, Schultz and Zachariah 1970) of A.castellanii has been described. Band (1963) and Griffiths and Hughes (1969) developed replacement media for inducing encystment of Acanthamoeba and Byers, Rudick and Rudick (1969) and Rudick (1970) have examined changes which occur in cultures of A.castellanii during the logarithmic and stationary phases.

Reports that Acanthamoeba was the causative agent in a number of cases of primary amoebic meningoencephalitis (Cerva and Novak 1968, Callicot 1968) have been disproved (Carter 1972). Nevertheless, experimental animals have proved susceptible to infection by some strains of Acanthamoeba (Cerva 1967, Culbertson 1961).

Species of Acanthamoeba are of interest both as easily cultured protozoa for biochemical or physiological studies and as facultative parasites. Some means of distinguishing between strains and species of this organism would be of considerable practical use. In view of the difficulties of classifying amoebae by morphological criteria, increasing use must be made of biochemical techniques. Detailed comparisons of the DNA of these organisms may provide a satisfactory method.

MATERIALS AND METHODS

M1 The amoebae.

The amoebae used in this study all belong to the genus Acanthamoeba as re-defined by Page (1967). The 11 strains or species examined are listed below.

Acanthamoeba castellanii (Douglas (1930)

Neff strain	Isolated from soil in California by Neff (1957) and identified as Acanthamoeba sp.
H strain	Isolated from soil in Scotland by Harrison (1957) and described as Hartmannella sp.
W1	Found as a contaminant in tissue culture (See Adam 1964).
Singh strain	Isolated from soil in England by Singh (1952) and identified as Hartmannella rhysodes.
p27 strain	Isolated from fresh water in USA by Page (1967) and identified as Acanthamoeba castellanii.

Acanthamoeba palestinensis (Reich 1933).

First described by Reich (1933) in Palestine and identified as Mayorella palestinensis.

Acanthamoeba polyphaga (Pushkarew 1913).

Isolated from fresh water in USA by Page (1967) and identified as A.polyphaga.

Acanthamoeba astronyxis (Ray and Hayes 1954).

Isolated from water in USA by Ray and Hayes (1954) and identified as Hartmannella astronyxis.

Acanthamoeba spp.

Strains A1 (Culbertson 1961), and A30 and A35 (Culbertson, Ensminger and Overton, 1965) are pathogenic strains of Acanthamoeba, isolated in the USA, and identified only as Acanthamoeba spp.

Tetranymena pyriformis W, was obtained from the Cambridge Culture Collection.

M2 Culture methods.

M2.1 Media

Amoebae were grown in 2% (w/v) mycological peptone (Oxoid) plus 0.05M maltose. The medium was made up complete and sterilised by autoclaving for 20 - 30 minutes at 15 p.s.i. and 120°C.

Tetrahymena pyriformis W was grown in 2% (w/v) bacteriological peptone (Oxoid) sterilised as above.

M2.2 Types of cultures.

Stock cultures of amoebae were maintained in 3ml of medium in 15mm x 160mm screw capped glass tubes, and were subcultured at approximately monthly intervals.

Tetrahymena stock cultures were maintained in 5 - 10ml of medium in screw capped tubes and subcultured weekly. Inocula for bulk cultures were grown in 10ml of medium in 50ml conical flasks. Bulk cultures were grown in either 250ml of medium in 500ml round flasks or 1.5 litres of medium in 5 litre round flasks. Flasks were plugged with cotton wool stoppers. The 250ml and 1.5 litre cultures were stirred with 1. inch or 2½ inch silicone rubber covered magnets, spun at 250 rpm by motors placed beneath the flasks. All cultures were incubated at 25°C.

M3 Harvesting cultures.

M3.1 Counting amoebae.

Amoebae were counted in a Fuchs Rosenthal 0.2mm haemocytometer. All the amoebae in $3mm^3$ were counted. When cell numbers were higher than 150 - $200/mm^3$ the amoebae in only $1mm^3$ were counted. The results of counts were always expressed as amoebae/mm^3.

M3.2 Harvesting small cultures.

Cultures of less than 500ml were harvested in weighed polypropylene centrifuge bottles by 5 minutes centrifugation at 3,000 rpm and 0C. The weight of the pelleted amoebae was determined by reweighing the bottle.

M3.3 Harvesting bulk cultures.

Bulk cultures were harvested when the numbers of amoebae reached between 300 and $600/mm^3$. Cultures at this stage of growth could be selected by eye, but samples were usually taken and counted.

The amoebae were collected in an MSE continuous action rotor precooled to $0^{\circ}C$. A 15cm filter funnel was connected by a collar of rubber tubing to a short piece of glass tube drawn out into a 1 - 2 mm jet, the jet was inserted into a length of thick walled rubber tubing which was connected to the inlet tube of the rotor. In this way a flow rate of about 500ml/minute was maintained independently of the height of the funnel. The effluent was run via a length of rubber tubing into a bucket on the floor.

The culture was poured into the funnel and allowed to run through the rotor at rotor speeds of 4,500 rpm. As soon as the outflow ceased the rotor was run down, braked, and taken off. The amoebae collected inside the sediment container and were washed off into the medium remaining in the rotor bowl by sucking some of the medium up into a 10ml pipette and blowing it out on to the packed amoebae. The amoebal suspension was transferred to a 500ml beaker and stirred for a few minutes to disperse the cells before pouring it into 2 weighed 250ml polypropylene centrifuge bottles. The amoebae were pelleted by centrifuging at 3,000 rpm for 5 minutes at 0°C. The bottles were then reweighed to determine the yield of amoebae.

Tetrahymena were recovered from bulk cultures in the same way except that the continuous action rotor was run at 9,000 rpm and the cells were pelleted at 6,000 rpm.

M4 Extraction of DNA.

M4.1 Solutions.

The composition and abbreviated names of commonly used solutions are listed below.

i) SSC, saline sodium citrate - 0.15M NaCl 0.015M sodium citrate in distilled water. SSC was made up as a stock solution at ten times its normal concentration (x10 SSC) and diluted as required. The pH of the diluted solution was adjusted to pH 6.8 - 7.0

ii) Tris, Trizma base (Sigma, reagent grade) 0.01M solution in distilled water. Tris was prepared as an 0.1M stock solution and diluted as required.

iii) SLS, 25%(w/v) solution of sodium lauryl sulphate in distilled water. Before use this solution was warmed to about 50°C to redissolve SLS which had precipitated at room temperature.

iv) Ethanol, 96% solution of industrial methylated spirits in distilled water. This reagent was used to precipitate DNA from solution and was always used in the proportion 2 volumes of ethanol to one volume of DNA solution.

v) 75% Phenol, was prepared by dissolving 25ml of the buffer used in the DNA extraction method in 75g phenol. This solution was prepared fresh for each experiment.

vi) 90% Phenol. Solution of aqueous 8-hydroxyquinoline in phenol, made up in the proportions; 0.1g 8-hydroxyquinoline, 10ml distilled water and 90g phenol.

vii) Chloroform. 24:1 mixture of chloroform:octanol.

viii) RNAase, Ribonuclease A from bovine pancreas (Sigma) was prepared as a stock solution in SSC at a concentration of 500µg/ml. The solution was heated for 5 minutes at 100°C then dispensed into Bijou bottles at 2ml/bottle and stored at-20°C.

ix) Pronase, Calbiochem grade B pronase was always used freshly prepared at a final concentration of 1mg/ml. The appropriate amount of pronase was weighed out and dissolved in 1ml of 0.01M Tris + 0.25% SLS, before addition to the sample solution.

M4.2 DNA extraction and purification.

The quantities of the solutions used in the various steps of extraction and purification procedures are expressed as volumes; one volume is equivalent to 1ml of solution per gram of amoebae in the original pellet.

M4.2a) The original procedure (Method I).

Initially DNA was prepared by the method of Adam Blewett and Flamm (1969). Pelleted amoebae were resuspended in 4 volumes of freshly prepared ice cold 0.3M sodium diethyldithiocarbamate + 0.015M phenolphthalein diphosphate pH 7.5.

The amoebae were lysed by the addition of 0.4 volumes of 25% SLS; 4 volumes of 75% phenol were added and the mixture was stirred for 30 minutes at 4°C. Following the phenol extraction, the mixture was centrifuged for 15 minutes at 0°C and 17,000 x g. The clear aqueous supernatant was removed to expose a thick viscous interfacial precipitate lying on top of the phenol phase. This precipitate was sucked up into a wide bore pipette, transferred to a glass stoppered tube and shaken with 3 changes of excess water saturated diethyl ether. The resulting gelatinous precipitate was dissolved in 2 volumes of SSC for 5 - 10 minutes at 60°C. The crude DNA solution was cooled and the DNA was precipitated with ethanol. The precipitate was spooled out on a glass rod and redissolved in 2 volumes of SSC. The DNA was precipitated with ethanol once more then redissolved in 0.01M Tris and stored at 4°C until required.

The crude DNA was purified on CsCl density gradients. These gradients were prepared by dissolving 4.59000 grams of CsCl (Analar) in 3.4ml of 0.01M Tris containing 50 - 200 µg of impure DNA. This gave an initial density of 1.720 - 1.725 g/cm^3. The solution was transferred to an MSE 10ml polypropylene centrifuge tube and overlaid with mineral oil to within a few mm of the top, the tube was then tightly capped. Gradients were centrifuged for 60 hours at 35,000 rpm and 25°C in a 10 x 10 angle rotor in an MSE superspeed 50 or 60 centrifuge. The gradients were fractionated as described in M5 below.

M4.2b) The modified procedure (Method II)

Pelleted amoebae were resuspended in 4 volumes of ice cold 0.3M sodium diethyldithiocarbamate and lysed by the addition of 0.4 volumes of 25% SLS. Four volumes of 75% phenol were added and the mixture was stirred for 30 minutes at 4°C. The phases were separated by centrifugation for 15 minutes at 1,500 rpm

and 0°C in 100ml glass centrifuge tubes or 250ml glass bottles. The interfacial layer was recovered as before, extracted with 3 changes of diethyl ether and resuspended in 2 volumes of SSC.

The crude DNA solution was heated for a few minutes at 60°C to drive off any residual ether, then pronase was added to a final concentration of 1mg/ml. The solution was incubated for 30 minutes at 60°C and then allowed to cool to room temperature. An equal volume of 90% phenol was added and the mixture was stirred for 15 minutes at room temperature. The aqueous phase was separated by centrifuging for 15 minutes at 1,500 rpm, the supernatant was collected using an inverted pipette and the DNA was precipitated with ethanol. The fibres were spooled out on a glass rod and redissolved in 0.5 volumes of 0.01M Tris. RNAase was added to a final concentration of 50µg/ml and the solution was incubated for 2 hours at 37°C. After RNAase digestion, 1mg/ml pronase was added and incubation was continued for a further 1 hour at 37°C. Following the enzymic treatments an equal volume of 90% phenol was added and the mixture was stirred and separated as before. DNA was precipitated from the supernatant with ethanol, spooled out and redissolved in 0.25 volumes of 0.01M Tris; this solution was dialysed against 2 litres of 0.01M Tris at 4°C for 24 - 48 hours. The dialysate was precipitated with ethanol and the DNA was redissolved in 0.25 Volumes of 0.01M Tris and stored at 4°C until required.

The purified DNA was fractionated by centrifugation using the rapid equilibration CsCl technique of Brunk and Leick (1969). A saturated solution of CsCl was prepared by dissolving 20g of CsCl in 10ml of 0.01M Tris at 25°C 2ml of this solution was pipetted into a 10ml polypropylene centrifuge tube (MSE) and overlaid with 3.5ml of mineral oil. 1.2ml of the same saturated CsCl solution was pipetted into a 10ml conical centrifuge tube and to this was

added to 0.8ml of DNA solution in 0.01M Tris (DNA concentration 100 - 200µg/ml). The solutions were mixed well then the mixture was layered on to the saturated CsCl solution by dropping it slowly through the oil layer. The tube was tightly capped and centrifuged for 24 hours at 38,000 rpm and 25°C in a 10 x 10 angle rotor as before.

M4.2c) Fractionation of CsCl gradients.

At the end of the run the rotor was allowed to decelerate (without braking) and the rotor was carefully removed from the drive shaft. The tubes were handled gently to avoid disturbing the gradient and all handling prior to fractionation was performed slowly keeping the tubes upright throughout.

The tube was held vertically in a clamp, a few inches above the bench, the grub screw in the centre of the cap was removed and an adaptor inserted in its place. The adaptor consisted on a 2cm tube, threaded on its lower half so as to screw into the centre of the tube cap, and tapered on its upper half to connect, via a 2mm diameter PVC tube, to an oil injection system. This system was made up of a vertical 20ml syringe above a horizontal 1ml syringe, the 2 syringes being connected through a 3-way tap. The 20ml syringe was filled with mineral oil and served as a reservoir for the 1ml syringe. The system was controlled by the 3-way tap which allowed oil to move between any 2 of the 3 interconnected parts.

The bottom of the centrifuge tube was pierced with a 19G hypodermic needle and mineral oil was injected into the tube from the 1ml syringe. Fractions of the gradient, displaced by the injected oil, were collected in small glass tubes. The fraction volume, 0.2ml, was controlled by the volume of oil injected, using the graduations on the syringe.

The fractions were diluted with 2ml of distilled water and the absorbance at 260mm of each fraction was measured in 1cm silica cells in a Beckman DB spectrophotometer. The DNA was recovered from the fractions of high absorbance either by precipitation with ethanol or by centrifuging the diluted fractions for 16 - 18 hours at 35,000 rpm and 25°C in an MSE Superspeed centrifuge.

M4.3 Preparation of ^{32}P labelled DNA from A.castellanii(Neff).

a) Culture.

Amoebae were grown in 30ml of medium in 100ml conical flasks which were incubated in a shaking water bath at 25°C. The cultures were inoculated with amoebae so as to give an initial concentration of 50 - 100 amoebae/mm^3. One millicurie of ^{32}P was added asceptically from a sterile solution of ^{32}P orthophosphate in dilute HCl (specific activity 1mCi/ml) obtained from the Radiochemical Centre, Amersham. The cultures were incubated for 3 days, by which time the cell numbers had risen to 400 - 600/mm^3.

b) Harvesting radio-active cultures.

The culture was pipetted into a 50ml glass centrifuge tube and centrifuged for 15 minutes at 1,500 rpm. The cell pellet was resuspended in 10ml of 0.5% NaCl and transferred to a 10ml graduated centrifuge tube. The amoebae were pelleted by centrifuging for 15 minutes at 1,500 rpm and 0°C, and the packed cell volume was measured from the graduations on the tube.

c) Extraction of DNA.

Because yields of amoebae from the radio-active cultures were small, the amounts of solutions used were based on packed cell volume not on weight.

The DNA was extracted from the radio-active amoebae by Method I. After washing with non-anhydrous diethyl ether, the DNA precipitate was incubated in 1 volume of 1mg/ml pronase for 30 minutes at 60°C. The digest was precipitated with ethanol, the DNA fibres were spooled out and redissolved in 1 volume of SSC. RNAase was added to a final concentration of 100 µg/ml and the solution was incubated for 2 hours at 37°C. Pronase was added, as a powder, to a final concentration of 1mg/ml and the mixture was incubated for a further hour at 37°C. The solution was then dialysed against 2 litres of 0.02M Tris for 48 hours at 4°C. The DNA was fractionated on a preparative CsCl density gradient using the technique described in Method II.

The gradient was fractionated as described above and the radio-activity in each fraction was determined by Cerenkov counting. The DNA containing fractions were pooled and were diluted about 10 fold; the DNA was recovered by pelleting for 16 hours at 35,000 rpm and 25°C.

M4.4 Preparation of DNA from T.pyriformis.

DNA was extracted from Tetrahymena using Method II, slightly modified, as follows;

i) the lysate-75% phenol mixture was centrifuged for 45 minutes at 1,500 rpm not 15 minutes.

ii) after treatment with 90% phenol the aqueous phase was separated by centrifuging for 10 minutes at 17,000 x g.

iii) the second pronase treatment was ommitted, after RNAase digestion the solution was treated immediately with 90% phenol.

iv) the final solution was in 0.5 volumes of 0.01M Tris, not 0.25 volumes.

M5 Sephadex Gel Filtration.

M5.1 Preparation of the column.

Two grams of dry Sephadex G-200 gel (Pharmacia Ltd.) were rehydrated in 100ml of buffer (0.1M NaCl + 0.01M Na cacodylate pH 6.9) for 72 hours at 4°C. After this time the excess buffer above the gel was drawn off with a Pasteur pipette and the gel suspension was poured into a 30cm x 2cm column fitted with a sintered glass plate and clamped at the base. The gel was left to settle and when a layer 5 - 7cm deep had formed at the bottom of the column the clamp was opened and the buffer allowed to drain off. The buffer level was allowed to fall to within a few mm of the gel surface and the column was clamped at the base. A small disc of filter paper (Whatman GFA, glass fibre filter) trimmed to the same diameter as the column was fitted snugly on to the top of the gel.

The space above the gel was filled with buffer and the column was stoppered with a rubber bung pierced with 2 holes. A narrow hole carried an inlet tube which was connected to an aspirator placed a few cm above the top of the column. A wide hole bore a short glass tube which ended just above the surface of the gel, and was topped with a collar of silicone rubber tubing closed with a gate clamp.

M5.2 Loading the column.

The inlet from the aspirator was closed and the collar on the wide tube was opened. 1 - 2ml of the test solution was mixed with 0.4 - 0.8ml of 2mg/ml sucrose in distilled water and the mixture was drawn up into a 5ml syringe. A length of fine teflon tubing was fitted on to the syringe needle and the free end was passed through the wide tube so as to discharge just above the gel surface.

The solution was slowly injected on to the gel, forming a layer between the gel surface and the overlying buffer. When all the sample had been injected the teflon tube was withdrawn and the collar on the wide tube was closed. The inlet from the aspirator was opened and the buffer was run through the column at a flow rate of about 20ml/hour.

2ml fractions were collected using an LKB collector and siphon assembly. The absorbance at 260 mμ of each fraction was measured in a Beckman DB spectrophotometer. Fractions were collected until the absorbance had fallen to background or until all the DNA containing fractions had been recovered. The column was washed with at least 500ml of buffer before being re-used.

M6 Hydroxyapatite chromatography.

M6.1 Preparation of hydroxyapatite.

Hydroxyapatite, (HAP), was prepared after the method of Miyazawa and Thomas (1965). The entire procedure was performed in a 3 necked Quickfit and Quartz 2 litre round bottomed flask mounted in an Electrothermal heating mantle. The central neck was fitted with a glass stirrer driven by an overhead electric motor at about 250 rpm. One of the side necks carried a water cooled condenser, the other was used for adding and removing solutions.

200ml of distilled water were added to the flask and 500ml each of 0.05M disodium hydrogen phosphate and 0.05M calcium chloride were siphoned into the flask at a rate of approximately 4ml/minute. The mixture was stirred throughout this stage. At the end of this lengthy step the precipitate was allowed to settle for 15 - 20 minutes then the supernatant was siphoned off and replaced with 1 litre of distilled water. The suspension was stirred briefly between washes.

After the fourth wash the HAP was resuspended in 1 litre of distilled water and 25ml of 40% NaOH (w/v) was added. The mixture was boiled with simultaneous stirring for 1 hour, then left to settle. The supernatant fraction was siphoned off and the precipitate was washed 4 times in distilled water as before.

The phosphate buffer used in the subsequent treatments was made by mixing equal moles of sodium dihydrogen phosphate and disodium hydrogen phosphate, the pH was 6.9. The precipitate was resuspended in 1 litre of 0.01M buffer and brought just to boiling. It was then allowed to settle, the supernatant fraction was removed and replaced with a further 1 litre of 0.01M buffer and boiled for 5 minutes. The precipitate was again resuspended in 1 litre of 0.01M buffer and boiled for 15 minutes. All the boiling treatments were accompanied by stirring. After each boiling some fine material was removed with the supernatant fraction.

After the final treatment the precipitate was resuspended in 500ml of 0.01M buffer and was dispensed into universal containers with a small amount of chloroform as a preservative. The filled containers were stored at 4°C.

M6.2 Fractionation of DNA on hydroxyapatite - test tube method.

a) Solutions.

Flasks containing 0.06M; 0.15M; 0.18M and 0.36M phosphate buffer pH 6.9 were maintained at 60°C in a water bath. The 0.15M and 0.18M buffers were dispensed using the following system. Each flask was connected via a short length of PVC tubing to a 3-way tap attached to a 10ml graduated syringe clamped to the end of the water bath. The other arm of the tap was joined to 1 metre of PVC tubing which was coiled up under the water in the bath, and ended in a short glass tube held just above water level. To dispense

buffer the syringe was filled from the flask, then a 3ml volume of buffer was injected through the 3-way tap into the coiled PVC tube. 3mls of buffer at 60°C were displaced from the coil and collected in the sample tube.

b) Centrifugation.

An MSE bench centrifuge was mounted inside a large incubator maintained at 60°C. HAP was separated from the eluting buffers by centrifuging for a few minutes at low speed in 10ml glass tubes.

c) Fractionation.

2ml of 0.06M buffer and 1ml of HAP suspension were pipetted into a 10ml tube and heated to 60°C in the water bath. The DNA sample was added and the solutions were mixed thoroughly using a vortex mixer. The tube was returned to the water bath and the sample volume was made up to 10ml by the addition of 7ml of 0.06M buffer at 60°C. The HAP was pelleted by a brief centrifugation at 60°C; the superatant fraction was discarded.

The pellet was resuspended in 3ml of 0.15M buffer, dispensed as described above, mixed well with a vortex mixer and centrifuged. The supernatant fraction was collected in a scintillation vial and the pellet was washed twice more in 0.15M buffer. After each wash the supernatants fractions were collected in the same vial. This procedure was repeated exactly for the 0.18M and 0.36M buffers, except that the 0.36M buffer was dispensed from a 10ml graduated pipette instead of by the syringe system. The pooled supernatants fractions were collected in separate vials. The activity eluted at each buffer concentration was determined by Cerenkov counting.

M6.3 Thermal denaturation of DNA on HAP columns.

a) The column.

The column used was a 1.5cm bore water-jacketed glass column fitted with a sintered plate and a stop-cock at its base. The jacket was connected to a Circon heating water pump circulating light mineral oil from a small oil bath positioned alongside the column. A glass thermometer (0 - 100 °C in 1/10th C divisions) was connected to the shaft of a small electric motor and suspended down the centre of the column; it was used to stir the eluant and to determine its temperature.

b) Preparation of HAP.

10ml of suspension in 0.01M buffer was pipetted into a 25ml beaker. 1.7mls of 0.36M buffer were added to raise the molarity to 0.06M. The DNA sample was dropped into the suspension and the mixture was stirred with a magnetic stirrer for 15 minutes. The slurry was poured into the column and stirred by the rotating thermometer while the column was heated to 25°C.

c) Elution.

The outlet of the column was connected to a peristaltic pump with a throughput of 1ml/minute. As soon as the slurry in the column reach 25°C the stop-cock was opened and the buffer was pumped out into a scintillation vial. When the meniscus fell to the top of the HAP bed the pump was stopped and 10ml of 0.12M buffer was added to the column. The fresh buffer was allowed to equilibrate to 25°C then a clean vial was placed under the pump outlet and the buffer was collected.

The column was heated in 5 - 10 °C steps up to 70 °C and in 2 - 3 °C steps thereafter, up to 97 - 100 °C. At each temperature 10ml of 0.12M buffer were pumped through the column and collected in a scintillation vial. After the

final temperature rise the DNA remaining on the HAP was removed by eluting with 10ml of 0.36M buffer.

M7 Capillary tube renaturation technique.

a) Preparation of DNA solutions.

DNA was extracted and purified using Method II (M4.2b). The nuclear DNA was collected from the appropriate fractions of the preparative gradients by precipitation with ethanol. The precipitated DNA was redissolved in 1ml of x2 SSC buffered to pH 6.9 with 0.015M phosphate buffer.

b) Sonication.

To keep the DNA solution cool during sonication the following device was adopted. The base of a 13.5ml Spinco cellulose nitrate centrifuge tube was forced into the top of an MSE 10ml polypropylene centrifuge tube to a depth of 1 - 1.5cm, then cut off flush with the top of the MSE tube. A 1 - 2mm hole was bored in the base of the MSE tube, then the whole assembly was inverted and the polypropylene tube was filled with water through the hole in its base. The water filled tube was stored inverted in a deep freeze at -20°C until required.

In use, the tube was held upright in a beaker filled with crushed ice and the DNA solution was pipetted into the well formed by the base of the Spinco tube. The probe of a Soni-probe ultrasonicator was carefully racked down into the DNA solution so that most of the solution was between the sides of the probe and the walls of the tube. The solution was sonicated for 20 seconds with the instrument set at mark 4 and tuned to 1.8mA. After treatment the solution was pipetted out of the cooling tube and dialysed against 2 litres of 0.012M phosphate buffer for 24 hours at 4°C.

c) Denaturation.

The DNA solutio was denatured in a modified Pasteur pipette which was designed to prevent loss by evaporation. A siliconised pipette was heated a short distance from its tip in a micro-burner, drawn out to a fine tip and heat sealed. The DNA solution was introduced into the pipette through the mouthpiece and the pipette was flicked gently to dislodge the bubble which was usually trapped just above the sealed tip. 1ml of mineral oil was then pipetted on top of the solution.

The filled pipette was immersed to the level of the oil-water interface in a boiling water bath for 15 minutes. The denatured DNA was quenched by transferring the pipette to a tube of iced water for 10 minutes.

A siliconised glass tube (7 x 50mm) was weighed and the DNA solution was transferred to it by cutting off the tip of the pipette with a pair of scissors and holding the broken end against the wall of the glass tube. The solution drained slowly into the tube, but the viscosity of the oil prevented it from flowing through the very fine jet thus allowing the complete separation of the aqueous phase. The tube was reweighed to determine the weight of the DNA solution; a volume of 1.2M buffer equivalent to 1/10th the weight of the DNA solution was added to raise the molarity of the buffer to 0.12M.

d) Preparation of samples.

The required number of 0.05ml samples of denatured DNA solution in 0.12M buffer were pipetted into siliconised 7 x 50mm tubes using Dispo 50µl capillary pipettes (Clay Adams Inc. Ny.) Siliconised micro-haematocrit tubes, heat sealed at one end, were placed open end down in the tubes. The rack of tubes was placed in a vacuum dessicator (without dessicant) and evacuated for a few

minutes until the air from the haematocrit tubes ceased to bubble through the solution. Air was then re-admitted into the dessicator, forcing the solution up into the haematocrit tubes.

The filled tubes were collected and the open ends were heat sealed in the flame of a micro-burner. The sealed tubes were incubated in tests tubes of water which were completely immersed in a water bath at 60°C.

e) Recovery of the samples.

A glass cutter was drawn firmly across the glass over the air space at one end of the haematocrit tube, and the end was broken off with a pair of forceps. The tip of a siliconised pasteur pipette, drawn out to a very fine tube, was inserted into the haematocrit tube and the contents were withdrawn and added to a predetermined volume of diluent.

M8 Thermal denaturation technique.

a) Apparatus

A Unicam SP 500 spectrophotometer was fitted with a double walled cell holder through which water was circulated by a heating water pump. A hole was drilled through the sample compartment lid exactly above the end cell position, so that a thermometer could be lowered through the hole into a water filled reference cell.

b) Preparation of solutions.

The DNA sample was diluted to a final volume of 1.5 or 2ml with either 1/10th SSC or 0.012M phosphate buffer. Both diluents were adjusted to pH 6.8 - 7.0 and filtered before use. A blank was prepared using 2ml of the appropriate diluent.

The sample and reference solutions were pipetted into 1cm silica spectrophotometer cells and stoppered with silicone rubber plugs pierced with 1mm diameter glass tubes. The cells were placed in a vacuum dessicator and the solutions were degassed under vacuum for 2 - 3 minutes. The rubber plugs were replaced with airtight stoppers and the cells were positioned in the cell holder of the spectrophotometer.

c) Experimental procedure.

The samples were heated from 25°C to 60°C to 70°C and then in 5°C steps up to 95°C. The absorbance at 260mμ of the DNA solution was measured at each temperature rise. The temperature of the sample was taken as being the same as that of the water in the reference cell. The system was allowed to stabilise for at least 5 minutes, after the temperature in the reference cell had reached the higher level, before taking the absorbance reading.

M9 Measurement of radio-activity.

a) Samples.

Fractions from preparative gradients were collected in small glass tubes and the tubes were placed directly into Packard scintillation vials.

Material from the HAP fractionation and denaturation procedures was collected in 10ml volumes of phosphate buffer in scintillation vials.

b) Scintillation counting.

Samples were counted in a Packard liquid scintillation spectrometer, programmed to detect Cerenkov radiation (25% gain, 50 - 1,000 window, background subtracted 24 counts/minute). Counting times varied for the different materials; HAP fractions were counted for 5 or 10 minutes, fractions from preparative CsCl gradients were counted for 1 or 2 minutes.

c) Cleaning scintillation vials.

Vials were cleaned by autoclaving for 20 minutes at 120°C in a dilute aqueous solution of the proprietary detergent Pyroneg and were rinsed overnight under a lowly running tap. The vials were then rinsed 12 times in hot running water, twice in distilled water and dried in a hot air oven.

M10 Analytical ultracentrifugation.

a) DNA samples.

DNA samples were prepared using Method I. All the DNA containing fractions from the preparative gradient (i.e. from the first fraction of the nuclear DNA peak to the last fraction of the minor DNA peak - inclusive) were pooled and the DNA was pelleted by centrifugation for 18 hours at 35,000 rpm and 25°C. 2ml of 0.01M Tris was added to the pellet and the tube was left overnight at 4°C. The DNA solution was adjusted to an optical density of 0.15 with 0.01M Tris and 2.7000g of CsCl was added to 2ml of this solution to give an initial density of 1.720g/cm^3 at 25°C. Finally 0.025ml of a reference DNA solution was added.

b) Centrifugation.

Samples were centrifuged in a Beckman model E analytical ultracentrifuge at 44,700 rpm and 25°C for 22 hours. Ultraviolet absorbtion photographs were taken when the system was at equilibrium and the negatives were scanned in a micro-densitometer.

The buoyant density of the sample was calculated from the formula;

$$Ps = Pm + 0.0092(r_s^2 - r_m^2) ,$$

where Pm is the density of the reference DNA, Ps is the density of the sample DNA, r_s is the distance of the sample DNA from the axis of the rotor and r_m is the distance of the reference DNA band from the axis of the rotor. The base composition of the sample DNA was calculated from its buoyant density using the relationship;

$$G + C = \frac{\text{density of sample} - 1.660}{0.098}$$

(Schildkraut, Marmur and Doty 1962).

M11 Electronmicroscopy of mitochondrial DNA.

a) DNA samples.

Mitochondrial DNA was collected from the minor bands of preparative CsCl gradients. Usually only 2 fractions were taken from the minor band and were combined. The solution was dialysed against 500ml of 1M ammonium acetate for 24 hours at 4°C, then adjusted to a DNA content of 3ug/ml by dilution with 1M ammonium acetate. 0.2ml of mitochondrial DNA solution in 1M ammonium acetate was mixed with 0.01ml of 1mg/ml cytochrome c in distilled water and with 0.02ml of 10% formalin (1 in 10 dilution of commercial preparation of 40% formaldehyde).

b) Spreading technique.

A clean plastic petri dish was positioned in a draught free corner of the laboratory. Two clean glass rods were laid side by side across the diameter of the dish which was then filled to overflowing with double distilled water. The glass rods were moved apart to the edges of the dish and a fine sprinkling of talc was dusted on to the water surface between them. A small clean wire loop was dipped in the DNA solution and touched gently on to the water surface.

As the sample spread the particles of talc were pushed aside leaving a clear area which marked the position on the water surface occupied by the DNA. The dish was left undisturbed for 10 - 15 minutes then a carbon coated grid was laid briefly but gently on the water surface. Excess water was absorbed on to a scrap of filter paper and the grid was left to dry. The dried grid was rotary shadowed with platinum and examined in a Siemens Elminskop electron microscope.

EXPERIMENTS AND RESULTS

Section E1 Techniques.

During the course of the experimental work it became necessary to modify techniques or to adopt new ones. Experiments which were carried out to test techniques are described in this section.

E1.1 Preparative CsCl density gradient centrifugation.

Earlier experiments, (Blewett 1969), had shown that CsCl centrifugation was the most suitable method for purifying and fractionating the DNA of Acanthamoeba. Brunk and Leick (1969) described a method for the isopycnic centrifugation of DNA on a 2 phase CsCl density system in which equilibrium was reached in only 12 hours. The advantages of such a system were so obvious that it was tested with amoebal DNA.

E.1.1.i) Centrifugation time.

A saturated solution of CsCl was prepared by adding 20g CsCl to 10ml of 0.01M Tris at 25°C. 2ml of this solution was pipetted into each of 2 MSE 10ml polypropylene centrifuge tubes and was overlaid with 3.5ml of mineral oil. 2.4ml of the same saturated CsCl solution was mixed with 1.6ml of purified Neff strain DNA in 0.01M Tris (DNA concentration 100µg/ml), in a conical centrifuge tube. 2ml of this solution was dropped slowly through the mineral oil in each tube to form a layer on top of the saturated CsCl solution. The tubes were capped tightly, balanced against CsCl solution blanks and centrifuged at 25°C and 38,000 rpm. One gradient was removed and fractionated after 15 hours and the second after 24 hours. The absorbance profiles obtained from the 2 gradients are shown in Fig.E1.i).

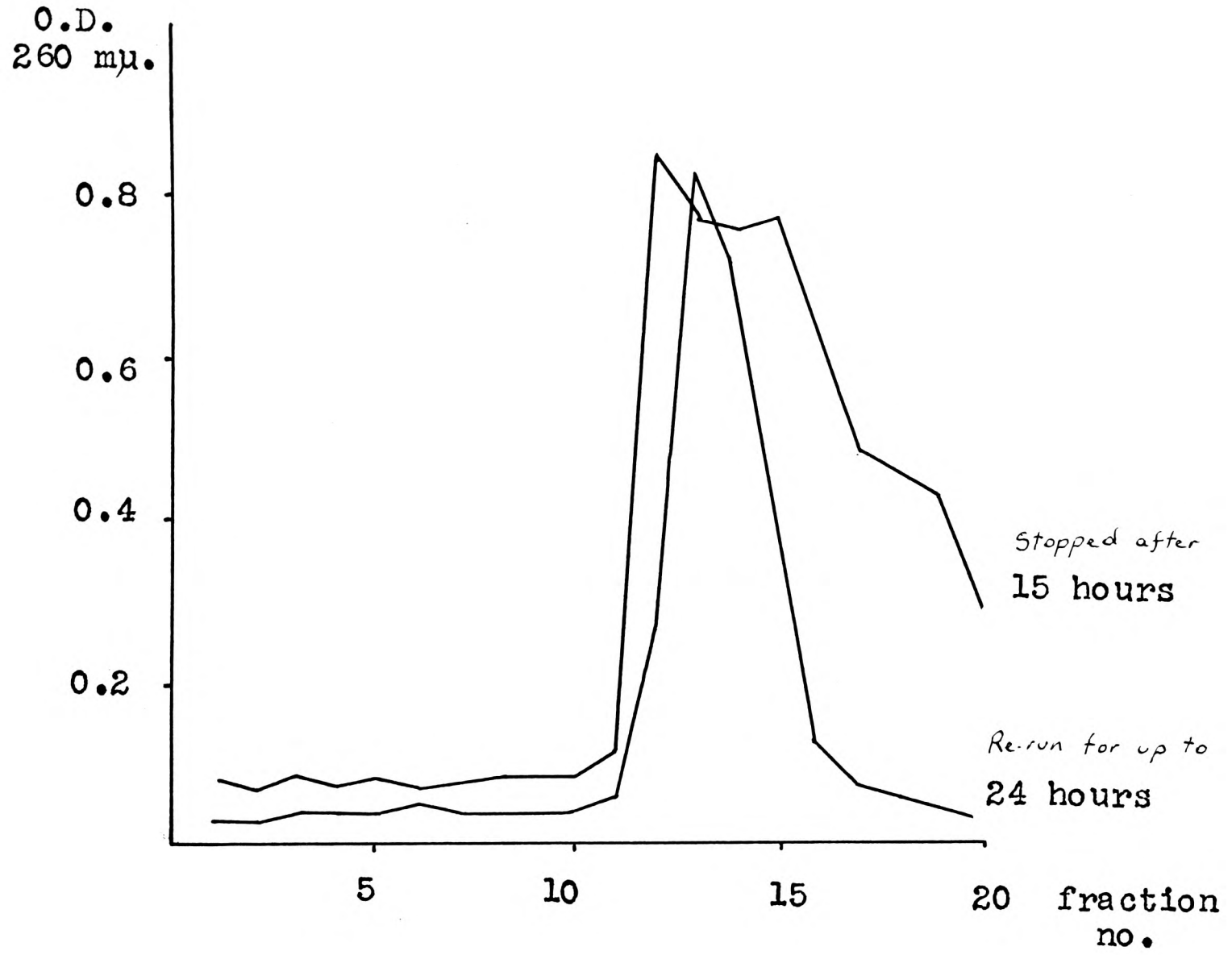


Fig. E 1.i) The effect of centrifugation time on the banding of A.castellanii(Neff) nuclear DNA on CsCl gradients prepared by the method of Brunk and Leick(1969).

E1.1.ii) The ability of the system to purify DNA.

The DNA used in experiment i) had been purified by 2 cycles on conventional CsCl gradients. To test the efficiency of the 2 phase method for the purification of DNA, the previous experiment was repeated exactly using a comparable amount of unpurified DNA (M4.2a). The gradients were fractionated as before, and their absorbance profiles are shown in Fig.E1.ii).

Results.

For both pure and impure samples the time required to reach equilibrium was between 15 and 24 hours. 24 hours was a convenient period and was therefore adopted as the standard time for preparative centrifugation.

The 2 phase system was effective in purifying DNA, but the yield of DNA recovered after centrifugation was less than expected. (See Appendix)

E1.2 Modifications to the extraction procedure.

The DNA extracted by the method of Adam, Blewett and Flamm (1969) was recovered as a protein-DNA mixture. When this DNA was prepared for 2 phase CsCl centrifugation it was dissolved in 0.8ml 0.01M Tris, not as previously in 3.4ml 0.01M Tris. The addition of saturated CsCl solution to such high concentrations of protein caused a heavy precipitation of protein. It was thought likely that this precipitate trapped a large amount of DNA and was responsible for the poor DNA recovery noted in experiment i). To make the most effective use of the shorter centrifugation times therefore, it was necessary to remove as much protein as possible from the crude DNA extracts.

E1.2.i) Purification procedures.

Freshly prepared "interface" DNA (M4.2a) was dissolved in 2 Volumes of SSC by heating for 10 minutes at 60°C. Pronase was added to a final concentration

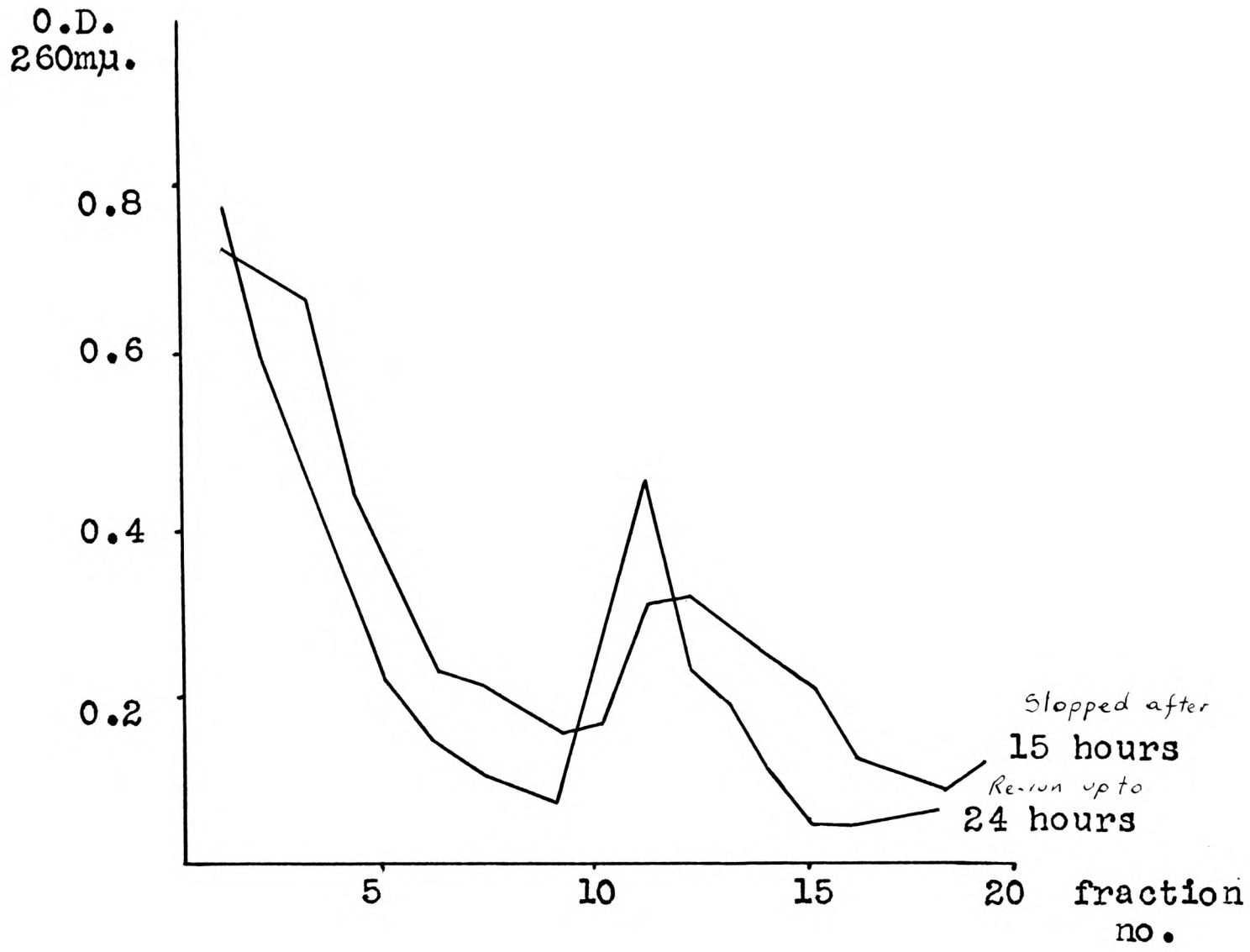


Fig. E 1.ii) The effect of centrifugation time on the banding of A.castellanii (Neff) unpurified DNA on CsCl gradients prepared by the method of Brunk and Leick(1969).

of 1mg/ml and the solution was incubated for 30 minutes at 60°C. After incubation, the solution was cooled on ice and was then subjected to one of the purification procedures outlined in E1.1. Each of the procedures was tested using separate batches of "interface" DNA prepared from 7 - 10 gram pellets of amoebae as described above.

The effectiveness of the different treatments was assayed by comparing the absorption profiles of the samples after centrifugation by the modified method. The results are shown in Fig. E1.2.

Table E1.1 Purification procedures tested.

Expt. No.	Treatments
1	ppt.x2...CsCl
2	90% phenol...ppt x2...CsCl
3	$CHCl_3$...ppt x2...CsCl
4	90% phenol...ether...ppt...dialysis...ppt...CsCl
5	90% phenol...ppt...RNAase...dialysis...ppt...CsCl
6	90% phenol...ppt...RNAase...Pronase...90% phenol... ...ppt...dialysis...ppt...CsCl.

Abbreviations

ppt	precipitation with 2 volumes of 96% ethanol; ppt x2 re-solution of precipitated DNA followed immediately by reprecipitation with ethanol.
90%	phenol; stirred for 15 minutes with 90% phenol at room temperature. DNA recovered in aqueous phase after centrifugation.
$CHCl_3$	shaken 5 - 10 minutes with an equal volume chloroform: octanol. DNA recovered in aqueous phase after centrifugation.
ether	shaken 5 - 10 minutes with an equal volume of water saturated diethyl ether. DNA recovered in aqueous phase after centrifugation.

RNAase digestion with 50µg/ml ribonuclease, 2 hours at 37°C.

Pronase digestion with 1mg/ml pronase, 1 hour at 37°C.

dialysis dialysed against 2 litres SSC for 36 - 48 hours at 4°C.

CsCl centrifuged on CsCl gradient.

Results.

The purification procedures used in experiment 6 were clearly the most effective. DNA purified by this method was apparently free of RNA and the contaminants which gave rise to the high base lines obtained in the other experiments. This method was therefore adopted as the standard purification procedure.

E1.2ii) Modified extraction procedure.

Either of the 2 reagents used in the DNA extraction procedure originally described, sodium diethyldithiocarbamate and phenolphthalein diphosphate, were effective in releasing intact DNA from acanthamoebae (Blewett 1969). Since phenolphthalein diphosphate is expensive, tests were carried out to discover if this salt could be omitted from the extraction solution.

A 10 gram pellet of amoebae was divided and DNA was extracted from one half by the standard method and from the other by using an extractant solution containing only 0.3M sodium diethyldithiocarbamate. The DNA from each pellet was purified by the method used in treatment 6 above, and centrifuged on modified CsCl gradients. The absorbance profiles obtained from the 2 preparations are shown in Fig. E1.3.

Results.

Omission of phenolphthalein diphosphate from the extractant solution neither reduced the amount of DNA recovered nor lowered its molecular weight.

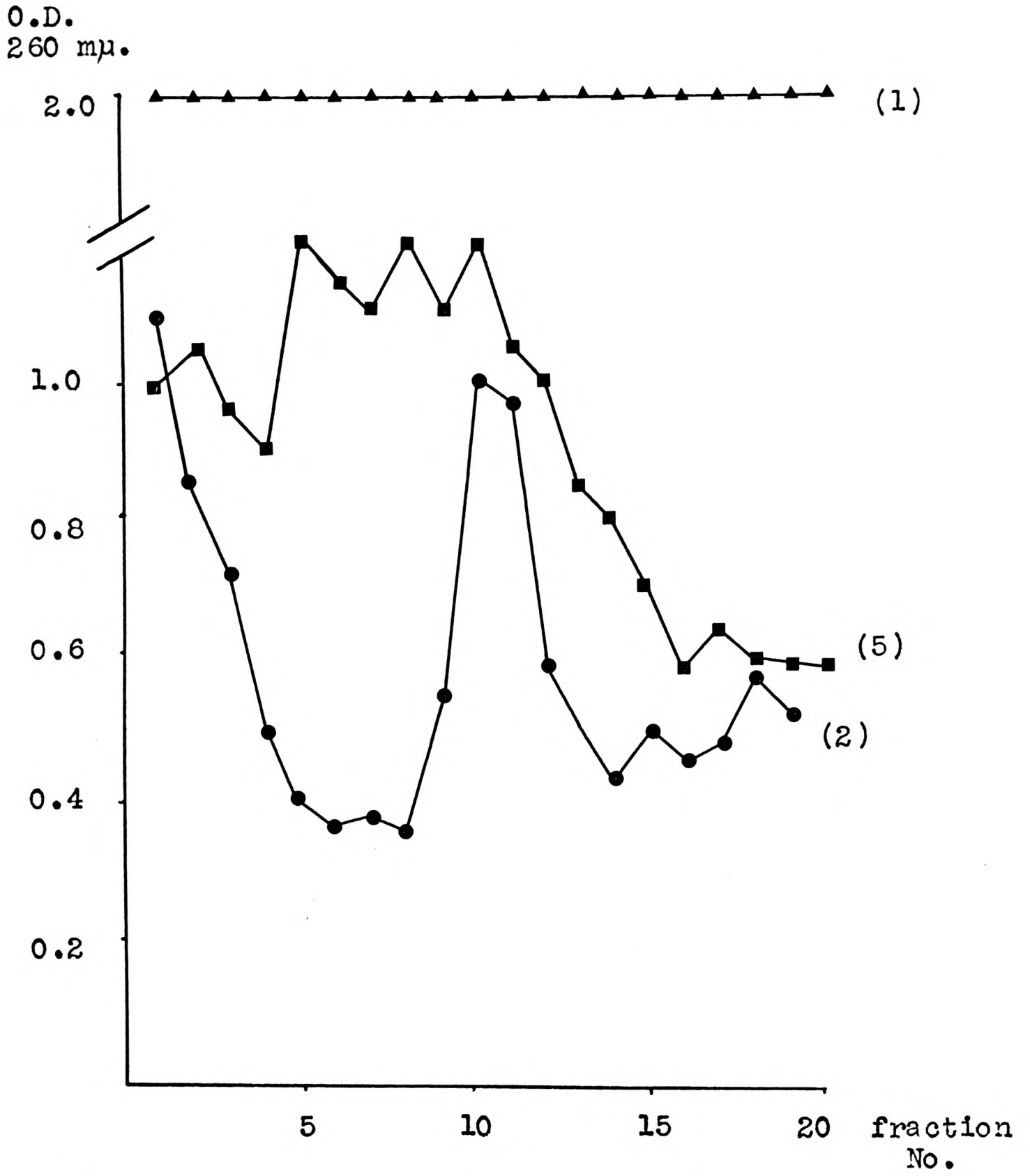


Fig. E 1.2. Absorbance profiles of CsCl gradients after centrifugation of DNA samples subjected to different purification procedures.

The numbers in brackets refer to experiment numbers in Table E 1.1

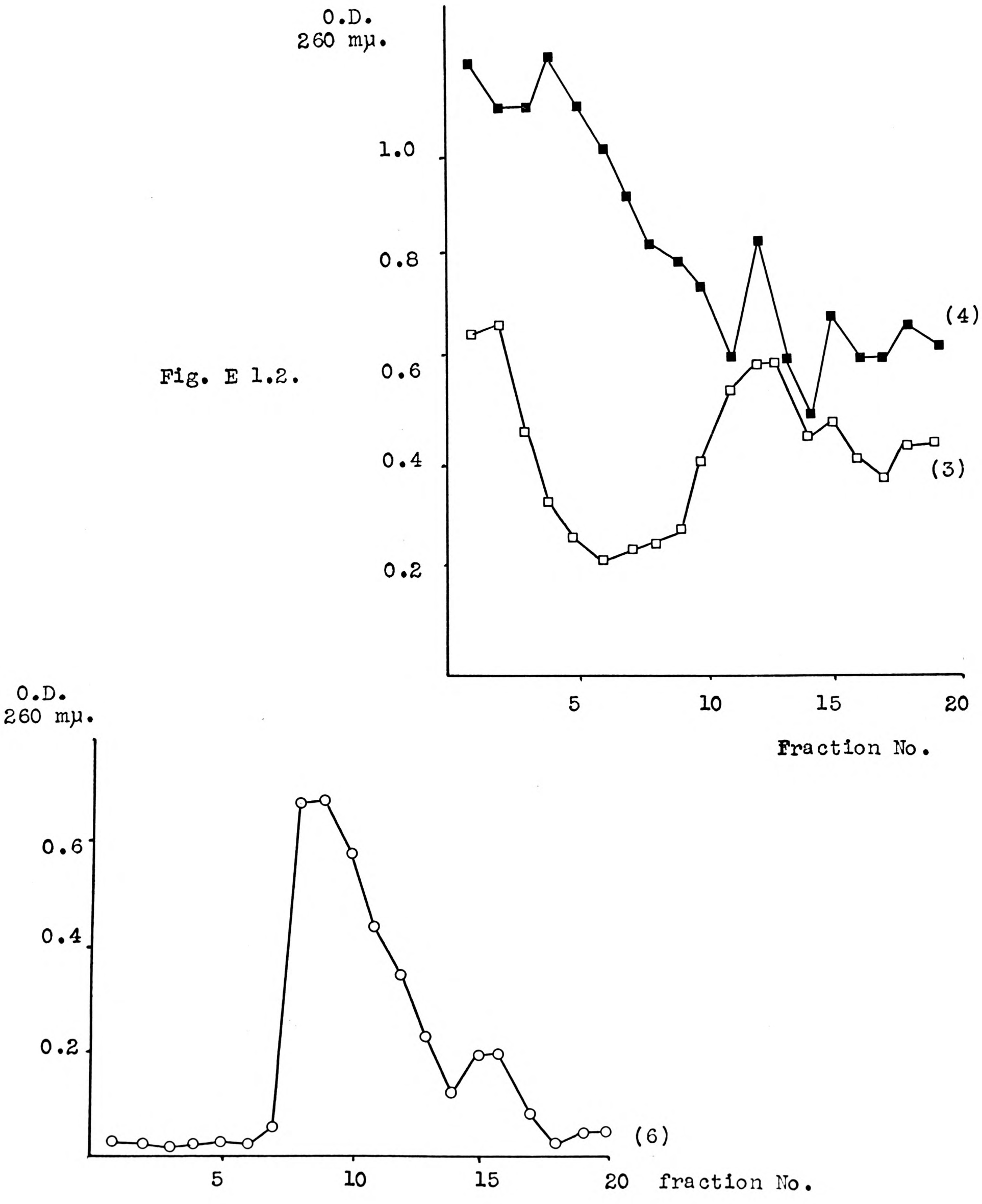

O.D.
260 mμ.
1.0
0.8
0.6
0.4
0.2
(4)
(3)
5
10
15
20
Fraction No.
O.D.
260 mμ.
0.6
0.4
0.2
(6)
5
10
15
20
fraction No.

Fig. E 1.2.

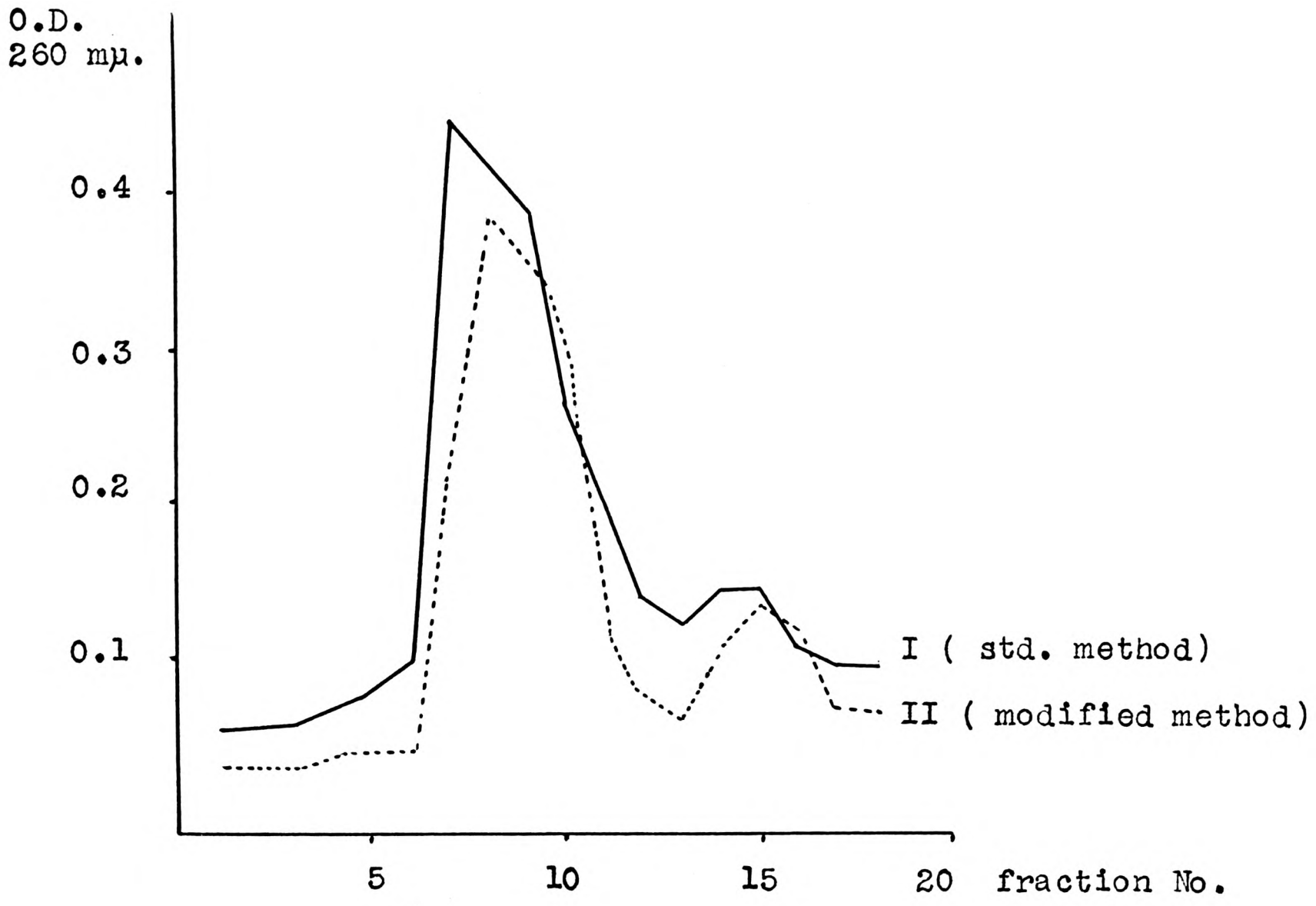


Fig. E 1.3. The effect of omitting phenolphthalein diphosphate from the DNA extraction solution. CsCl centrifugation of DNA prepared by extraction methods I and II.

The difference in the mitochondrial DNA peaks was probably not significant since similar variation had been found between DNA samples prepared by Method I.

As a result of these experiments on extraction and purification, the original method of Adam et al (1969) was replaced by the modified methods. These methods are described fully in materials and methods (M4.2b).

E1.3 Preparation of radio-active DNA.

When A.castellanii Neff strain, was grown in 250ml stirred cultures in peptone maltose medium containing 1mCi of ^{32}P orthophosphate, the isotope was incorporated into the DNA (Adam and Blewett, unpublished data). The specific activity of DNA prepared in this was low (about 100 cpm/µg), and much higher specific activities were required for hybridisation studies. To avoid the necessity of handling large amounts of isotopes, experiments were performed to attempt to grow amoebae to high densities in small agitated cultures containing 1 mCi of ^{32}P.

E1.3.i) The Growth of Acanthamoeba in small agitated cultures.

Two 100ml conical flasks, each containing 30ml of medium were inoculated with 1ml of amoebae from a 10ml culture. The flasks were shaken gently and duplicate samples were taken aseptically from each flask and counted in a Fuchs Rosenthal haemocytometer. The cultures were incubated in a shaking water bath at 25°C and shaken at about 60 excursions per minute. Every 24 hours duplicate samples were taken from each flask and counted as before. Sampling was continued for 6 days, by which time the cultures were approaching stationary phase.

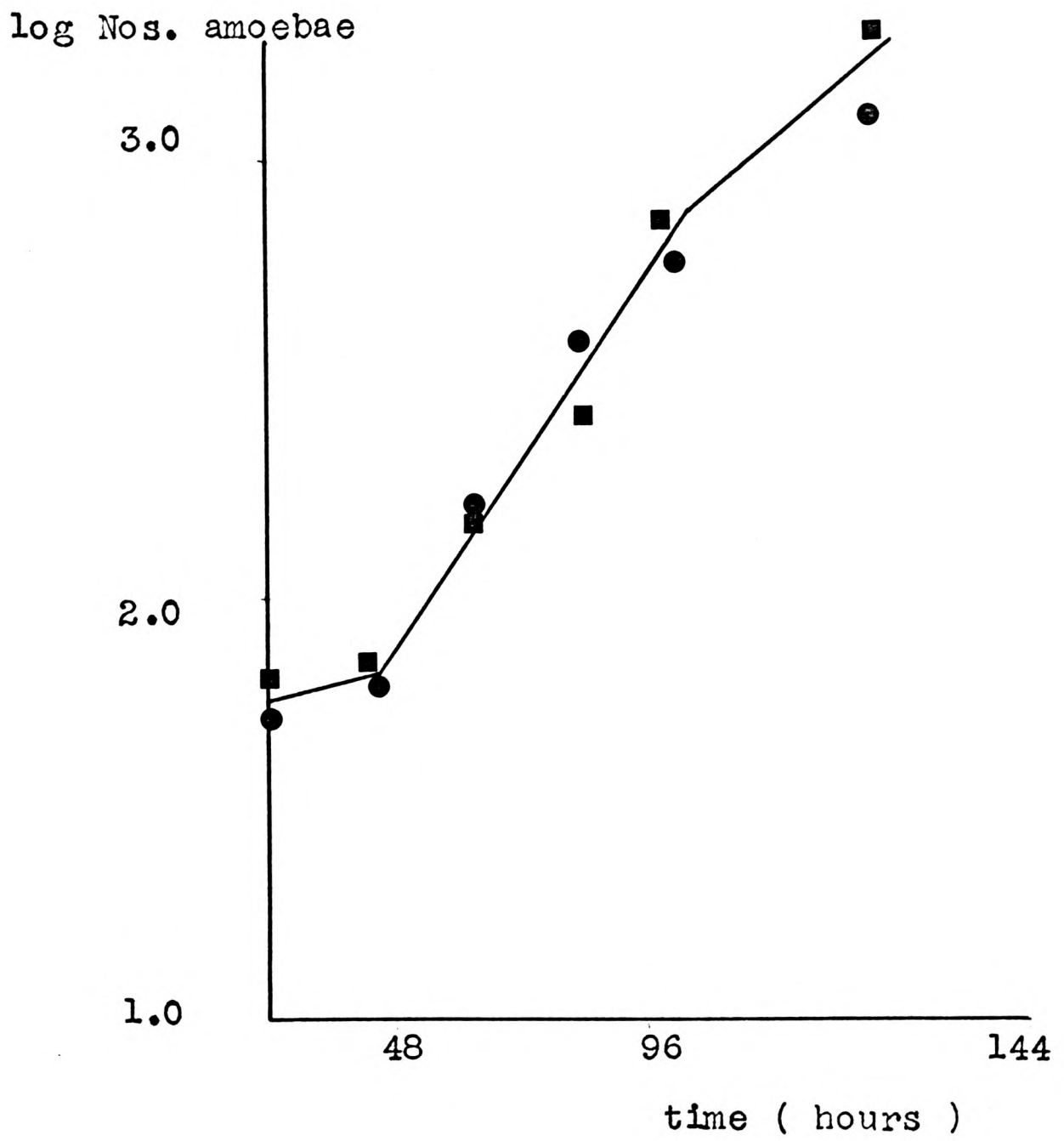


Fig. E 1.4. The growth of A.castellanii(Neff) in 30ml shaken cultures in peptone-maltose medium. Points (●) and (■) represent the means of duplicate counts taken from replicate cultures.

Results.

The growth curves obtained from the 2 cultures are shown in Fig. E1.4. After a 24 hour lag phase the amoebae grew rapidly with a mean generation time of approximately 24 hours and reached a final density of 600 - 700 amoebae per mm^3 without the formation of cysts. Within 48 hours of inoculation the amoebae began to form large clumps, each of which was made up of several hundred amoebae. The presence of these clumps apparently did not inhibit growth of the amoebae, but it did make it difficult to obtain accurate cell counts.

E1.3.ii) Preparation of high specific activity ^{32}P labelled DNA from amoebae.

One millicurie of ^{32}P orthophosphate was added to 30ml of medium in a 100ml conical flask. Amoebae were added so as to give an initial count of 50 - 100 mm^3, and the culture was incubated as described above.

After 3 days, when the numbers of amoebae had reached between 400 - 600/mm^3, the culture was transferred to a 50ml glass centrifuge tube and centrifuged for 15 minutes at 1,500 rpm. The cell pellet was resuspended in 10ml 0.5% NaCl and pipetted into a 10ml graduated centrifuge tube. The amoebae were pelleted by centrifuging for 15 minutes at 1,500 rpm and the packed cell volume was measured from the graduations on the tube. The DNA was extracted by Method I (M4.2a), the proportion of extractant used was based on packed cell volume, assuming the cells had a density of 1g/ml.

The unpurified DNA was centrifuged on a CsCl gradient using the modified technique (Method II). The gradient was fractionated and the absorbance at 260mμ of each fraction was measured. The fractions were then transferred to scintillation vials and the radio-activity in each fraction was measured by Cerenkov counting. The results are shown in Fig. E1.5i).

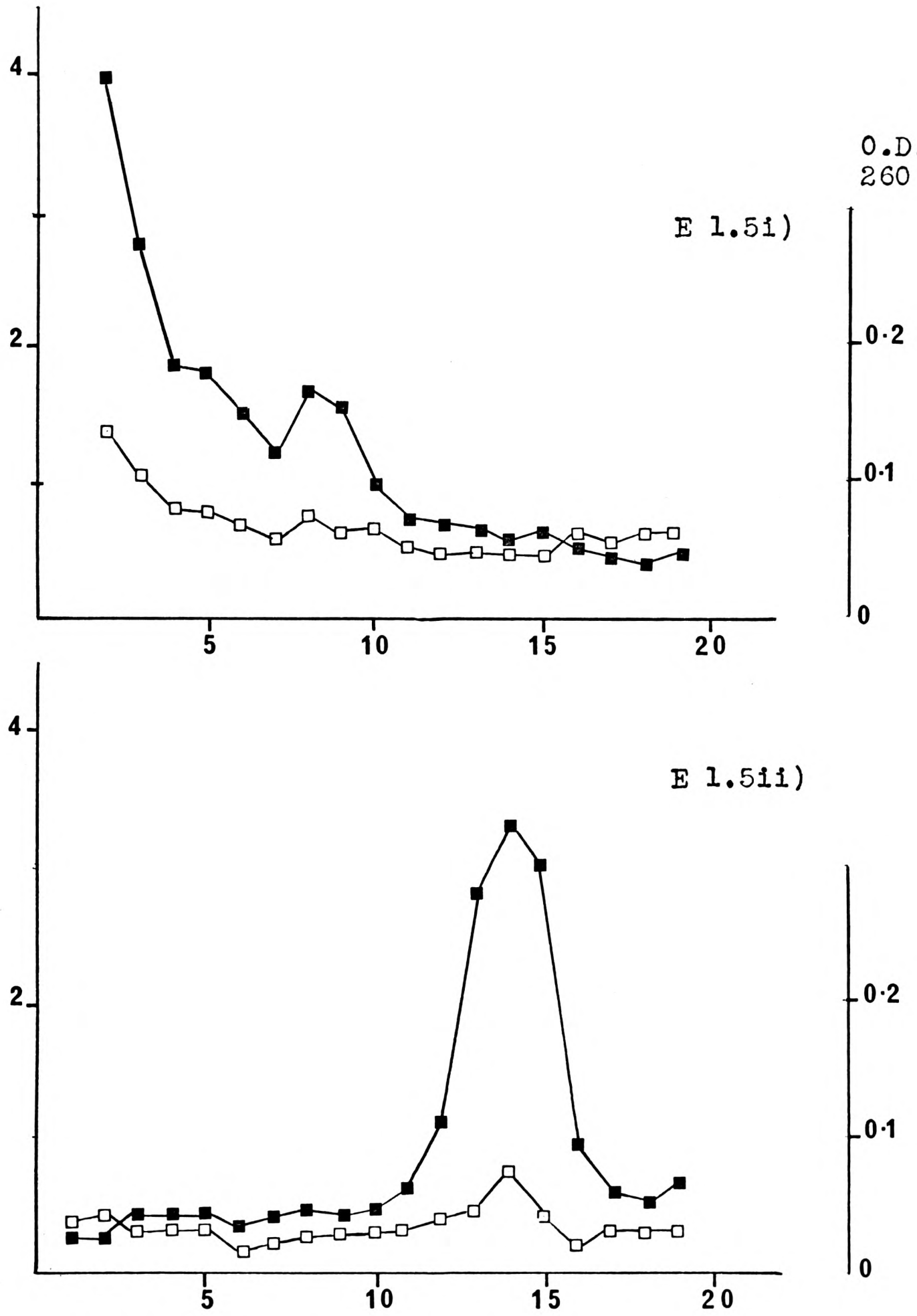


Fig. E 1.5. The preparation of P 32 labelled DNA from A.castellanii(Neff). CsCl absorbance (□) and activity (■) profiles of unpurified (i) and purified(ii) DNA.

This experiment was repeated, but the DNA was subjected to the purification procedure as in E1.2i) but adapted to suit the very small volume of the sample.

The crude DNA was incubated with 1mg/ml pronase for 30 minutes at 60°C then precipitated with ethanol. The precipitate was redissolved in 1ml of SSC. RNAase was added to a final concentration of 100µg/ml and the solution was incubated for 2 hours at 37°C. The RNAase digestion was stopped by the addition of 1mg of dry pronase followed by a further 1 hour incubation at 37°C. The solution was dialysed against 2 litres of 0.01M Tris for 48 hours at 4°C, then centrifuged on CsCl as before. The gradient was fractionated and the absorbance and activity of each fraction were measured as described. The results are shown in Fig. E1.5ii).

Results.

The amount of DNA recovered from the radio-active amoebae was too low to be detected by its absorbance at 260 mµ. The profiles obtained from measurements of radio-activity in the fractions in both experiments were very similar to the absorbance profiles obtained with purified and impure DNA in experiments E1.i) and ii). The extraction and purification techniques used in this experiment were effective in producing high specific activity DNA from A.castellanii Neff strain, and were adopted as the standard preparative procedure.

E1.4 Hydroxyapatite (HAP) fractionation of DNA.

At certain phosphate buffer concentrations single stranded DNA is not adsorbed to HAP, whereas native DNA remains firmly bound. As it was intended to use this property of HAP to analyse DNA hybridisation mixtures, the conditions were determined for the release from HAP of single stranded amoebal DNA.

E1.4.i) Stepwise elution of single stranded DNA from HAP.

a) Preparation of radio-active DNA.

Amoebae were grown in two 30ml shaken cultures containing 1mCi of 32P orthophosphate as described. A 1ml pellet of amoebae was obtained and the DNA from this pellet was extracted and purified as described in experiment 3ii) above. The DNA was fractionated on a CsCl gradient and the activity in each fraction was determined by Cerenkov counting. The profile obtained is shown in Fig. E1.6. The fractions containing the nuclear and mitochondrial DNA were collected as indicated in the Figure.

The nuclear DNA fractions were pooled, diluted to 10ml and centrifuged at 35,000 rpm and 25°C for 16 hours. The pelleted DNA was redissolved in 1ml of x2 SSC and sonicated for 20 seconds at 0°C as described (M7.b). The solution was then dialysed for 24 hours against 2 litres of 0.06M phosphate buffer at 4°C.

0.5ml of the sonicated DNA solution in 0.06M buffer was diluted with 1ml of 0.06M buffer, heated for 10 minutes at 100°C and then quenched in iced water.

b) Preparation of the HAP column.

The HAP suspension used was prepared exactly as described in section M6.1. 1ml of the solution of denatured DNA was added to 9ml of HAP suspension and stirred for 15 minutes at room temperature. The slurry was poured into a 1cm glass water-jacketed column fitted with a sintered plate and closed at the bottom with a stop-cock. Water was circulated through the jacket to maintain the column at 60°C.

c) Elution procedure.

A series of phosphate buffer solutions was prepared rising in 0.015M steps from 0.06M to 0.24M, pH 6.8 - 7.0. Once the column had equilibrated at 60°C the supernatant fraction was drained off, down to the top of the HAP and was

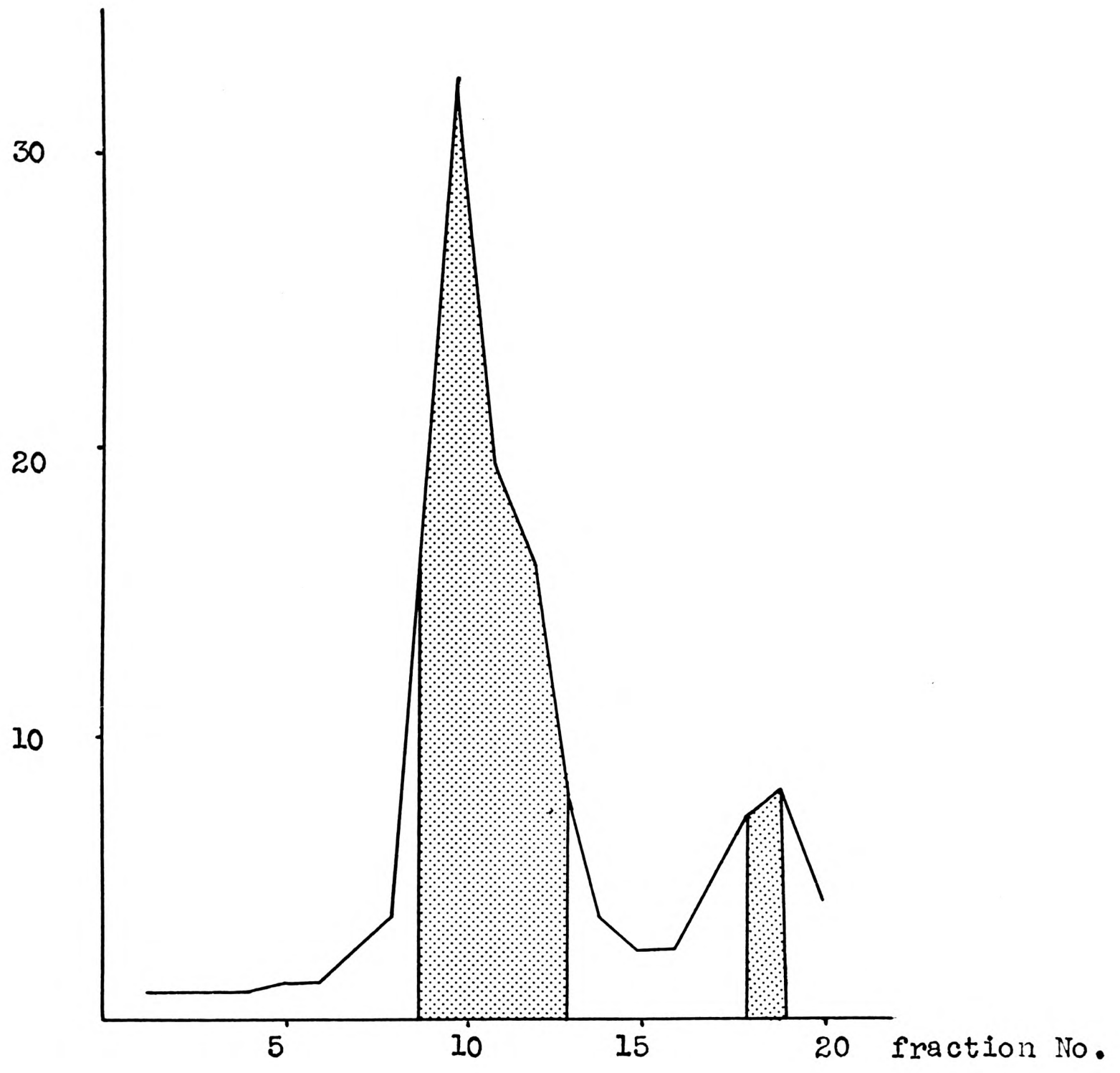


Fig. E 1.6. Preparation of P 32 labelled DNA from A.castellanii (Neff), activity profile from CsCl gradient. Shaded areas represent the DNA containing fractions collected.

replaced with 10ml of 0.06M buffer at 60°C. A few minutes were allowed for the buffer to equilibrate then it was run off into a scintillation vial. The column was washed in the same way using two 5ml washes of 0.06M buffer. The washings were collected together in a second scintillation vial. This procedure was repeated for each of the phosphate buffer solutions.

The activity eluted in each sample was determined by Cerenkov counting; the results are shown in Fig. E1.7.

Results.

Most of the single stranded DNA was eluted from HAP at phosphate buffer concentrations between 0.09M and 0.135M. This suggested that washing with 0.15M buffer would be effective in eluting single stranded DNA from HAP at 60°C.

E1.4.ii) Elution of single stranded and native DNA from HAP at selected buffer concentrations.

a) The DNA samples.

The remaining 0.5ml of the denatured DNA solution prepared for experiment 4i) above was used as the single stranded DNA sample. The native DNA sample was 0.25ml of the sonicated DNA solution in 0.06M buffer prepared for the previous experiment.

b) Preparation of HAP columns.

Both DNA samples were made up to 1ml with 0.06M buffer, then were adsorbed on to HAP and were packed into columns exactly as described in experiment 4i).

c) Elution procedure.

Using the same elution method as before the columns were washed with 0.15M, 0.18M and 0.36M phosphate buffer at 60°C. The activity in the samples

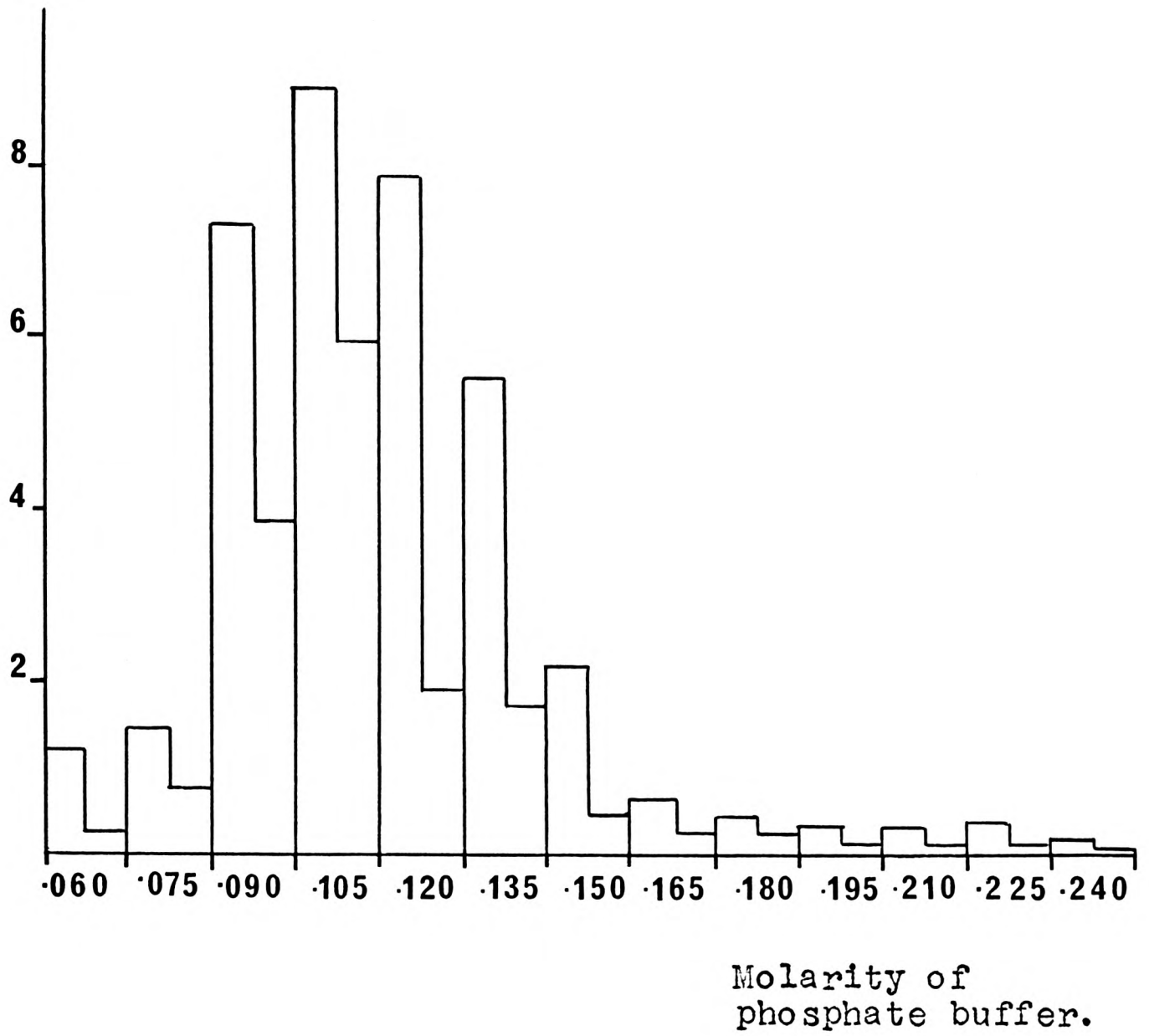


Fig. E 1.7. The elution of denatured DNA from HAP at different phosphate buffer concentrations.

was determined by Cerenkov counting and the total activity released from the columns at each buffer concentration was recorded.

Results.

The percentage of the total activity released by the different buffer concentrations are shown in Table E1.2.

Table E1.2

	% 32P eluted at -		
DNA sample.	0.15M PB	0.18M PB	0.36M PB
single stranded	82	12	6
double stranded	10	6	83

Single stranded DNA was eluted from HAP at 60°C by washing with 0.15M and 0.18M phosphate buffer.

Double stranded DNA remained bound to HAP at buffer concentrations up to 0.18M, there was however a proportion of this DNA that eluted at a buffer concentration of 0.15M. Whether this was due to breakdown of the DNA during preparation or fractionation was not clear.

E1.4.iii) Thermal denaturation of nuclear and mitochondrial DNA on HAP columns.

a) Nuclear DNA.

The nuclear DNA sample used was from the remaining 0.2ml of the sonicated native DNA in 0.06M buffer prepared in 4.i). The sample was adsorbed on to HAP as described in M6.3b) and poured into a column. The HAP was washed with 10ml or 0.12M buffer, then the column was filled with fresh 0.12M buffer and heated up to 85°C. A reservoir of 500ml of the same buffer was connected to the column and the buffer was pumped through with a peristaltic pump at a

flow rate of 20ml per hour. At the same time the thermostat of the heating water pump was geared to an electric clock motor to give a constant temperature rise of 5.5°C per hour. The eluant was collected in 2ml fractions using an LKB fraction collector and siphon assembly adapted to hold scintillation vials.

The system was run for 2 hours by which time the temperature of the heating water had reached 96°C. The activity in each fraction was determined by Cerenkov counting.

b) Mitochondrial DNA.

The mitochondrial DNA was obtained from the 2 fractions collected from the preparative gradient shown in Fig. E1.6. The fractions were pooled and were layered directly on to a Sephadex G-200 column and eluted with 0.06M buffer. 2ml fractions were collected using the modified LKB assembly described above. The activity in each fraction was determined and the profile is shown in Fig. E1.8. The fractions indicated in figure were collected and pooled.

The pooled sample was added to 10ml of HAP suspension plus 1.7ml of 0.36M buffer and stirred for 15 minutes. The slurry was poured into the column and prepared for denaturation in exactly the same manner as the nuclear DNA sample except that the column was heated to a starting temperature of 75°C.

Using the same temperature control, elution and fraction collecting systems as before, the sample was heated from 75 - 90°C. The activity in each fraction was determined in the usual way.

Results.

The activity eluted in each fraction was expressed as a cumulative percentage of the total activity eluted during each experiment. The resulting

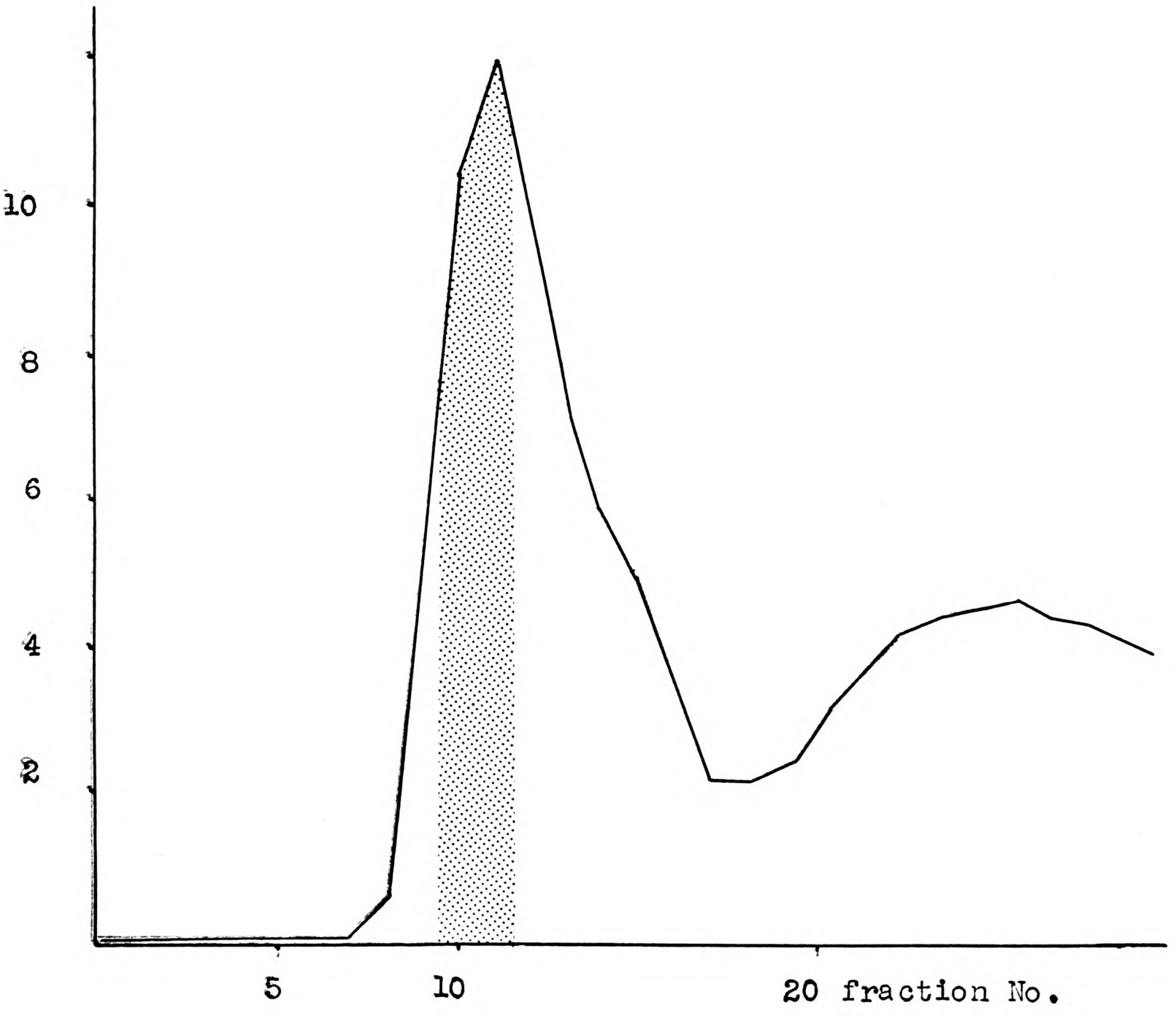


Fig. E 1.8. Sephadex gel filtration of radio-active mitochondrial DNA. The shaded area represents the fractions collected.

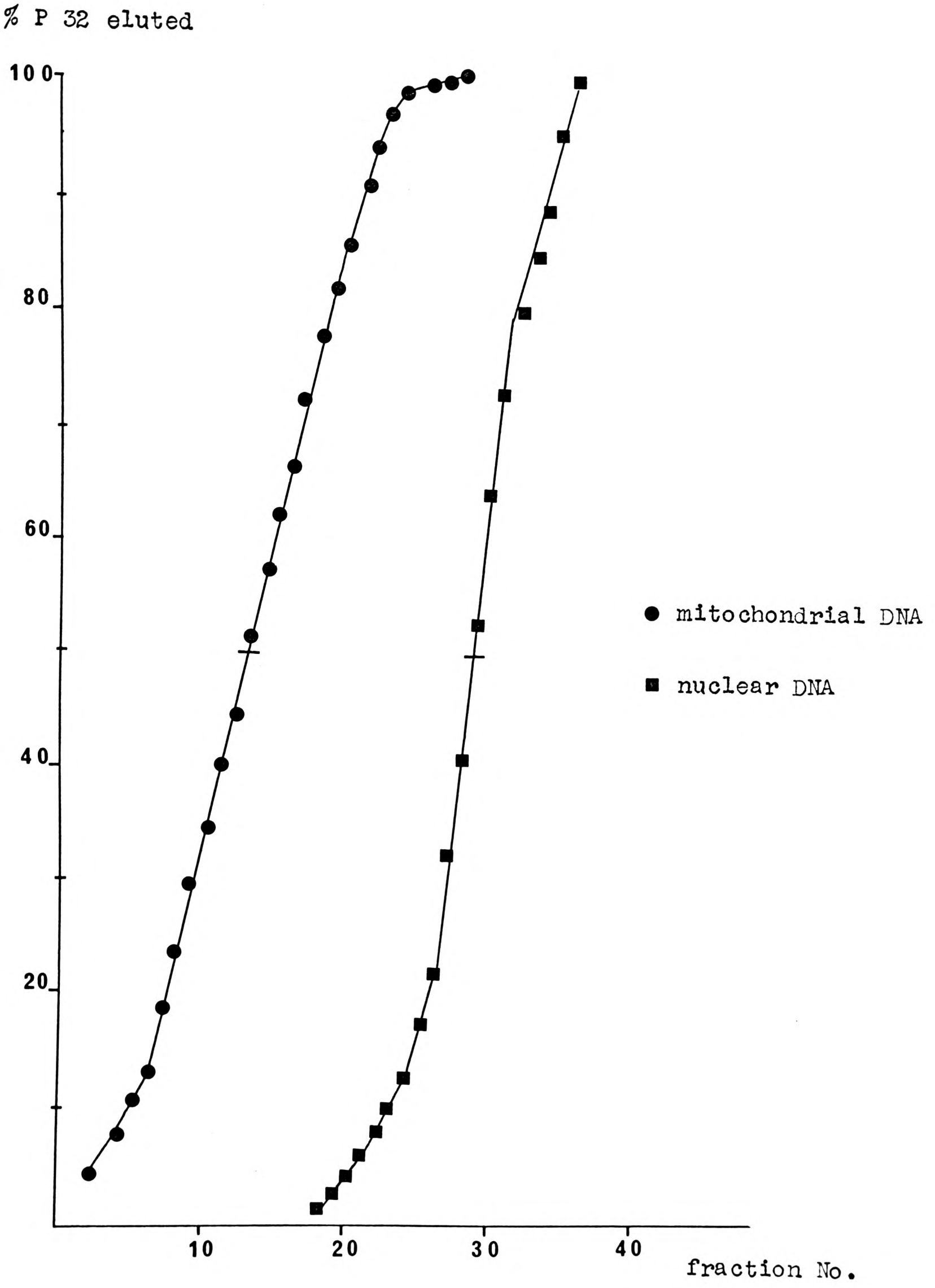


Fig. E 1.9. The thermal denaturation of nuclear and mitochondrial DNA on HAP columns.

denaturation curves are shown in Fig. E1.9. Both samples denatured, showing that the radio-active material which banded on the preparative gradients was DNA. The Tm for the mitochondrial sample, 82°C, was in good agreement with the base composition of this DNA (34% GC). The nuclear DNA had a Tm of 90°C, a value some 5°C lower than expected, but this low value was due to the inability of the water heating system to raise the temperature of the column above 96°C.

Section E2. Characterisation of Acanthamoeba DNA

The DNA base composition was determined of 6 strains or species of Acanthamoeba. Denatured Neff strain nuclear DNA was prepared for use as a reference DNA in these determinations.

a) Preparation and calibration of denatured Neff strain DNA.

DNA was extracted by method I and purified on a CsCl gradient. The high absorbance fractions of the major peak were collected, diluted precipitated with ethanol and redissolved in 1/10th SSC. The DNA was denatured by heating for 10 minutes at 100°C, and was then quenched by immersing the tube in iced water. The solution was dialysed against 2 litres of 0.01M Tris for 18 hours at 4°C, after dialysis the solution was made up to 3.4ml with 0.01M Tris. Caesium chloride was added to give a solution with a density of 1.740g/cm^3 and the solution was centrifuged for 60 hours at 35,000 rpm and 25°C. The gradient was fractionated and the absorbance at 260mμ of each fraction was measured. The absorbance profile is shown in Fig.E2.1.

The fractions of high absorbance were pooled, 2.7 grams of CsCl was dissolved in 2ml of purified native, Neff strain DNA in 0.01M Tris (at a concentration of 5μg/ml). 0.075ml of the denatured DNA solution was added and a sample of the mixed solutions was centrifuged in a Beckman Model E analytical ultracentrifuge. A densitometer tracing of the uv photograph obtained is shown in Fig.E2.2. Neff strain nuclear DNA has a buoyant density of 1.720g/cm^3 relative to E.coli DNA (1.710g/cm^3), (Adam et al 1969). From its relative banding position, the buoyant density of the deantured DNA was calculated to be 1.735g/cm^3.

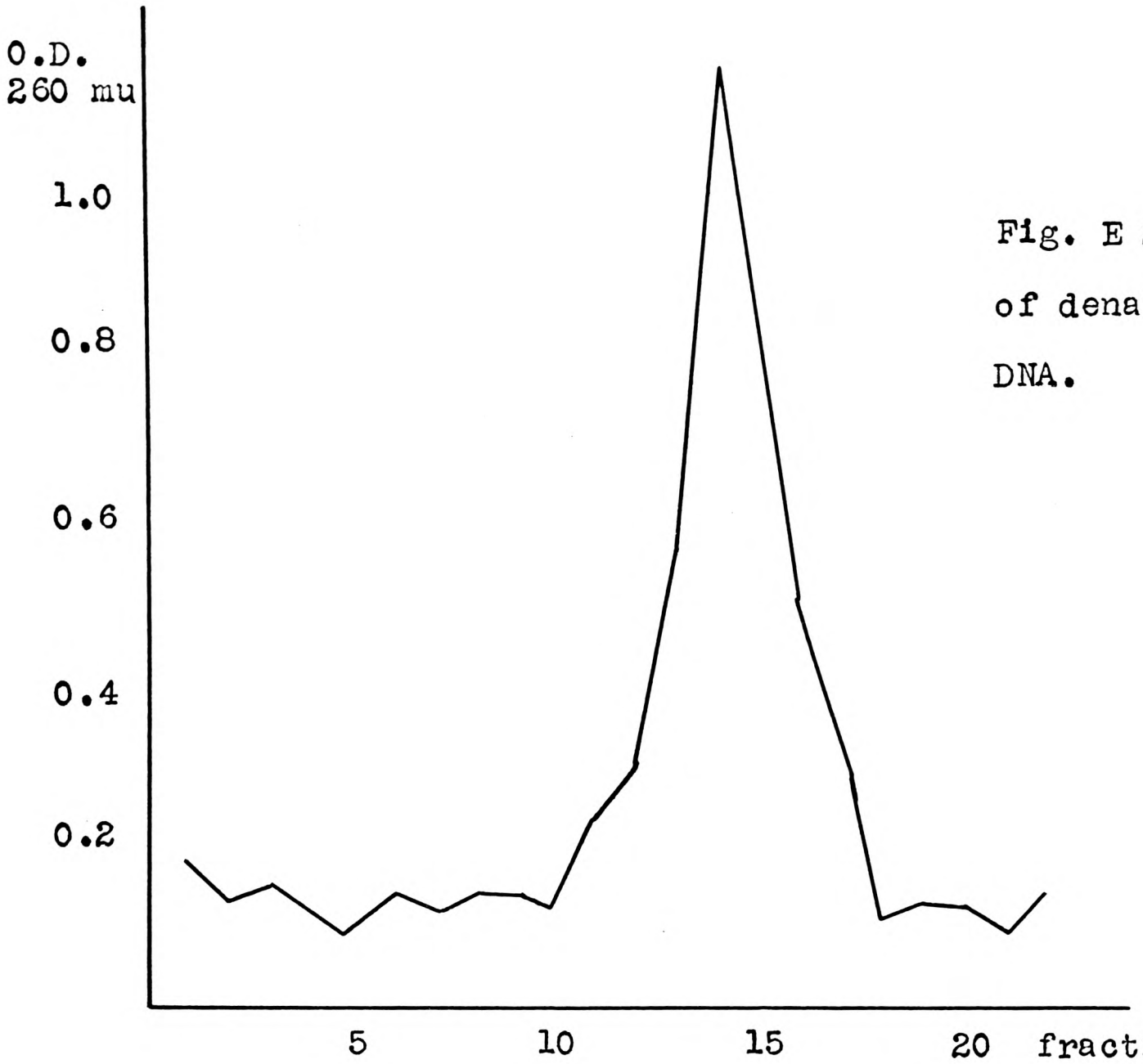


Fig. E 2.2 Preparation of denatured nuclear DNA.

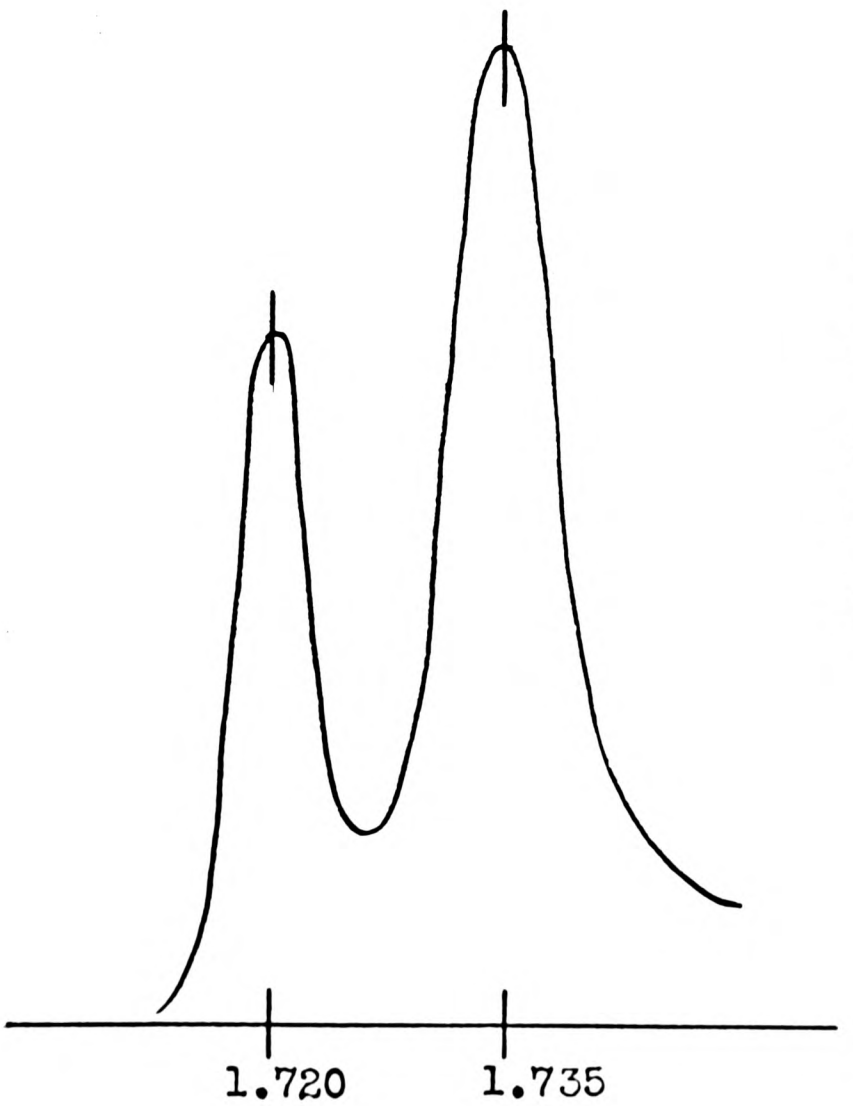


Fig. E 2.2. Analytical ultracentrifugation of A.castellanii (Neff) native and denatured DNA.

b) The base composition of Acanthamoeba DNAs.

DNA from 6 strains or species of Acanthamoeba was extracted and purified by method I and characterised by analytical ultracentrifugation as described in Section M10. The results obtained are shown in Table E2.1 and Fig. E2.3.

Table E2.1 Buoyant densities and base composition of Acanthamoeba DNAs.

Amoeba	Buoyant density		% GC	
A.castellanii	major DNA	minor DNA	major DNA	minor DNA
Singh strain	1.720	1.693	61	34
W1 strain	1.720	1.693	61	34
P27 strain	1.720	1.693	61	34
Isolates "				
A1	1.715	1.697	56	37
A35	1.720	1.693	61	34
A30	1.720	1.693	61	34

" strains from other laboratories identified only as Acanthamoeba sp.

The DNA of six different acanthamoebae was characterised, of these six strains, three (Singh, W1 and P27) are considered to be strains of A.castellanii (Page 1967, Adam 1964). The results of the DNA base composition analysis support the inclusion of these three strains within a single species. Isolate A1 has been shown to be serologically distinct from the Neff, W1 and Singh strains of A.castellanii (Adam 1964) and its different DNA base composition confirms its specific status. The GC contents of the DNA of the A30 and A35 strains indicate that these amoebae ~~should~~ could be included in the species A.castellanii, but identity in base composition does not prove synonymity and the specific status of these strains requires further investigation.

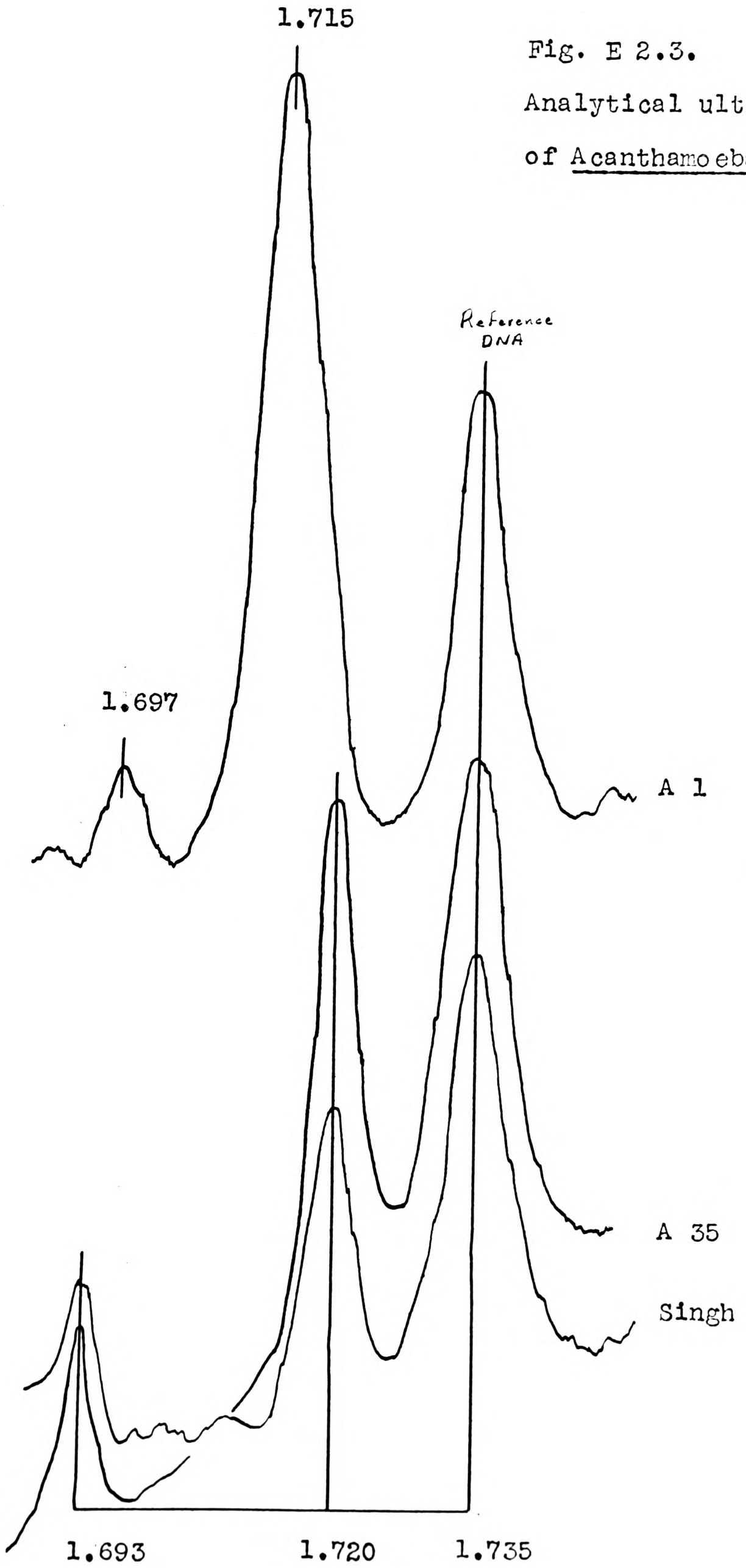


Fig. E 2.3.

Analytical ultracentrifugation of Acanthamoeba DNA.

Section E3 Renaturation of Acanthamoeba DNA.

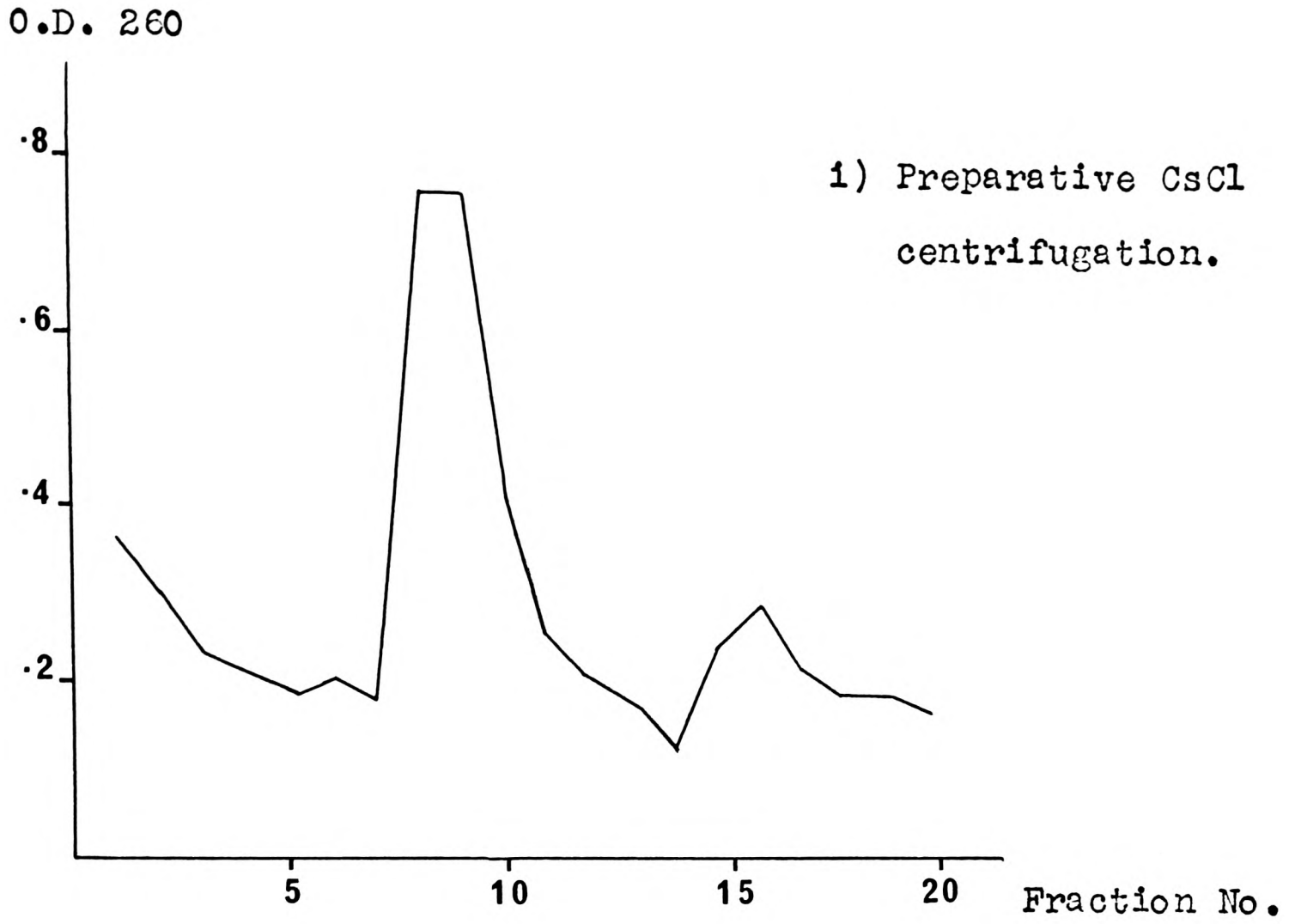
DNA-DNA hybrids are formed by the renaturation of heterologous DNA's. Before attempting hybridisation studies it was necessary to establish conditions for achieving a high percentage renaturation of homologous DNA. Experiments were therefore carried out to examine the renaturation rate of Acanthamoeba DNA and to determine how far the renaturation reaction would proceed.

E3.1 Renaturation in the spectrophotometer.

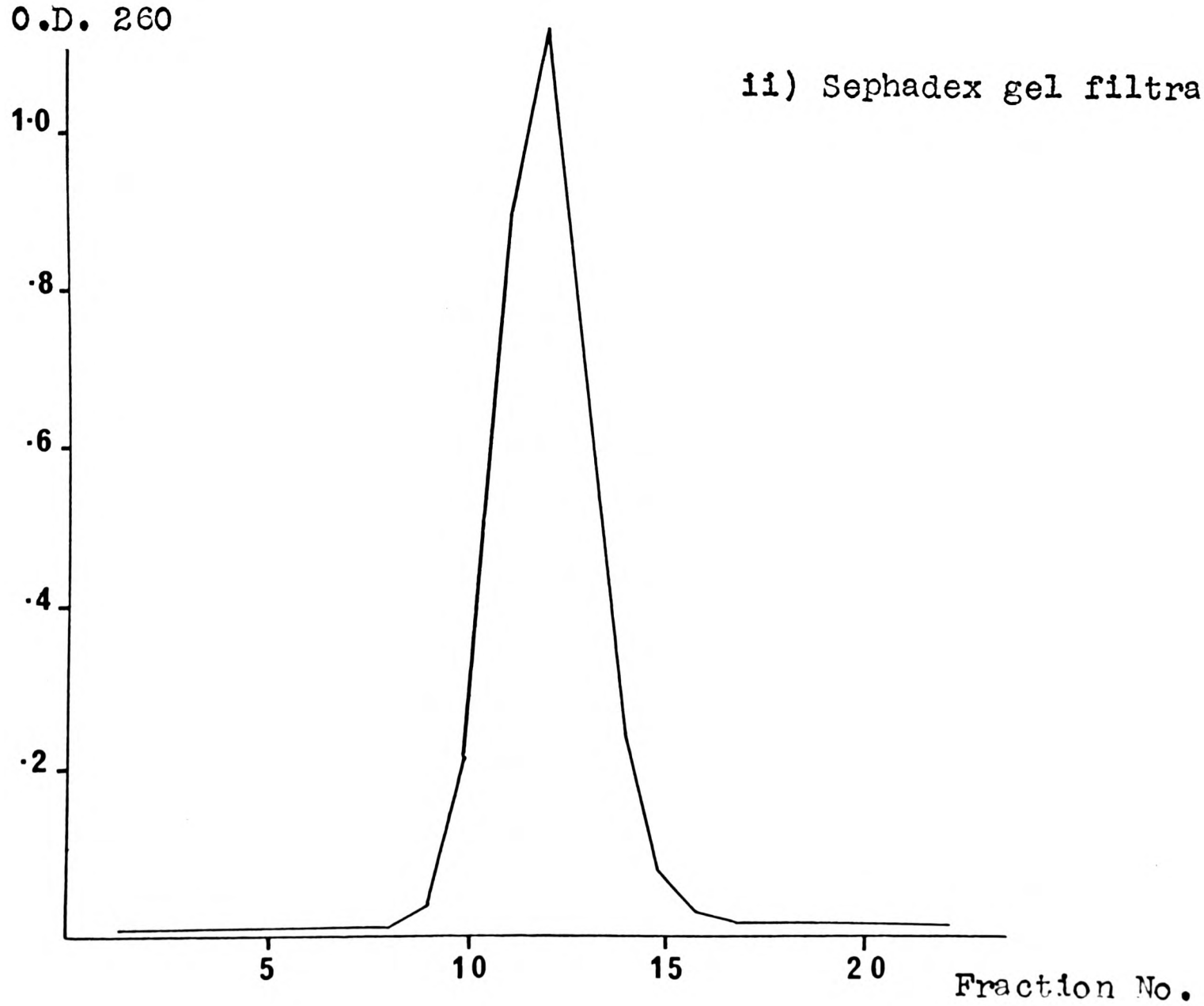
The simplest technique for monitoring the progress of renaturation is to follow the decrease in absorbance at 260mμ of a sample of denatured DNA incubated at 60°C in a spectrophotometer. Accordingly, this technique was used for the first experiments to determine the rate and extent of renaturation of Acanthamoeba DNA.

The DNA used in the first series of experiments was prepared by Method I (M4.2a) and was sonicated as described in M7b). The sonicated DNA was transferred to 0.1M NaCl 0.01M Na cacodylate buffer by filtration through Sephadex G-200(M5).

2ml of DNA solution in NaCl-Na cacodylate buffer at a concentration of 30μg DNA/ml, was heated for 5 minutes in a boiling water bath. The solution was then immediately transferred to a 1cm silica cell already in position in the sample compartment of a Zeiss spectrophotometer fitted with a water-jacketed cell holder maintained at 60°C. A matched reference cell containing boiled buffer at 60°C was also positioned in the sample compartment. The absorbance of the DNA at 260mμ was measured immediately and at selected intervals for up to 18 hours.



i) Preparative CsCl centrifugation.



ii) Sephadex gel filtration.

Fig. E 3.1. The preparation of DNA for renaturation experiments.

The data were treated by the method of Wetmur and Davidson (1968). The reciprocal of the absorbance due to the hyperchromicity of the remaining denatured DNA was plotted against time to give the renaturation rate of the DNA.

The percentage renaturation in the DNA samples was calculated by expressing the fall in absorbance over a given time as a percentage of the difference in absorbance between the fully denatured and native DNA.

Experiment I. Renaturation of Neff strain DNA.

DNA was prepared from 10 grams of amoebae as described. The absorbance profiles of the CsCl gradient and Sephadex gel filtration procedures are shown in Fig. E3.1.

2ml samples of the DNA solution were denatured and allowed to renature in the spectrophotometer as described above. The test was repeated 3 times on samples from the same DNA preparation. The results are shown in Table E3.1 and Fig. E3.2.

Results.

Fig. E3.2 shows that there was considerable variation in the results obtained from replicate samples. There was no evidence of continuous renaturation during the experiment, but the fall in absorbance during the first 10 minutes of incubation indicated the presence of a rapidly re-associating fraction.

Table E3.1

OD 260 native DNA	denat DNA	hyper-chromicity	% renat'd 10 mins	% renat'd final	duration expt
0.600	0.815	1.36	6.9	12.2	6hrs
0.600	0.795	1.33	9.9	6.6	4hrs
0.600	0.809	1.36	9.3	7.1	4hrs

DNA concentration 30ug/ml

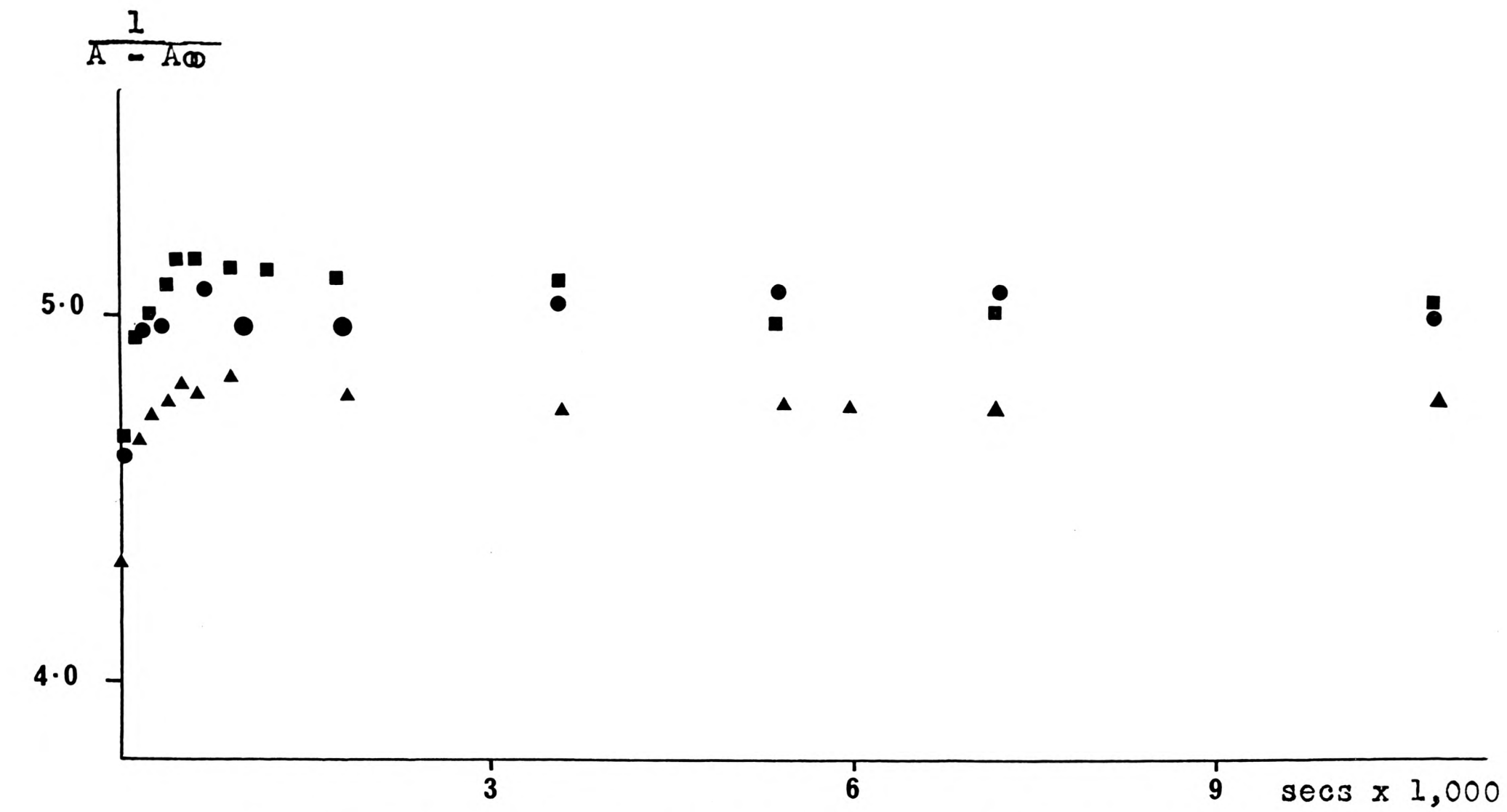


Fig. E 3.2 Renaturation of A.castellanii (Neff) DNA.

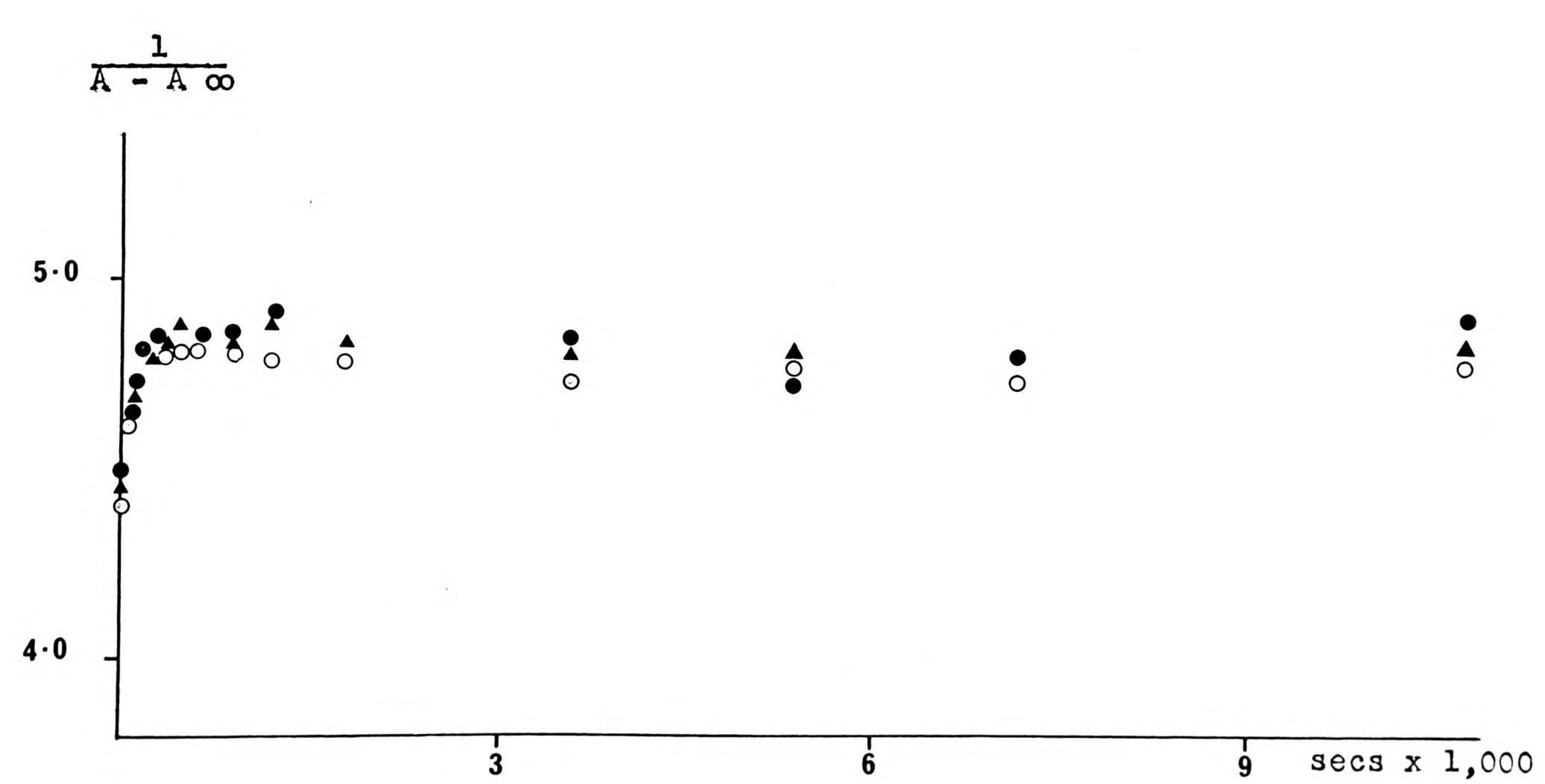


Fig. E 3.3 Renaturation of A.castellanii (Neff) DNA.

Experiment II. Effect of further purification on the renaturation of Neff strain DNA.

It was possible that the variable results obtained in experiment I were due to impurities in the DNA. To test this, the experiment was repeated using more highly purified DNA.

DNA was extracted from 10 grams of amoebae using Method I and was subjected to a 2nd cycle on preparative CsCl gradients before sonication and Sephadex gel filtration. 2ml samples of this DNA preparation were renatured in the spectrophotometer as before. The results of 3 tests are shown in Fig. E3.3 and Table E3.2.

Results.

More consistent results were obtained using highly purified DNA, but there was still no evidence of continuous renaturation.

Table E3.2.

OD 260 native DNA	OD 260 denat DNA	hyper-chromicity	% renat'd 10 mins	% renat'd final	duration expt
0.622	0.857	1.37	8.4	2.0	4hr
0.622	0.847	1.36	7.1	5.1	4hr
0.622	0.834	1.34	8.1	8.0	4hr

As in experiment I there was a small rapidly reassociating fraction, comprising 7 - 8% of the DNA, which renatured in the first 10 minutes incubation.

Experiment III. Renaturation of Neff strain DNA over longer periods.

In experiments I and II the DNA was incubated for periods of 4 or 6 hours. Apart from the rapidly reassociating fraction there was no indication that the DNA had renatured during this time. The experiments were therefore repeated using much longer incubation times.

Fig. E 3.4. Renaturation of A.castellanii DNA.

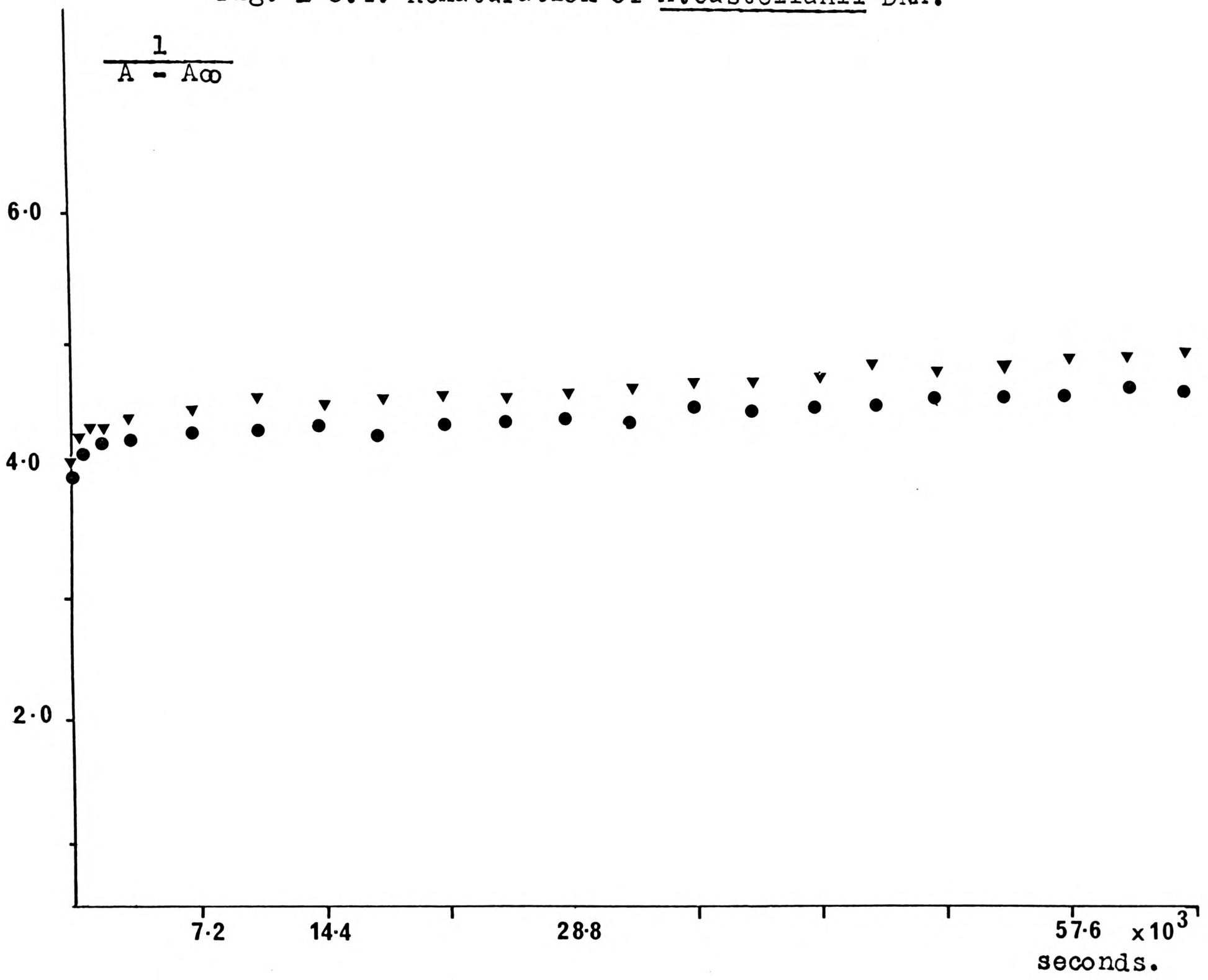


Table E 3.4

O.D. 260 native DNA	O.D. 260 denat DNA	hyper-chromicity	% renat'd 10 mins	% renat'd final	duration expt.
0.630	0.889	1.36	4	14	18 hours
0.630	0.890	1.36	5	12	18 hours

DNA was prepared from 15 grams of Neff strain amoebae exactly as described above. 2ml samples of this DNA, at a concentration of 33µg/ml in NaCl-Na cacodylate buffer, were renatured in the spectrophotometer for 18 hours. The results of 2 experiments are shown in Fig. E3.4 and Table E3.3.

Results.

In common with other renaturation experiments a small proportion of the DNA renatured very rapidly in the first few minutes of incubation. Throughout the incubation period there was a very slow renaturation, and after 18 hours 12 - 14% of the DNA had reassociated.

The renaturation rate of DNA is increased if the DNA concentration and ionic strength of the medium are increased. DNA solutions at concentrations significantly higher than those used in the renaturation experiments would have absorbance values beyond the optimum range on the spectrophotometer scale. Renaturation under relaxed conditions, such as high salt concentrations, would probably increase the percentage of DNA which reassociated but a large proportion of this DNA might prove to be unstable in the low ionic strength buffers required for HAP fractionation. For this reason it was thought inadvisable to increase the ionic strength during renaturation and it was concluded that alternative methods must be sought for studying the renaturation of the amoeba DNA.

E3.2 The renaturation of amoeba DNA at high concentrations.

The results of the experiments just described suggested that up to 90% of the DNA of the amoebae might be single copy DNA. Under the conditions chosen for renaturation DNA of this type will only reassociate after prolonged

incubation at high concentrations. The spectrophotometric technique was not suitable for these conditions and a method was therefore developed for preparing concentrated DNA solutions, for incubating them over long periods and for analysing the reaction mixture. The method which was adopted, the capillary tube renaturation technique, is described in Section M7. Experiments I and II below were designed to test the method and to compare techniques for analysing the reaction mixture. The DNA used in both experiments was taken from a single batch extracted from 28 grams of amoebae using method II, and was prepared for capillary tube renaturation exactly as described in Section M7.

Experiment I: The estimation of percentage renaturation from the hypochromicity of diluted samples.

Sealed tubes containing 0.05ml of DNA solution (DNA concentration 450µg/ml in SSC) were incubated at 60°C for 0; 0.5; 1.0; 3.0; 18; 27; 43; and 51 hours before sampling. The samples were prepared for analysis as follows. A clean dry, spectrophotometer cell containing 1ml of 1/10th SSC was weighed to 5-places. The sample tube was broken open and the solution withdrawn with a fine pipette. Without touching the spectrophotometer cell, which was still in position on the balance pan, the DNA solution was dropped directly on to the surface of the 1/10th SSC in the cell. The cell was then reweighed stoppered and transferred to the heated sample compartment of a Unicam SP 500 spectrophotometer. The cell was left in the sample compartment at 60°C for 15 - 20 minutes, then the absorbance at 260mµ was measured against a blank of 1/10th SSC. The results are shown in Table E3.4.

Table E3.4

Time hrs.	Cell wt. g	Cell wt and DNA g	DNA soln. wt g	OD 260 60°C	undiluted OD 260	% renat'd
0	6.64388	6.66988	0.02600	0.275	10.57	0
0.5	6.64070	6.68837	0.04767	0.445	10.0	20
1.0	6.63750	6.67000	0.03250	0.300	9.5	38
3.0	6.63758	6.67595	0.03837	0.357	9.7	31
18	6.63590	6.68227	0.04637	0.405	9.1	52
27	6.63802	6.67617	0.03815	0.338	9.2	48
43	6.63919	6.67931	0.04012	0.361	9.3	45
51	6.63839	6.68349	0.04510	0.390	9.0	56

The absorbance of the samples before dilution was calculated by multiplying the absorbance at 60°C by the appropriate dilution factor. The percentage renaturation was calculated by expressing the hypochromicity of a given sample as a percentage of the hypochromicity were the time 0 sample to be 100% reassociated.

Experiment II: The estimation of percentage renaturation from the hyperchromicity of diluted samples.

Sealed tubes of DNA solution were prepared exactly as before and incubated for 0; 0.5; 1.5; 3.5; 7.5; 15.5 and 40 hours. The tubes were sampled as before except that the solution was dropped into 1ml of 1/10th SSC in small siliconised glass tubes which were weighed before and after the addition of SSC as well as after the addition of the DNA.

The diluted samples were transferred to stoppered spectrophotometer cells and the DNA was denatured by heating slowly up to 95°C. The absorbance at 260mu was measured at 20, 60 and 70°C and then at 5°C steps up to 95°C.

The percentage reassociation was calculated by expressing the hyperchromicity of the solution between 60 and 95°C as a percentage of the calculated maximum hyperchromicity for that solution. The results of the experiment are shown in Fig. E3.5 and Table E3.5.

Table E3.5

Time hrs.	Wt. SSC g	Wt. DNA g	Dilut'n	OD 260 dil.	OD 260 orig'n	% reassociated OD	Tm
0.0	1.00938	0.05003	20.58	0.494	10.33	0	15
0.5	0.99140	0.05052	20.21	0.457	9.23	40	20
1.5	0.99488	0.04391	23.21	0.411	9.54	29	25
3.5	0.99522	0.04781	21.36	0.443	9.46	32	38
7.5	1.00404	0.04651	22.04	0.441	9.72	22	39
15.5	1.00207	0.04757	21.54	0.429	9.24	39	42
40	1.00865	0.04329	23.70	0.399	9.45	32	45

Results of Experiments I and II.

Direct measurements of absorbance at 260mμ and 60°C of carefully diluted samples of the reaction mixture did not prove to be a satisfactory method for estimating the percentage reassociation. This was probably due to a multiplication of errors incurred in measuring the absorbance of the samples and in determining their dilution.

The use of restoration of hyperchromicity as a measure of reassociation was more successful. This method was less susceptible to the errors noted above, since the measurements which were taken were relative not absolute. But, because the dilution technique was not precise, it was difficult to determine the original abosrbance of the native DNA in each sample, this was therefore calculated from the hyperchromicity factor (1.36) and the absorbance of the fully denatured DNA (at 95°C in 1/10th SSC).

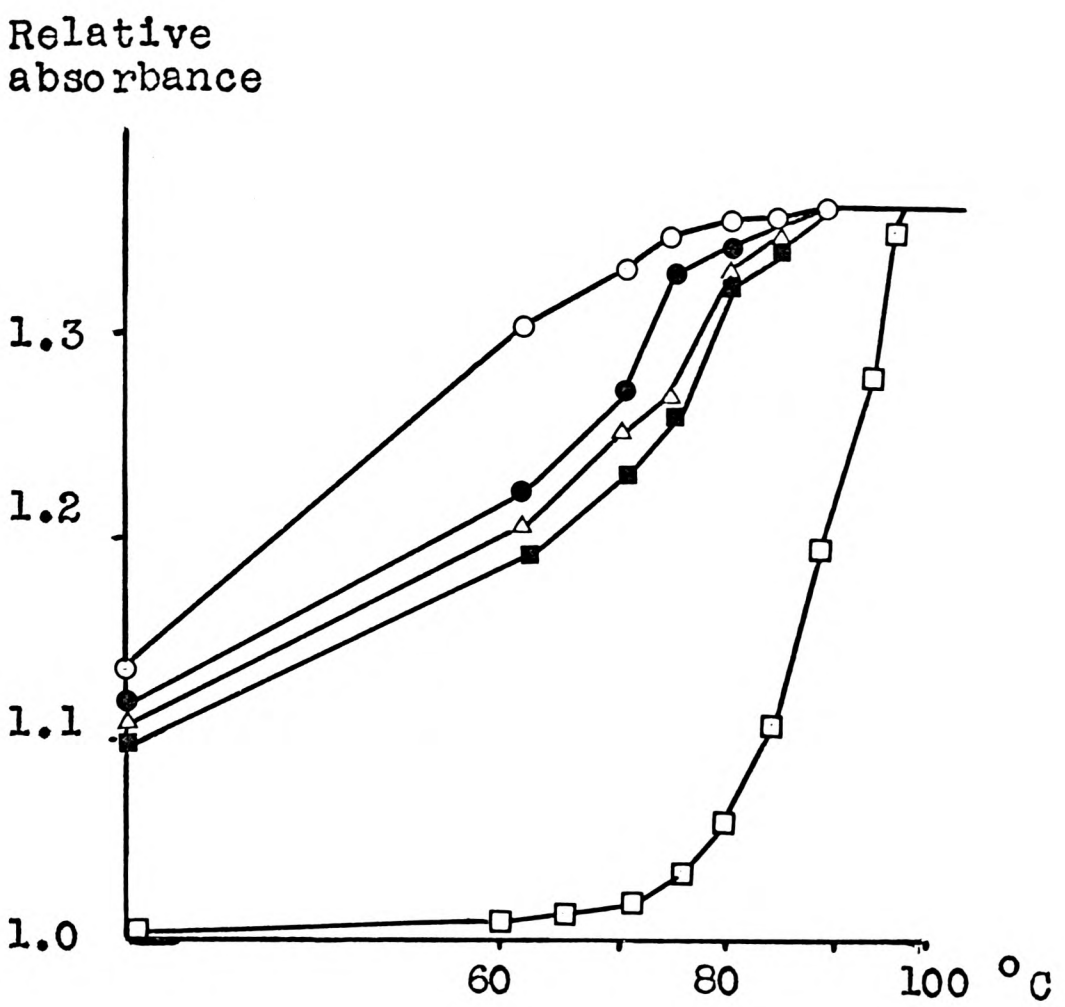


Fig. E 3.5. Estimation of renaturation by the restoration of hyperchromicity. Thermal denaturation of renatured DNA.

A.castellanii(Neff) DNA renatured for ;

0 hours ○
3.5 hours ●
7.5 hours △
40.0 hours ■

(□ native DNA)

E3.3 Hydroxyapatite fractionation of the reaction mixture.

To test the assay technique used in Experiment II a sample of renatured DNA was fractionated on HAP into duplex and single stranded fractions. The denaturation curve of each fraction was compared to those of an unfractionated and a native DNA sample. Tetrahymena DNA was used for this experiment because it was more easily prepared in the high concentrations required.

1.5mg of DNA was extracted from 8 grams of Tetrahymena as described in Section M4.4. An 0.05ml sample was set aside before denaturation and the remainder of the DNA was prepared for renaturation exactly as described. The denatured DNA at a concentration of 1,284µg/ml was sealed into capillary tubes and incubated for 18 hours at 60°C.

The native and the 18 hour renatured DNA samples were diluted in 2ml of 0.012M phosphate buffer and denatured as in Experiment II. The sample to be fractionated was diluted in 2ml of 0.012M buffer at 60°C, then mixed thoroughly with 0.5ml of HAP suspension in a 10ml centrifuge tube. The suspension was centrifuged at 60°C and the supernatant fraction was discarded. The subsequent fractionation was carried out at 60°C as described in Section M6.2. The pellet was subjected to 3 washes each of 0.7ml in 0.12M phosphate buffer and the washings were pooled to form the 0.12M fraction. The DNA still adsorbed to the HAP was removed in a single wash in 0.36M buffer to give the 0.36M fraction.

The 0.36M fraction was diluted with 1.4ml of distilled water to give 2.1ml of solution at a buffer concentration of 0.12M. The 0.12M and 0.36M fractions were then denatured in the spectrophotometer; the thermal denaturation curves obtained from the 4 samples tested are shown in Fig. E3.6.

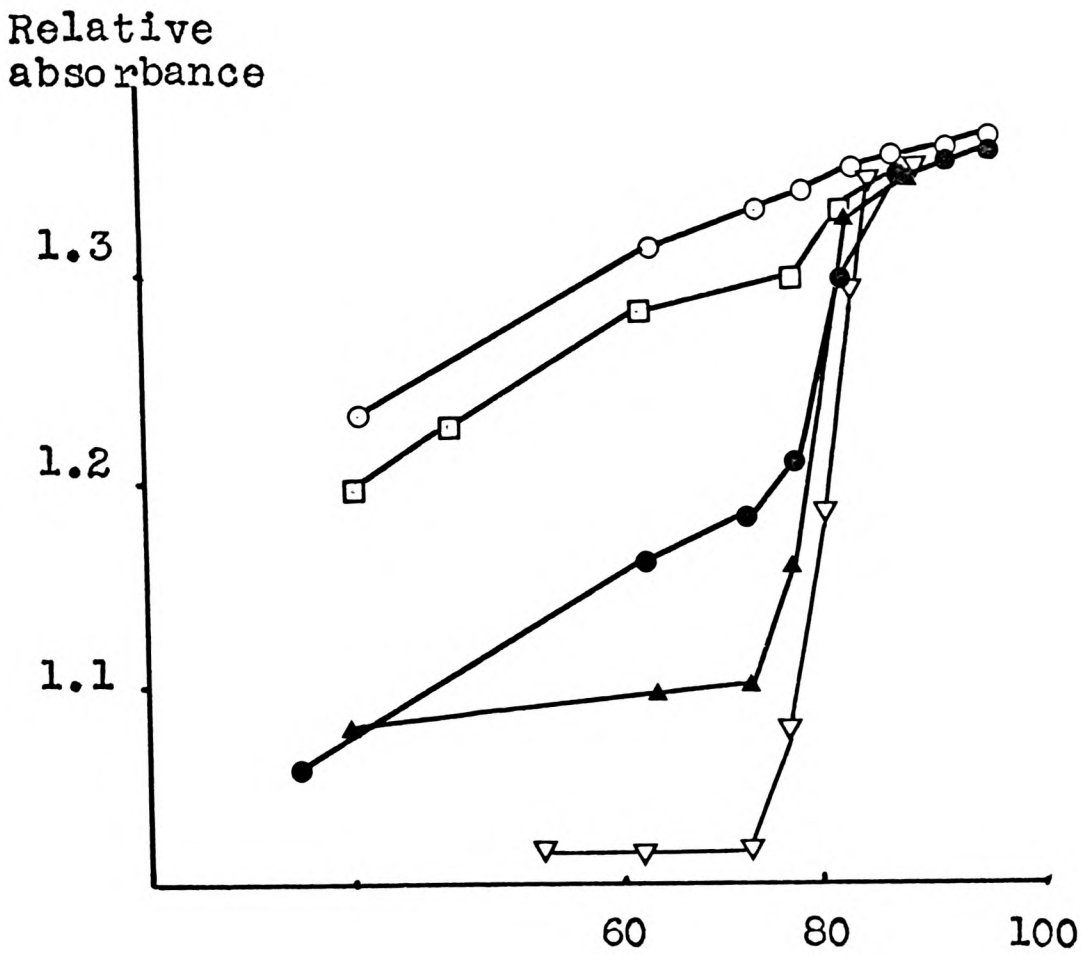


Fig. E 3.6. Hydroxyapatite fractionation of the reaction mixture. Thermal denaturation curves of native, fractionated and renatured Tetrahymena DNA.

□ 0.12 M fraction
▲ 0.36 M fraction
● renatured DNA
▽ native DNA
(○ denatured DNA)

Results.

The 18 hour sample was calculated, from its hyperchromicity, to contain 50% duplex DNA. Using HAP it was possible to select from this sample a fraction of the DNA which had a thermal denaturation curve very similar to that of native DNA. It was calculated that 73% of the DNA in this fraction was double stranded. In contrast, the 0.12M fraction showed an indefinite melting curve and was estimated to contain 22% double stranded DNA. From the absorbance values of the 2 fractions at 260mμ and 95°C it was estimated that 55% of the DNA had eluted in the double stranded fraction. The results of Experiment E3.2II had showed that up to 45% reassociation of amoeba DNA could be achieved by using the capillary tube renaturation technique, and estimating the percentage reassociation from the restoration of hyperchromicity A determination of the percentage reassociation based on HAP fractionation of the reaction mixture gave a value in agreement with that obtained from measurement of hyperchromicity.

E3.3 The renaturation of DNA from A.palestinensis, A.polyphaga and A.castellanii, Neff, H and W1 strains.

The capillary tube renaturation technique had been used successfully to demonstrate the reassociation of Neff strain DNA. To test the technique further it was used to renature the DNA of A.palestinensis, A.polyphaga and the Neff, H and W1 strains of A.castellanii.

Samples of DNA were prepared from the amoebae using method II and were prepared for renaturation exactly as described (M7). The DNA from A.palestinensis was divided into 0.02ml samples, the DNA from the other amoebae was divided into 0.05ml samples. The samples were sealed into capillary tubes and incubated at 60°C for the times indicated in Table E3.6.

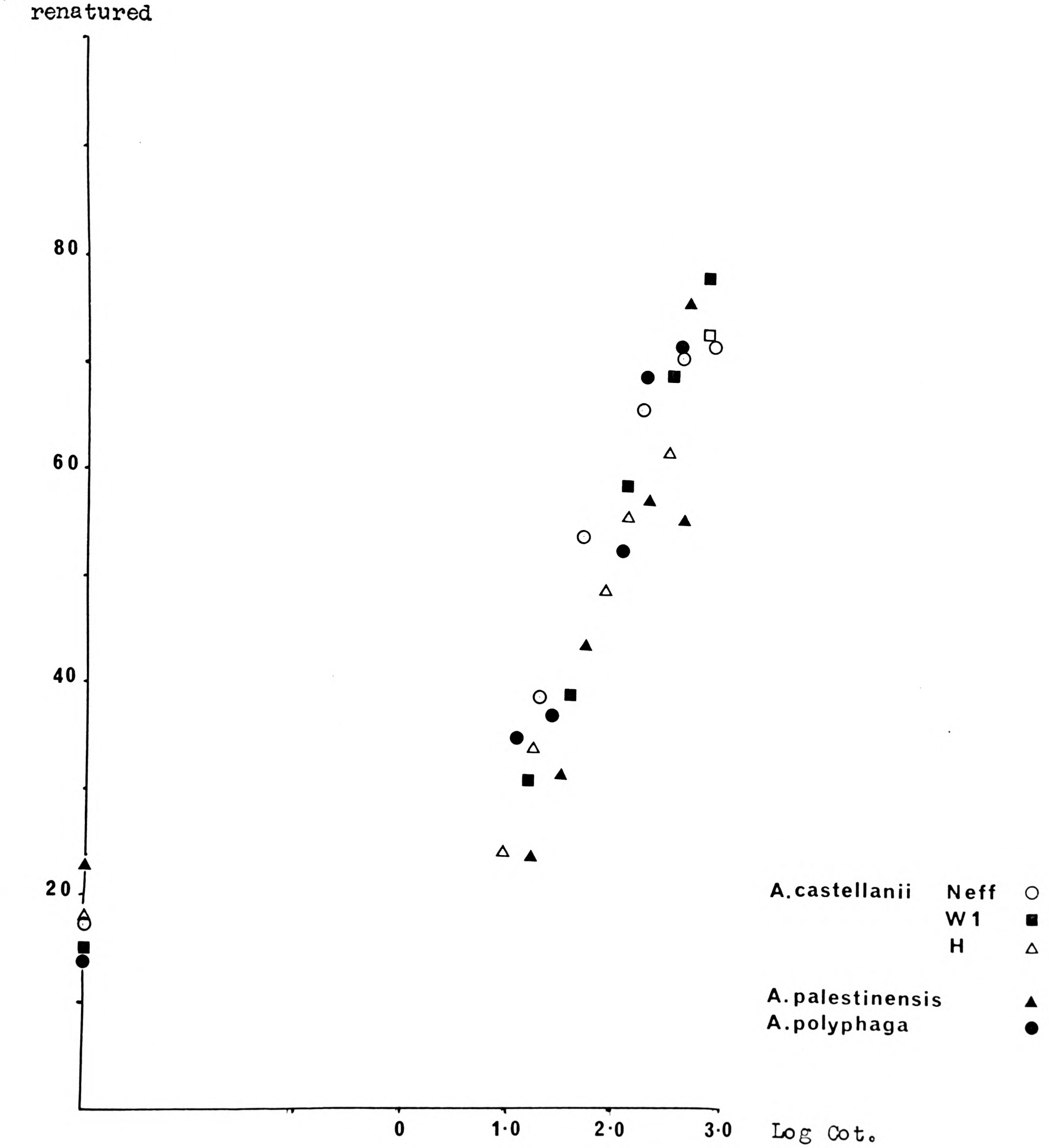


Fig. E 3.7. Renaturation of amoebal DNAs.

At selected times the samples were opened and diluted in 2ml of weighed 1/10th SSC and denatured in the spectrophotometer. The absorbance values were corrected for thermal expansion and the percentage renaturation was calculated from the hyperchromicity. (Table E3.6). The percentage renaturation of each DNA at the selected times was plotted against DNA concentration x time of incubation (Cot) on a logarithmic scale, Fig. E3.7.

Results.

All 5 of the amoebal DNA samples tested showed a high percentage renaturation.

The factor Cot combines the important variables of DNA concentration and time of incubation, and therefore allows direct comparisons of the percentage reassociation of DNA's incubated for different times and at different concentrations. Figure E3.7 shows that for all the amoebae tested the DNA renaturation rates were similar.

The Cot value at which 50% of the DNA is reassociated (Cot $\frac{1}{2}$) is related to the complexity of the DNA (Britten and Kohne 1966). The Cot$\frac{1}{2}$ of the amoebal DNA's tested was between 50 and 100, values consistent with the whole genome forming a single slow renaturing fraction. This supports the tentative conclusion from the spectrophotometric determinations that up to 90% of the DNA of Acanthamoeba was single copy DNA. Since the Cot values of the samples incubated in the spectrophotometer were less than 10, it also explains why this technique was unsuccessful.

Table E 3.6 Denaturation rates of amoebal DNAs.

* OD_{260} of native DNA calculated from

$$\frac{\text{OD 260 at 95° C}}{1.36}$$

** Undiluted OD_{260} of native DNA calculated from

$$\frac{\text{OD 260 at 95° C}}{1.36} \times \text{dilution factor}$$

† Cot calculated from

$$\frac{\text{OD 260 undiluted DNA} \times 50 \times \text{times(secs)}}{330 \times 1{,}000}$$

† Cot values were calculated for DNA of 500,000 molecular weight. High molecular weight DNA is rapidly broken down by sonication to fragments of approximately 500,000 molecular weight, but thereafter the fragments are increasingly resistant to disruption by ultrasound. (Doty, McGill and Rice, 1958). The amoebal DNAs were sonicated under standard conditions to yield fragments of approximately 500,000 molecular weight.

Cot	Time hrs.	Calculated OD 260 native DNA*	Calculated undiluted OD 260 native DNA**	% renat.
Neff strain				
0	0	0.388	16.79	17
18.0	2	0.394	16.59	39
45.0	5	0.392	16.47	54
167.2	18	0.415	17.03	66
407.2	45	0.387	16.59	71
822.7	97	0.313	15.55	72
Wl strain				
0	0	0.300	12.75	15
14.5	2	0.314	13.32	31
35.8	5	0.304	13.14	39
128.1	18	0.291	13.05	59
317.9	45	0.281	12.95	69
694.7	97	0.304	13.13	78
H strain				
0	0	0.176	7.69	17
8.2	2	0.171	7.50	24
17.9	4	0.182	8.23	34
76.8	18	0.176	7.82	49
115.4	30	0.154	7.05	56
294.5	72	0.168	7.50	62
A. polyphaga				
0	0	0.253	10.60	14
11.0	2	0.223	10.10	35
22.2	4	0.251	10.18	37
101.7	18	0.218	10.36	53
173.9	30	0.223	10.63	69
420.6	72	0.254	10.71	71
A. palestinensis				
12.9	2	0.133	11.84	23
26.2	4	0.141	12.01	31
52.8	8	0.144	12.09	44
151.6	24	0.131	11.58	57
347.7	48	0.136	13.28	55
407.6	72	0.095	10.38	76

Section E4. Renaturation and hybridisation experiments.

E4.1 The renaturation of DNA from the Neff, H and singh strains of A.castellanii, A.polyphaga and T.pyriformis, in the presence of ^{32}P labelled DNA from the Neff strain of A.castellanii.

A high percentage renaturation of amoebal DNA could be achieved using the capillary tube renaturation technique, To test the efficacy of this method for the production of DNA-DNA hybrids, amoeba DNA was renatured in the presence of low concentrations of ^{32}P labelled Neff strain DNA. T.pyriformis was included in the experiment as a control; little sequence homology should exist between a ciliate and an amoeba, especially as the DNAs differ by almost 30% GC in base composition.

The following experimental procedure was used. Radio-active DNA was added to a test DNA and the mixed solution was prepared for renaturation by in the capillary tubes and, after selected times of incubation, duplicate tubes were removed. The contents of one tube were used for an optical determination of the homologous reaction, the contents of the other tube were fractionated on HAP to estimate the amount of hybrid DNA.

a) Preparation of DNA.

DNA was prepared from 15 - 20 gram pellets of the amoebae using extraction and purification method II. Tetrahymena DNA was prepared from 5 grams of cells as described in Section M4.4.

Radio-active Neff strain DNA was extracted from amoebae which had been grown in two 30ml shaken cultures, each of which contained 1mCi of ^{32}P orthophosphate. A 2 gram pellet of amoebae was obtained and from this 40ug of radio-active DNA was prepared as described in Section M4.3.

b) Preparation of renaturation/hybridisation solutions.

The non-radio-active DNA samples were each dissolved in 1ml of x2 SSC, the DNA concentration in each solution was approximately 500µg/ml. 0.2ml of the radio-active DNA (40µg/ml in x2 SSC) was added to each solution. The solutions were then prepared for renaturation by the capillary tube renaturation technique exactly as described (Section M7). Twelve 0.05ml samples were taken from each DNA solution and sealed into capillary tubes. The tubes were incubated at 60°C and pairs of tubes were removed at times, - 0; 2; 4; 18; 30;172 hours.

c) Estimation of percentage reassociation of homologous DNA.

One sample tube from each pair was opened and its contents diluted into 1.5ml of 0.012M phosphate buffer. The tubes of diluent were weighed before and after the addition of the DNA solution. The diluted samples were denatured in the spectrophotometer, and after correction for thermal expansion the percentage reassociation was calculated from the hyperchromicity of the sample. The results are shown in Table E4.1 and Fig. E4.1.

d) HAP fractionation of the radio-active DNA.

The second capillary tube from each pair was broken open and its contents were added to 2ml of 0.06M phosphate buffer in a 10ml centrifuge tube. 1ml of HAP suspension was added and the sample was thoroughly mixed by holding the tube lightly against a vortex mixer. After mixing 8ml of 0.06M buffer was added and the suspension was centrifuged briefly at 60°C. The supernatant was discarded and the HAP pellet was washed 3 times in 3ml volumes of 0.15M; 0.18M and 0.36M buffer. All the washings were carried out at 60°C as described in Section M6.2. The washings at each buffer concentration were pooled in scintillation vials and the radio-activity of the eluates was determined by Cerenkov counting. The results are shown in Table E4.2.

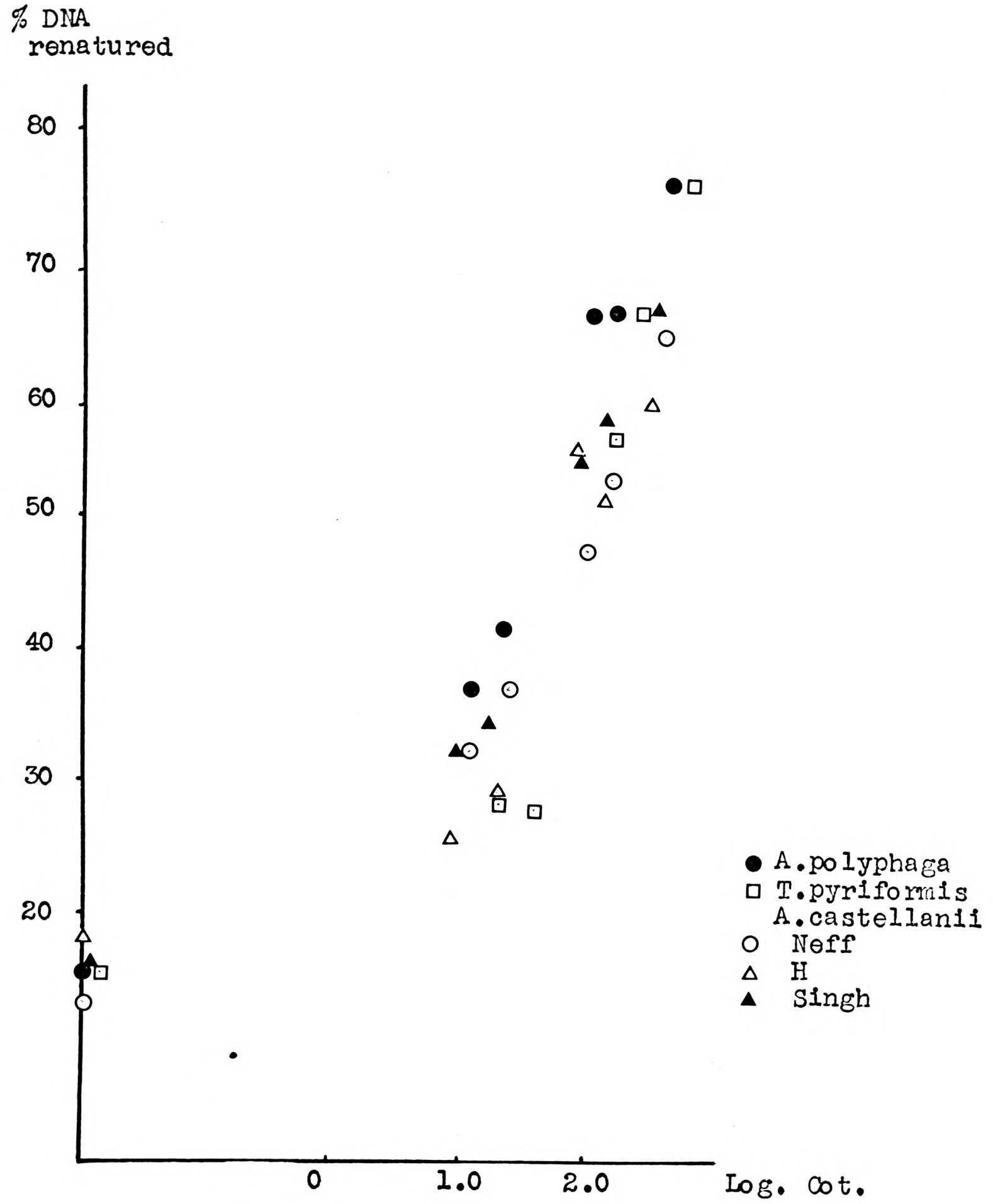


Fig. E 4.1. The renaturation of homologous DNAs.

Table E4.1 Renaturation of amoebae DNA's optical determination of homologous reactions.

Time (hrs)	Neff % renat	Cot	Singh % renat	Cot	H % renat	Cot	Polyphaga % renat	Cot	Tet % renat	Cot
0	12.6	0	15.7	0	17.5	0	32.0	0	15.0	0
2	32.0	10.6	32.0	7.4	25.5	8.2	37.6	10.8	27.9	18.1
4	37.4	21.3	34.6	14.8	29.5	16.5	43.0	21.6	26.3	36.2
18	48.3	96.1	55.5	67.0	55.8	74.1	67.0	97.4	56.7	163.0
30	54.0	160.2	58.6	111.6	51.7	123.5	67.0	162.3	66.6	271.1
72	65.0	384.6	67.1	268.0	60,5	296.5	77.0	389.5	77.5	652.1

Table E4.2 HAP fractionation of hybridisation mixtures.

	%^{32}P in -				
	0.15M	0.18M	0.36M	0.18+0.36	Cot
Neff	92.2	4.0	3.8	7.8	0
	78.5	10.9	10.6	21.5	10.6
	69.3	16.2	14.5	30.7	21.3
	39.5	35.9	24.6	68.7	160.2
H	94.3	3.2	2.5	5.7	0
	86.1	6.9	7.0	13.9	8.2
	78.8	14.3	6.9	21.2	16.5
	66.1	22.2	11.7	33.9	74.1
	60.3	26.2	13.5	39.7	123.5
	59.3	26.3	14.4	40.7	296.5
Singh	95.7	1.4	2.9	4.3	0
	91.1	6.9	2.0	8.9	7.4
	81.7	12.9	5.4	18.3	14.8
	64.0	27.4	8.6	36.0	67.0
	62.5	26.5	11.0	37.5	111.6
	55.8	30.6	13.6	44.2	268.0
A.polyphaga	89.3	5.5	5.2	10.7	0
	86.6	8.6	4.8	13.4	10.8
	83.3	11.4	5.3	16.7	21.6
	68.0	24.2	7.8	32.0	97.4
	64.8	26.1	9.2	25.2	162.3
	60.3	26.9	12.8	39.7	389.5
Tet	89.6	5.2	5.2	10.4	0
	88.9	5.4	5.7	11.1	18.1
	89.8	6.1	5.1	11.2	36.2
	87.1	7.0	5.9	12.9	163.0
	85.4	8.4	6.2	14.6	271.1
	84.6	8.9	6.5	15.4	652.1

It was apparent that an increasing proportion of the DNA in each sample eluted in 0.18M buffer. It seemed probable that the 0.18M fraction from the hybridisation experiments contained partially renatured DNA; that is, DNA strands held together by short lengths of stable duplex. To test this supposition the 0.18M and 0.36M fractions were denatured on HAP columns and their thermal stability was measured.

e) The thermal stability of the 0.18M and 0.36M fractions.

The five 0.36M fractions from the Neff strain samples (Table 2) were diluted with 100ml of distilled water to lower the buffer concentration to 0.12M. 10ml of HAP was added and the suspension was stirred for 15 minutes at room temperature and then allowed to settle for 30 minutes. Approximately 2/3 of the supernatant fraction was decanted and the remaining slurry was poured into a water-jacketed column. The column was packed and washed with 10ml aliquots of 0.12M buffer at increasing temperatures as described in Section M6.3. The eluants were collected in scintillation vials and the activity eluted at each temperature was determined by Cerenkov counting. The cummulative total of percentage activity eluted at each temperature was plotted to give a denaturation curve.

The 0.18M fractions were treated in the same way, except that the pooled fractions were diluted with only 25ml of distilled water to lower the buffer concentration to 0.12M. As a control, 0.1ml of 32P DNA in 0.012M buffer, from the preparation used in the hybridisation solutions, was denatured by heating for 15 minutes at 100°C then quenched in iced water and diluted to 100ml in 0.12M buffer. The DNA was then adsorbed on to HAP, packed into a column and denatured as before. The results are shown in Fig. E4.2.

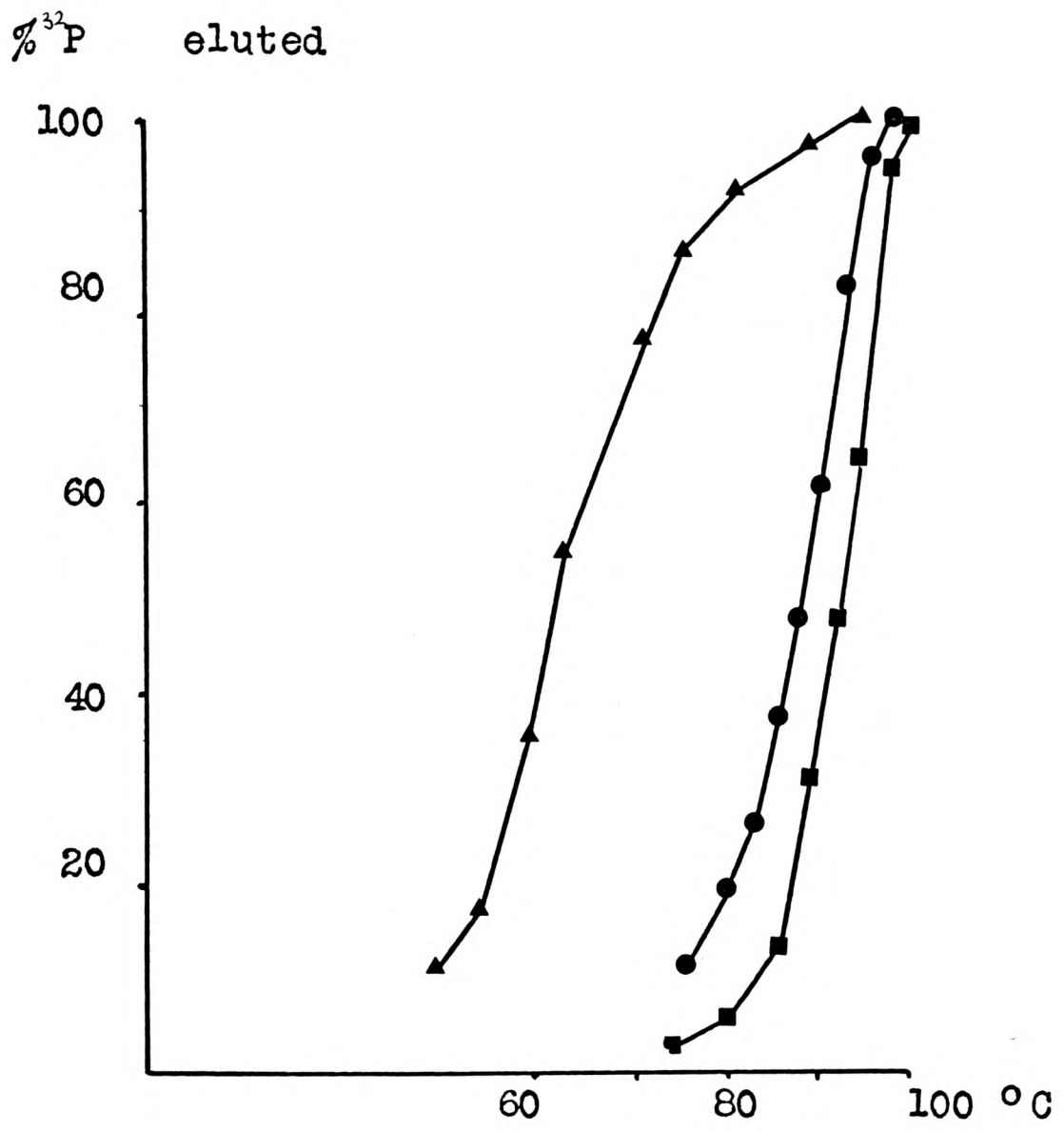


Fig. E 4.2. The thermal stability of the 0.18 and 0.36 M fractions. Hydroxyapatite thermal chromatograms.

▲ denatured DNA
● 0.18 M fraction
■ 0.36 M fraction

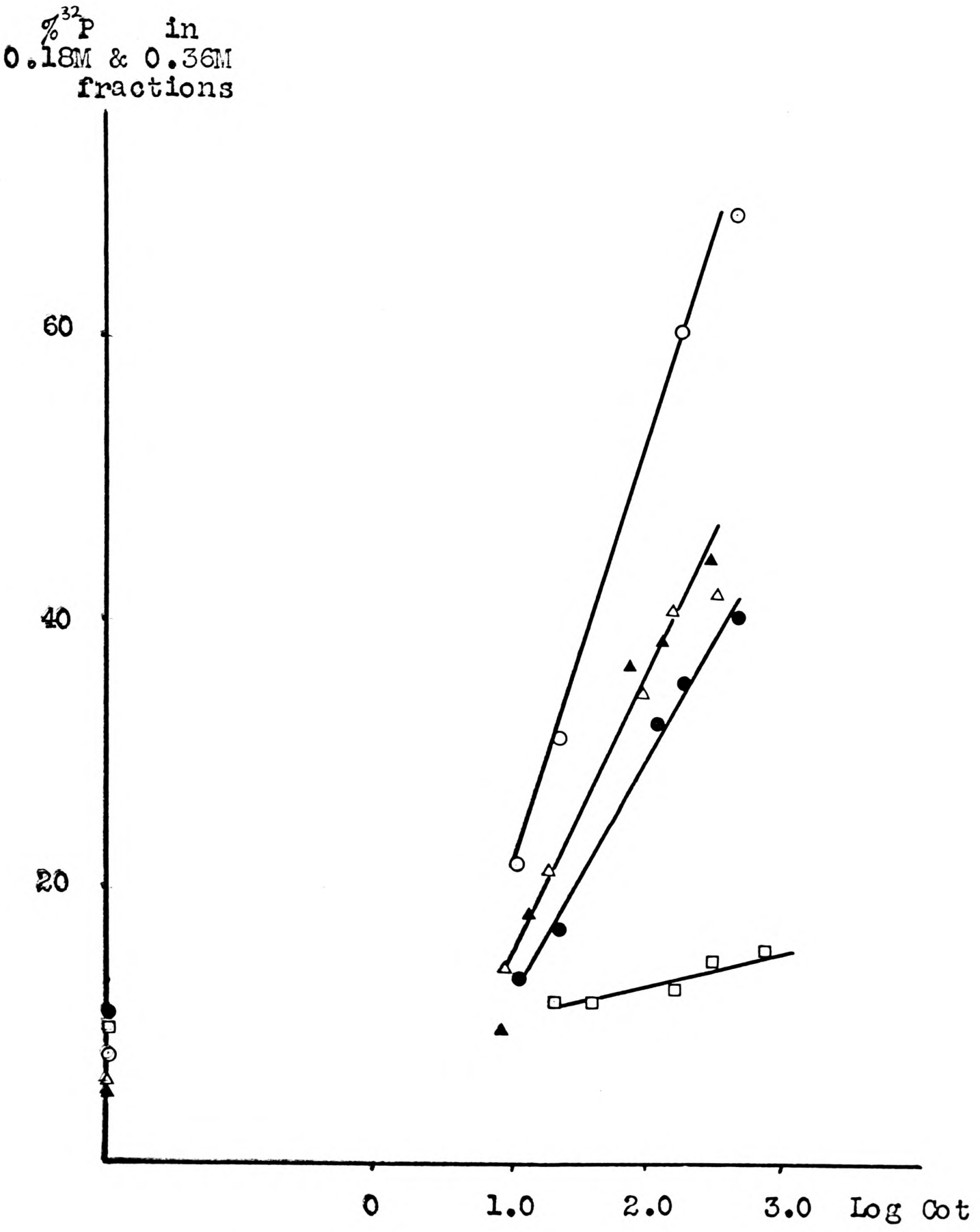


Fig. E 4.3. The rate of formation of hybrid DNAs.

A.castellanii (Neff) DNA hybridised with DNA from ;

△ H strain
▲ Singh strain
● A.polyphaga
□ T.pyriformis
(○ homologous reaction)

Results.

i) The homologous reaction.

The values for Cot $\frac{1}{2}$ of the DNA's tested fell within the range 50 - 100, as in experiment E3.3, and support the view that Acanthamoeba DNA consists mainly of slow renaturing single copy DNA. The nucleus of Acanthamoeba contains approximately 0.5 picograms of DNA (Blewett 1969) representing in the order of 10^8 base pairs) and mammalian DNA (5×10^9 base pairs). Similarly, the Cot $\frac{1}{2}$ of Acanthamoeba DNA (50 - 100) is intermediate between that of E.coli (Cot $\frac{1}{2}$ 10) and calf thymus single copy DNA (Cot$\frac{1}{2}$ about 3,000).

ii) The thermal stability of the 0.18M and 0.36M fractions.

Estimations of the percentage hybrid formed were more difficult than anticipated, and it was necessary to decide which fraction of the hybrid DNA was good duplex and which was not. The elution conditions for HAP fractionation had been selected without recognising that the hybrid molecules would vary from short poorly matched sequences to stable, well matched duplex molecules.

A comparison of the thermal denaturation curves of the 0.18M fraction and denatured DNA showed that the 0.18M fraction contained a large proportion of double stranded DNA. Although, as expected, the DNA in the 0.18M fraction was less stable than that in the 0.36M fraction, (the thermal denaturation temperature was depressed by 4°C), it was decided that the hybrid DNA in the 0.18M fraction was sufficiently stable to warrant its inclusion with that in the 0.36M fraction to represent the percentage of hybrid DNA.

iii) The formation of hybrid DNA.

The capillary tube renaturation technique was successful in producing hybrid DNAs. The data in Table E4.2 show that the amounts of hybrid formed with

the H and Singh strains of A.castellanii and with A.polyphaga were very similar, though these amounts were considerably less than that formed in the homologous reaction. The rates of hybrid formation are shown in Fig. E4.3. Although the heterologous reaction between amoebal DNA reached similar final levels the rate of hybrid formation appeared to be faster for the Singh and H strains than for A.polyphaga. There was no appreciable reaction between Tetrahymena DNA and Acanthamoeba DNA; the small amount of hybrid produced (15%) represented the homologous reassociation of the Neff strain DNA.

E4.2 The thermal stability of hybrid DNA's.

The previous experiment had shown that comparatively high levels of hybridisation could be attained using the capillary tube renaturation technique. It also confirmed that comparisons of DNA based on the final levels of hybrid produced were not reliable guides to sequence homologies. A series of experiments were therefore carried out to test the quality of hybrid DNA's.

The capillary tube renaturation technique was used to produce as much hybrid as possible between radio-active DNA from A.castellanii Neff and the DNA of the W1, Singh and H strains of A.castellanii, and of A.palestinensis, A.polyphaga and T.pyriformis. The hybrid DNA's were then denatured on HAP to determine the thermal stability.

a) Preparation of DNA.

DNA was prepared from 15 - 20 gram pellets of amoebae using method II, and from 10 grams of Tetrahymena as described in Section M4.4.

Radio-active DNA was prepared as described in Section M4.3 from Neff strain amoebae. A separate sample of radio-active DNA was prepared from a single 30ml culture for each hybridisation experiment.

b) Preparation of the hybridisation mixture.

The non-labelled DNA was prepared for renaturation by the capillary tube renaturation technique. The denatured low molecular weight DNA in 1ml of 0.012M phosphate buffer was dialysed for 48 hours against 2 changes of 2 litres of distilled water. The solution was then transferred to a siliconised scintillation vial and dried in a vacuum desiccator over silica gel.

The radio-active DNA at a concentration of 30µg/ml was sonicated and denatured as described for the capillary tube renaturation technique. After denaturation the solution was adjusted to 0.12M phosphate buffer and the dried DNA was redissolved in 0.05ml of this solution. The hybridisation mixture was sealed into a capillary tube and incubated at 60°C for 90 hours.

c) Analysis of the hybridisation mixture.

After incubation the sample was recovered and diluted into 1ml of 0.012M buffer. The tube of diluent was weighed before and after the addition of the DNA. 0.2ml of the diluted sample was pipetted into 1.5ml of 0.012M buffer and denatured in the spectrophotometer. The remaining 0.8ml of sample was adsorbed on to HAP, packed into a column and denatured exactly as described in Section M6.3.

The percentage renaturation in the homologous reaction was calculated from the hyperchromicity of the sample, and the DNA concentration in the original mixture was calculated as described in E3.2II.

i) The homologous reaction and the thermal denaturation of native DNA.

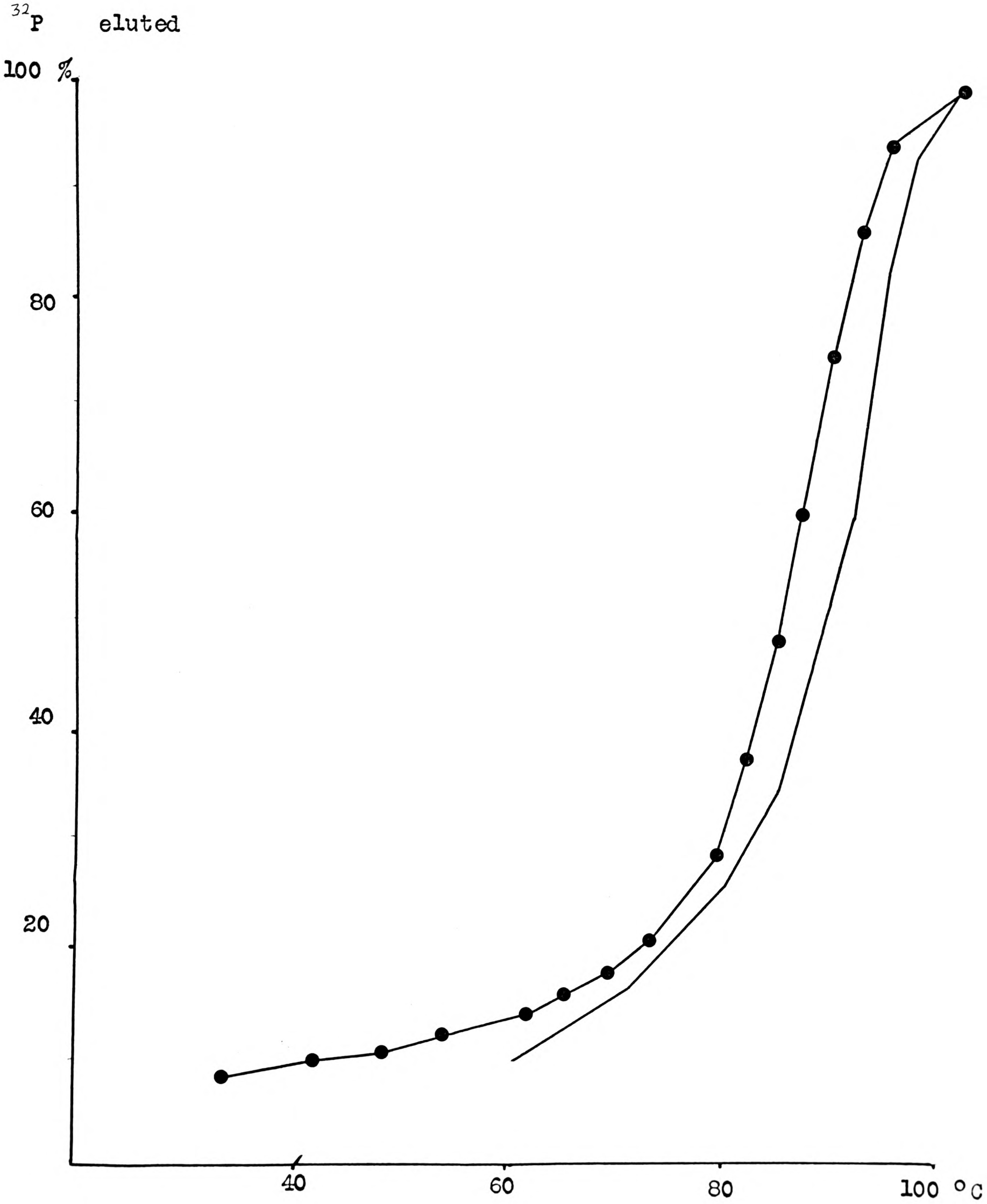
An 0.05ml sample of radio-active DNA was taken after sonication and dialysis, diluted in 2ml of 0.12M phosphate buffer and adsorbed on to HAP.

Fig. E 4.4.

Hydroxyapatite thermal chromatograms of *A.castellanii*(Neff) native (—) and renatured (•) DNA.

Tm native DNA 91°C

Tm renatured DNA 86.5°C


^{32}P eluted
100 %
80
60
40
20
40
60
80
100 °C

The HAP was packed into a column and slowly heated to denature the DNA. Non-labelled Neff strain DNA was renatured in the presence of radio-active DNA as described.

The HAP thermal denaturation curves of native and reassociated Neff strain DNA are shown in Fig. E4.4. The DNA concentration in the hybridisation mixtures and the percentage reassociation are shown in Table E4.3.

ii) Hybridisation of amoebal DNA's.

A.castellanii Neff strain DNA was renatured with excess non-labelled DNA from the amoebae listed in Table E4.3. The DNA concentrations, Cot values and percentage renaturation of the homologous DNA are shown in this Table. The denaturation curves of the hybrid DNA's are shown in Figs. E4.5, E4.6.

iii) The hybridisation of Neff strain DNA with Tetrahymena DNA.

As a control, Neff strain DNA was hybridised with excess Tetrahymena DNA under the conditions used for the amoebal DNAs. The hybridisation mixture was analysed as before; the results are shown in Table E4.3 and Fig. E4.6.

Table E4.3

DNA source	DNA conc µg/ml	Cot	% renat homol.	% renat hybrid	depression Tm °C
A.castellanii					
Neff	2909	2513	88	83	0
H	2025	1555	65	79	2
Singh	3272	2707	85	74	2
W1	1111	967	78	79	2
A.palestinensis	1001	1045	74	65	6
A.polyphaga	3010	2547	82	63	5
A.astronyxis	2849	2972	74	27	30
T.pyriformis	4272	3691	98	20	30

Fig. E 4.5.

Hydroxyapatite thermal chromatograms of A.castellanii(Neff) DNA hybridised with DNA from the Singh (■), W1 (▼) and H (●) strains of A.castellanii.

(O) thermal denaturation curve of renatured Neff strain DNA.

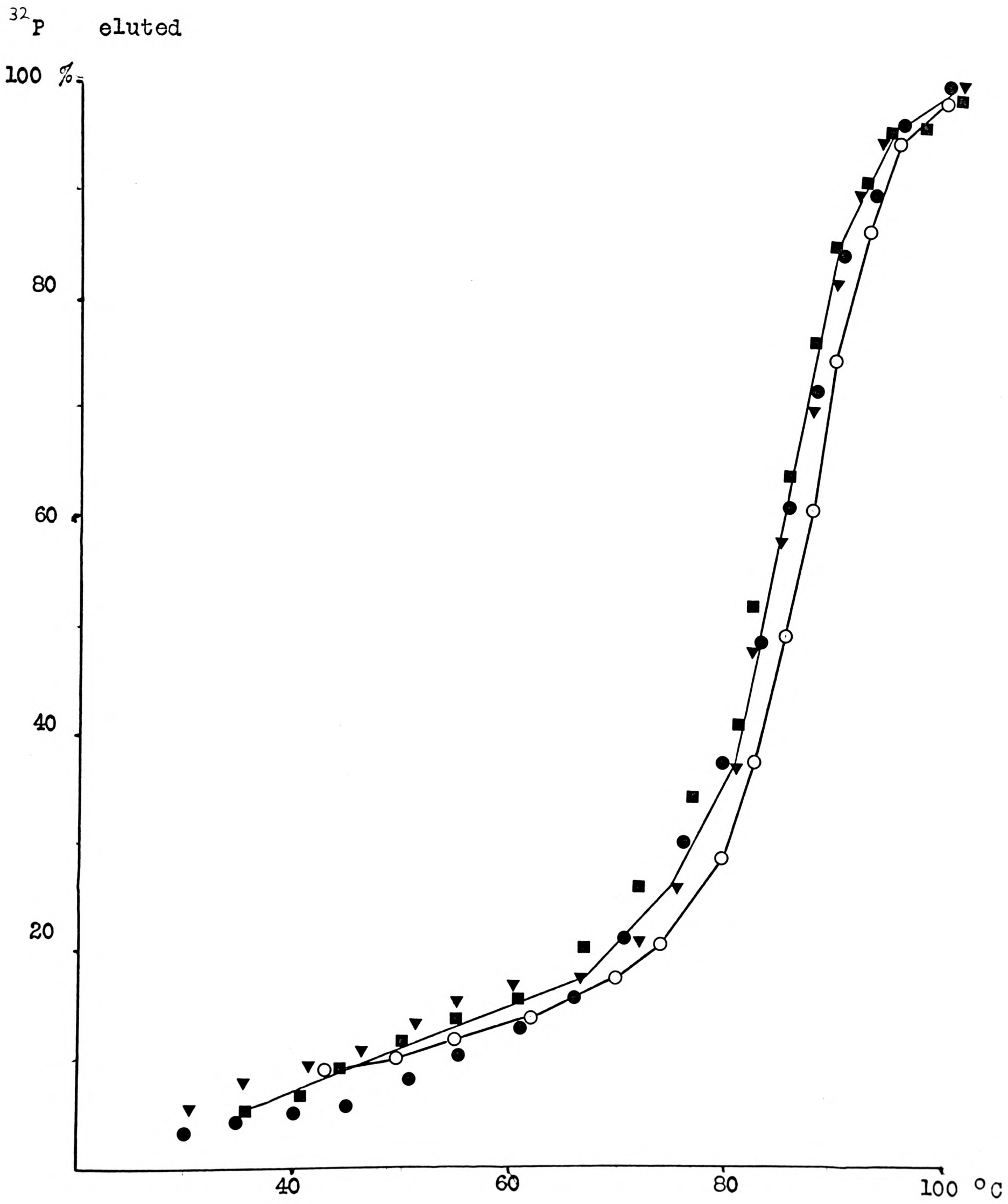
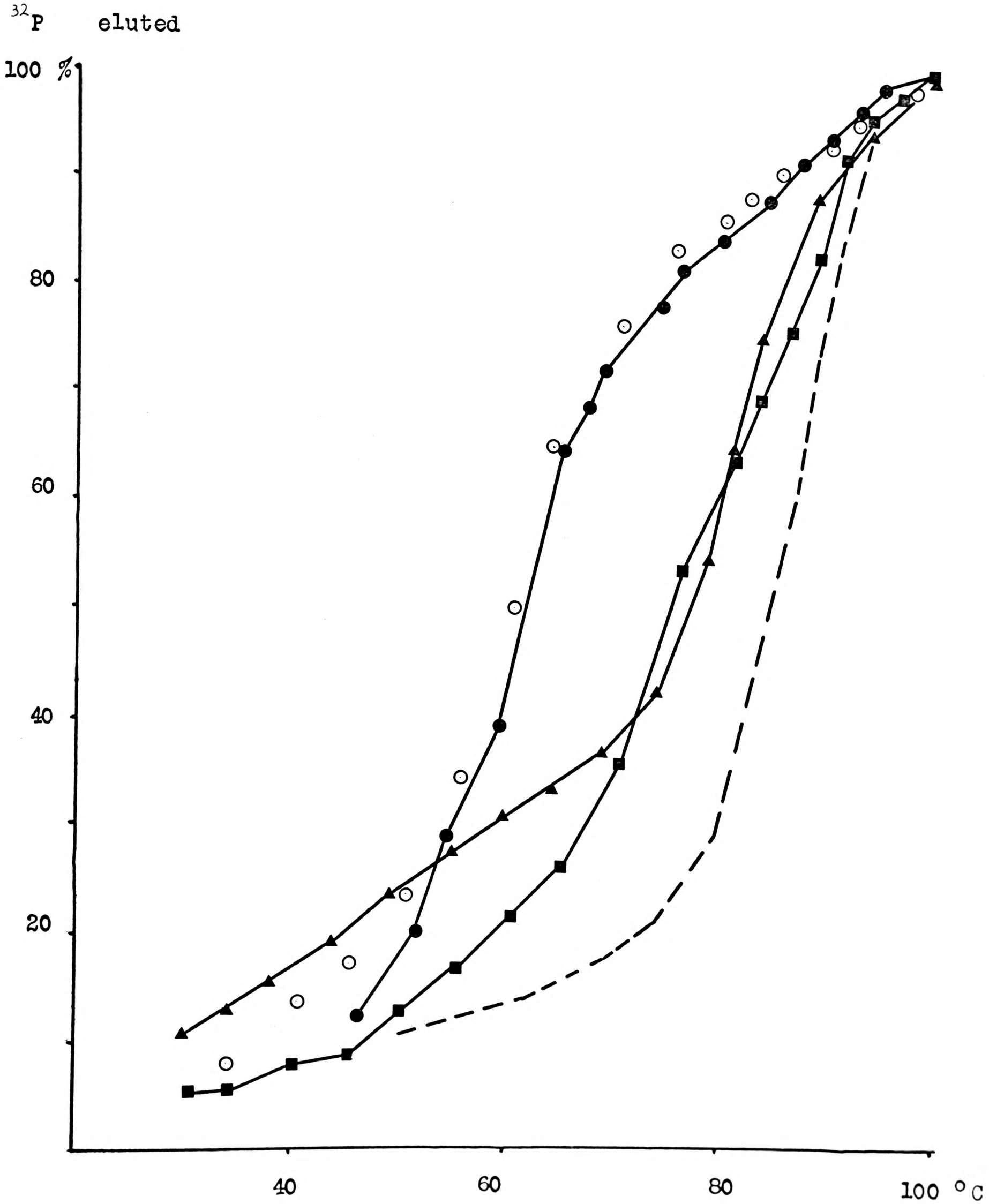

^{32}P eluted
100 %
80
60
40
20
40
60
80
100 °C

Fig. E 4.6.

Hydroxyapatite thermal chromatograms of A.castellanii(Neff) DNA hybridised with DNA from A.palestinensis(■), A.polyphaga (▲), A.astronyxis(●) amd T.pyriformis (o). Dashed line, thermal denaturation curve of renatured Neff strain DNA.


^{32}P eluted
100 %
80
60
40
20
40
60
80
100 °C

Results.

The technique of dehydration and resolution of DNA used in these experiments, enabled very high Cot values to be achieved (Table 3), As a result homologous reactions of up to 80% were obtained. Similarly, estimations of the amount of hybrid DNA (calculated from the amount of activity still bound to HAP at 70°C in 0.12M buffer) indicated that a high percentage of hybrid molecules had been formed.

The data show that the DNA of the H, Singh and W1 strains is very similar to that of the Neff strain. The final levels of hybrid produced are almost the same for all four amoebae, and the difference between the thermal denaturation temperatures of the homologous and heterologous hybrids was very small. There appeared to be less similarity between the DNA of A.palestinensis and A.polyphaga and of A.castellanii (Neff). Hybridisation reached a lower final level, and the hybrid formed was less stable than that produced between strains. The DNA of A.astronyxis has a base composition 11% GC lower than that of A.castellanii, and as expected the DNA from this species showed almost no similarity to that of A.castellanii.

Section E5. Mitochondrial DNA.

The DNA of Acanthamoeba contains a minor component, presumed to be of mitochondrial origin (Adam et al 1969). To examine more closely the origin of the minor component, DNA was extracted from isolated mitochondria.

E5.1 Extraction of DNA from isolated mitochondria.

Mitochondria were isolated from A.castellanii (Neff) using the method of Klein and Neff (1960). A 20 gram pellet of amoebae was resuspended in 80ml of ice cold 0.25M sucrose 0.01M EDTA and homogenised for 30 seconds at top speed in an MSE homogeniser. A sample of the homogenate was removed and examined under the microscope to check that all the cells had been disrupted, then the homogenate was made up to 200ml with cold sucrose EDTA solution. Large debris was removed by centrifuging for 8 minutes at 3,000 rpm and 0°C. The supernatant fraction (I) was collected and kept on ice while the pellet was resuspended with 100ml of cold sucrose EDTA solution and centrifuged once more. The second supernatant was collected and combined with supernatant I. The mitochondria were pelleted from the combined supernatant fractions by centrifuging for 10 minutes at 10,000 rpm and 0°C.

The mitochondrial pellet was resuspended in 10ml of 0.25M sucrose and 1mg of solid DNAase (Sigma Ltd. DNAase I) was added. The suspension was stirred for 15 minutes at 25°C, then a 0.5ml sample was removed for electron microscopy. An electron micrograph of the material in the sample is shown in Fig. E5.1. The remainder of the suspension was centrifuged for 10 minutes at 10,000 rpm and 0°C, and the resulting pellet was weighed. DNA was extracted from the mitochondrial pellet and purified on a CsCl gradient as described in M4.2a.

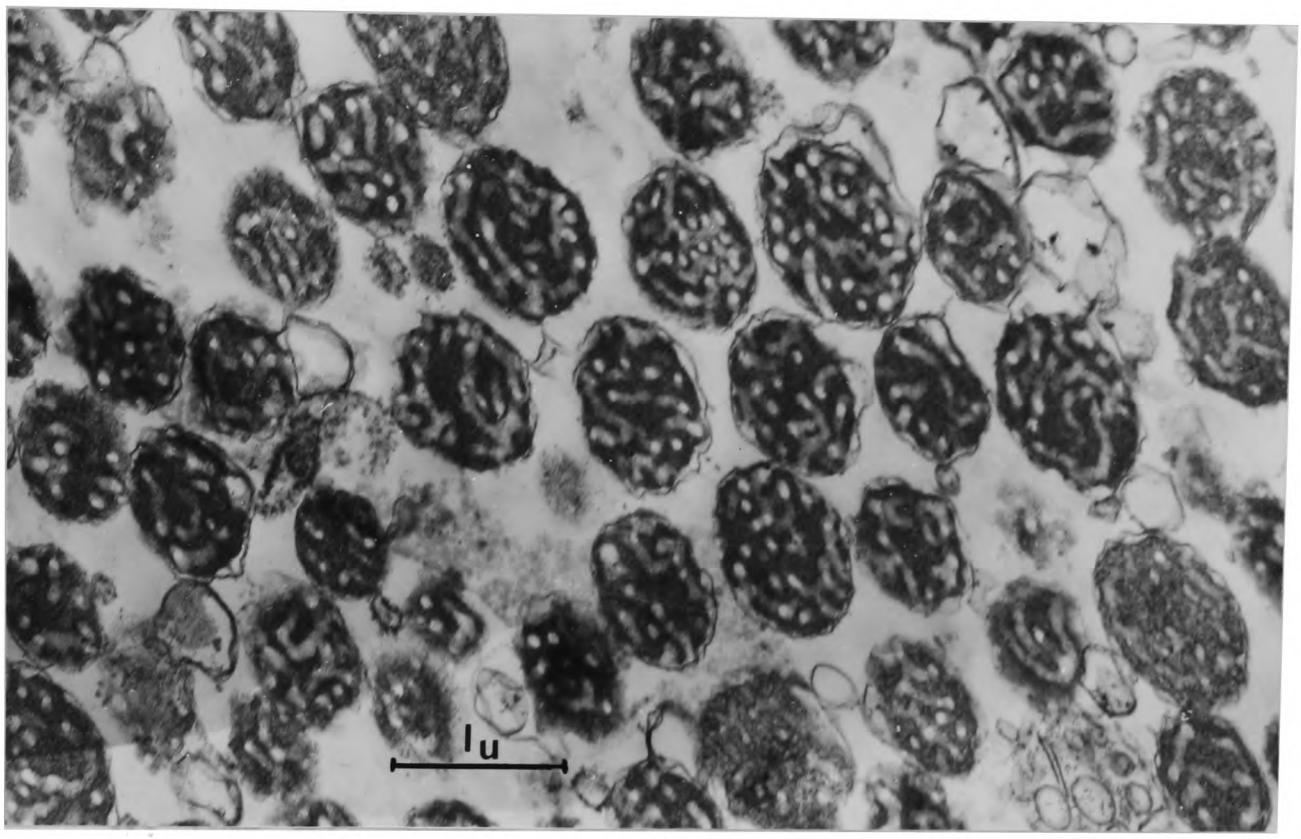


Fig. E 5.1. Electron micrograph of mitochondria isolated by the method of Klein and Neff (1960). Fixed in gluteraldehyde and sections stained with uranyl acetate and lead citrate.

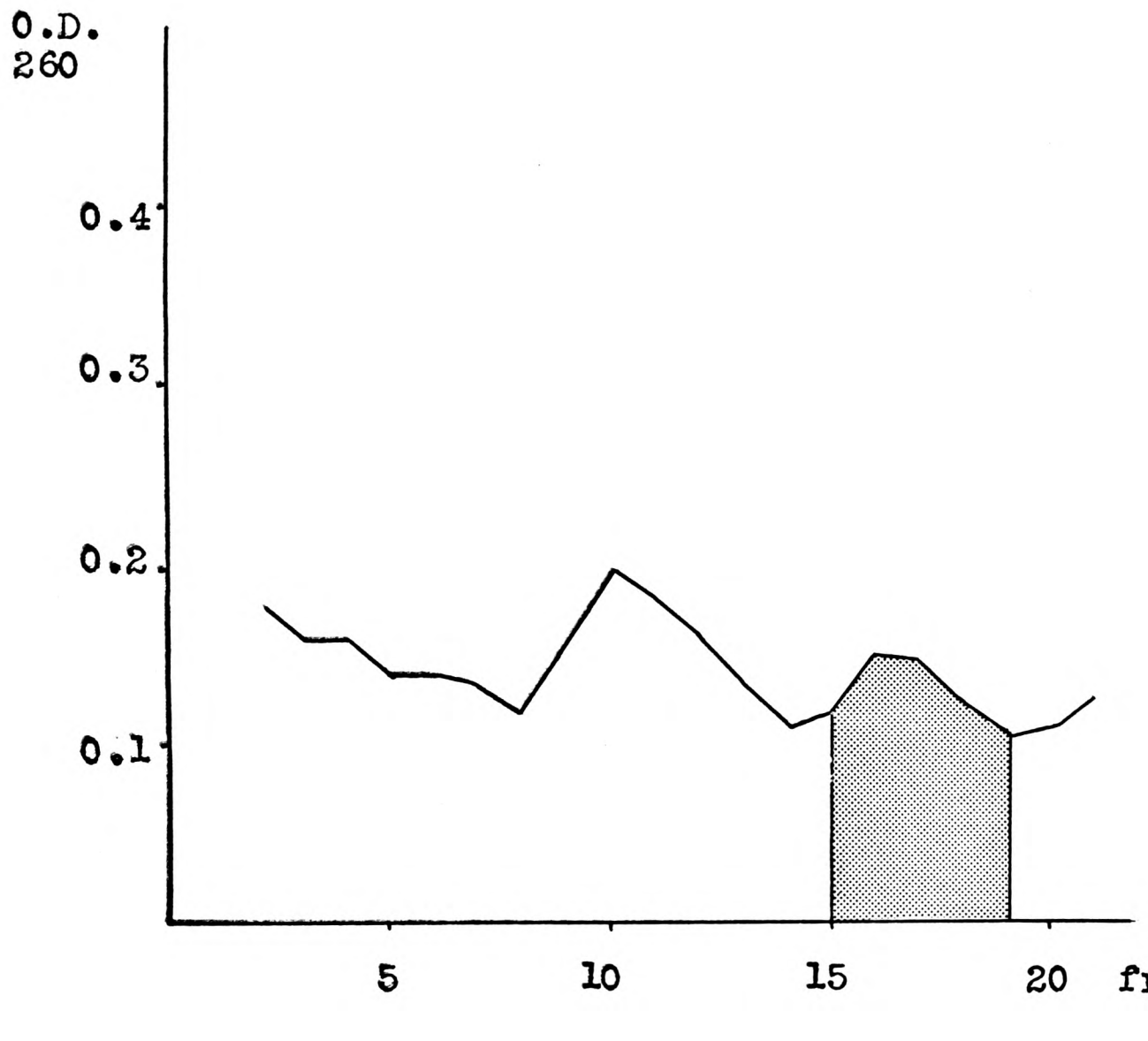


Fig. E 5.2. CsCl density gradient centrifugation of DNA extracted from isolated mitochondria. Shaded area represents fractions recycled.

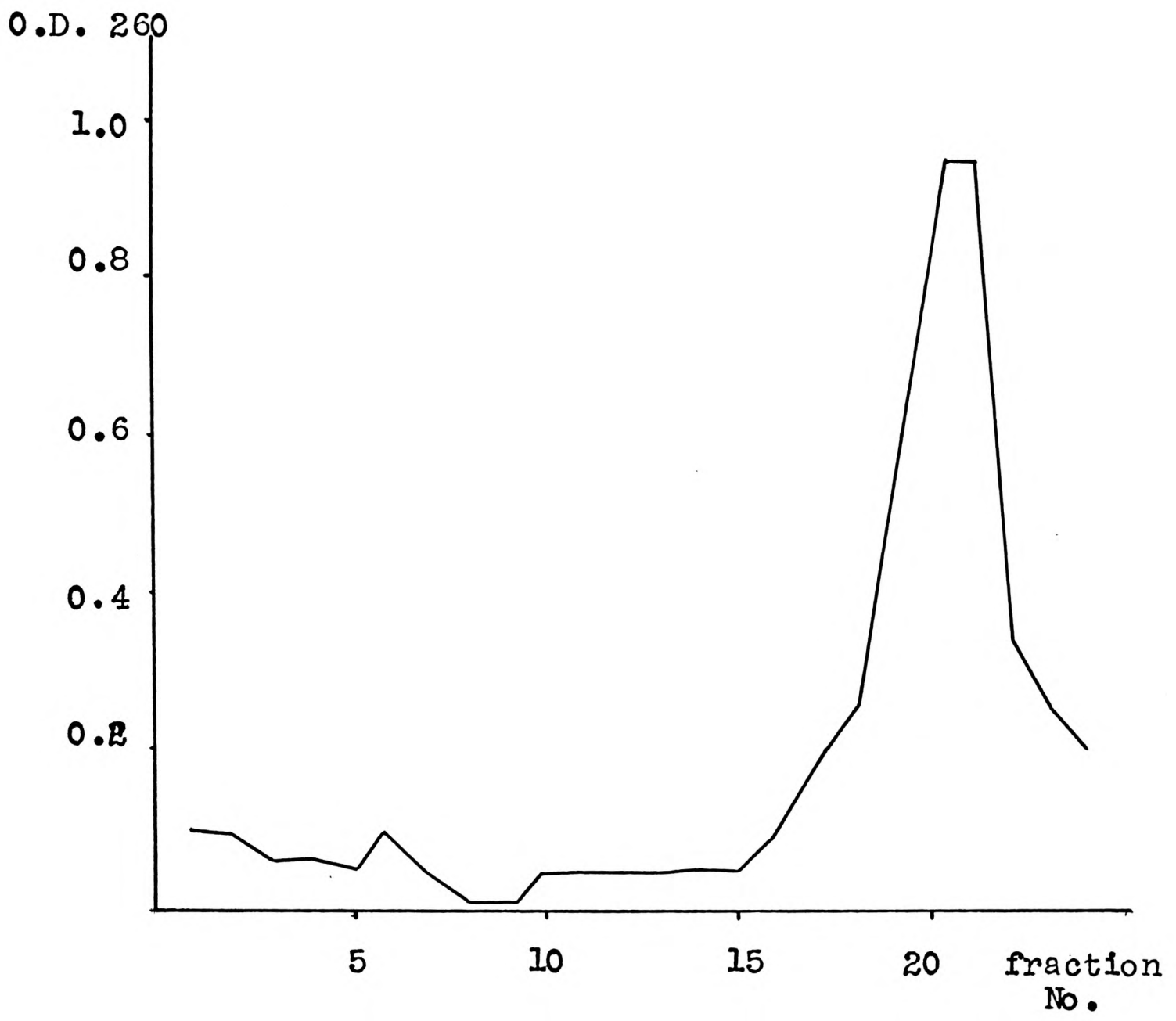


Fig. E 5.3. CsCl density gradient absorbance profile of recycled minor DNA.

The gradient was fractionated and the absorbance at 260mμ of each fraction was measured, the absorbance profile obtained is shown in Fig. E5.2. The high absorbance fractions of the second peak were collected (as shown in the Figure) and the DNA was recovered by centrifugation. The pelleted DNA was redissolved in 3.4ml of 0.01M Tris and CsCl was added to give a solution with density of 1.720g/cm^3. The solution was centrifuged for 60 hours at 35,000 rpm and 25°C, then the gradient was fractionated and the absorbance at 260mμ of each fraction was measured using Beckmann microcells to hold the undiluted fractions. The results are shown in Fig. E5.3.

Results.

From a 20 gram pellet of amoebae a 1 gram mitochondrial pellet was obtained, and the DNA was extracted from this, with some difficulty, using extraction method I. The absorbance profile after the first cycle on CsCl showed a small minor DNA peak and an only slightly larger nuclear DNA peak. The mitochondrial pellet therefore contained more minor DNA than would a comparable weight of intact amoebae. When the minor DNA was recycled on a second CsCl gradient it formed a sharp band in the upper part of the gradient.

The presence of enhanced amounts of the minor component in DNA extracted from isolated mitochondria indicates that the minor component consists of mitochondrial DNA. To test this conclusion samples of minor DNA were examined with the electron microscope for the presence of circular DNA molecules, since circular mitochondrial DNA has been discovered in a wide range of eucaryotic cells.

E5.2 Electron microscopy of mitochondrial DNA.

Samples of mitochondrial DNA were collected from CsCl gradients used for the routine preparation of amoebal DNA. The samples were treated as

described in M 11, and the prepared grids were examined in the electron microscope for the presence of circular DNA molecules.

Results.

Samples of mitochondrial DNA were examined from the Neff, H, W1 and Singh strains of A.castellanii, and from A.palestinensis, A.polyphaga and A.astronyxis. Figure E5.4 shows the appearance of a general field of W1 strain mitochondrial DNA, which was typical of all the preparations examined. A possibly circular molecule (arrowed) is surrounded by a large number of linear molecules and "rosettes" of unspread DNA. Although circular molecules were found in samples of mitochondrial DNA from all the different amoebae examined the actual numbers of clearly circular molecules which could be found in any given preparation were very small.

Estimations of contour length.

The photographic plates from the electron microscope were projected, using a photographic enlarger, on to a sheet of white paper and the image of the circular molecule was traced on the paper. Similarly, plates showing latex reference spheres (2640 A diameter), photographed in the same working session as the DNA samples, were also projected on to the paper and the diameter of 5 - 10 spheres was marked. The length of the tracing was determined using a map measuring device, and the contour length of the DNA molecule was determined from the relative lengths of the tracing and the diameter of the reference spheres.

The estimated contour lengths of mitochondrial DNA from the Neff, H and W1 strains of A.castellanii and from A.palestinensis are shown in Table E5.1 and electron micrographs of circular DNA molecules are shown in Figs. E5.5, 5.6 and 5.7.

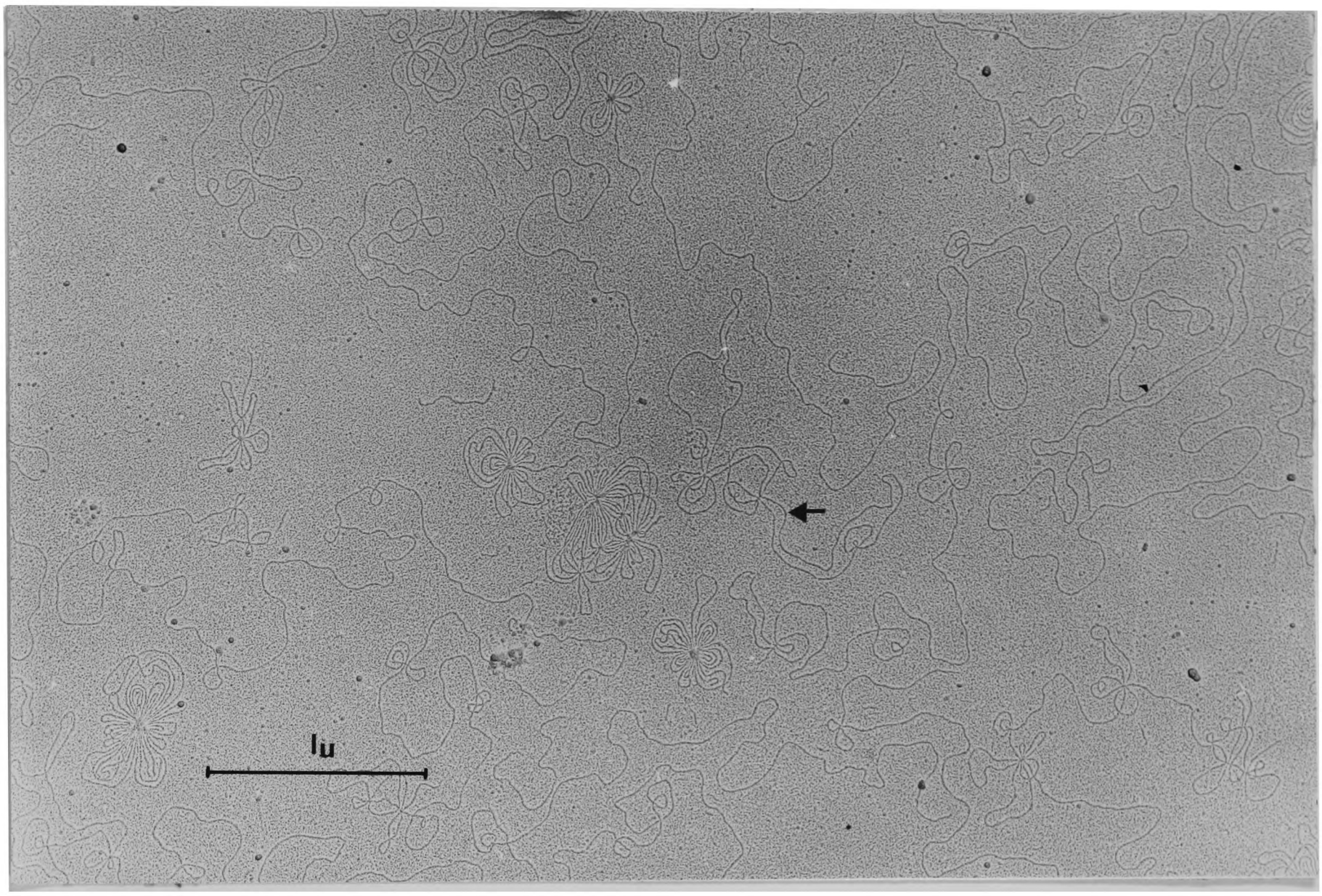


Fig. E 5.4. A.castellanii(W1) mitochondrial DNA spread, showing a possible loop of DNA (arrowed) and many linear molecules.

Table E5.1 Contour lengths of amoebal mitochondrial DNA.

Amoeba	Contour length (μ).
A.castellanii	
Neff	14; 12; 13; 16.
W1	17; 14; 14; 14.
H	15
A.palestinensis	14
A.castellanii	15
Singh*	15
A.polyphaga*	24
A.astronyxis*	16

* estimated from the indicated magnification on the electron microscope, micrograph of reference spheres not prepared.

Clearly circular molecules were found in all the minor DNA samples that were examined, confirming the conclusion drawn from experiment E5.1 that the minor DNA was of mitochondrial origin. The low frequency of circular molecules was probably due to breakage during extraction and purification since Acanthamoeba appears to contain potent nucleases which make DNA extraction difficult. The contour length of the circular DNA molecules from Acanthamoeba castellanii (14-15μ) while much higher than that of metazoan mitochondrial DNAs, is similar to that reported for the mitochondrial DNAs of other micro-organisms.

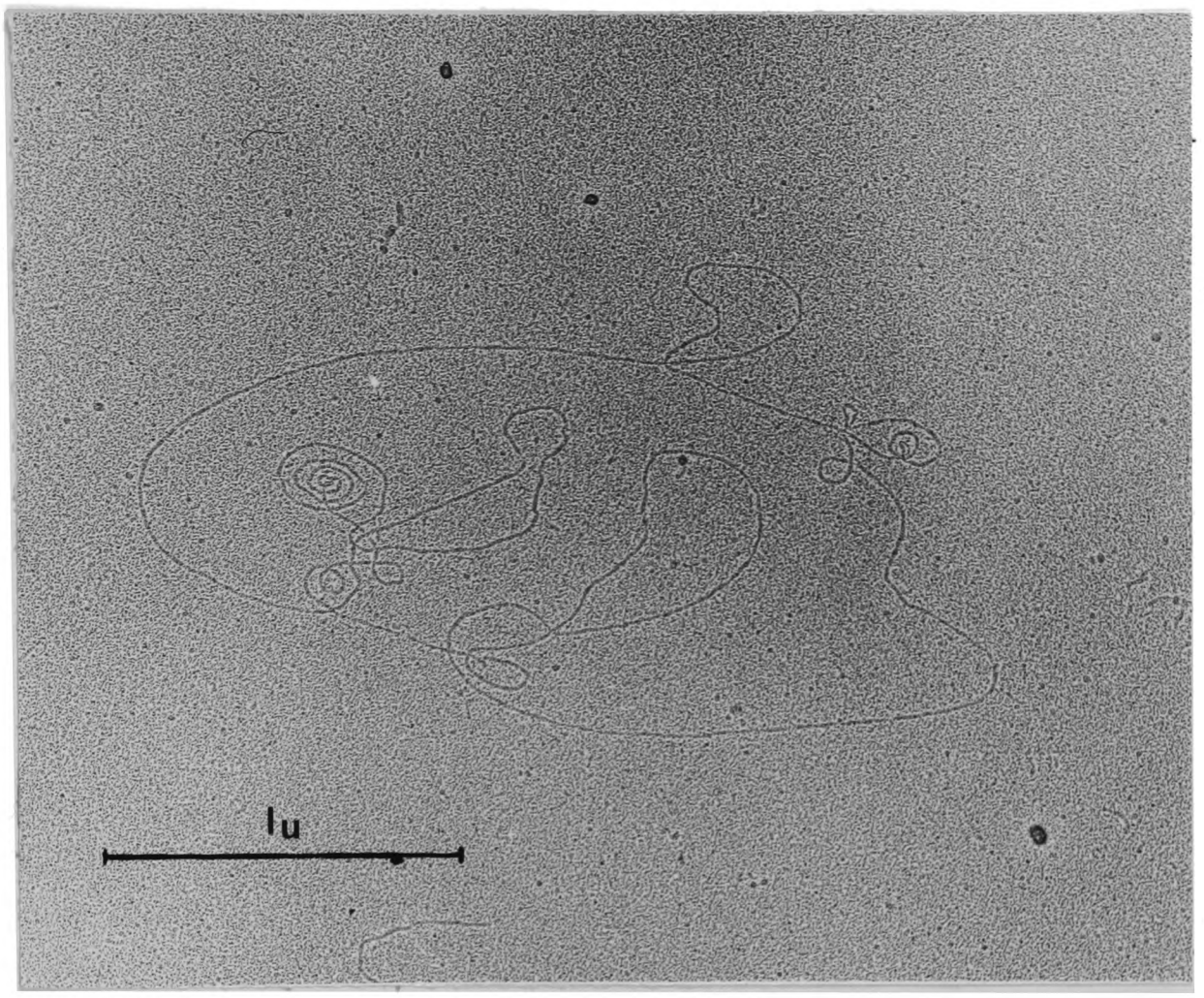


Fig. E 5.5. Circular mitochondrial DNA molecule from A.castellanii (Neff).

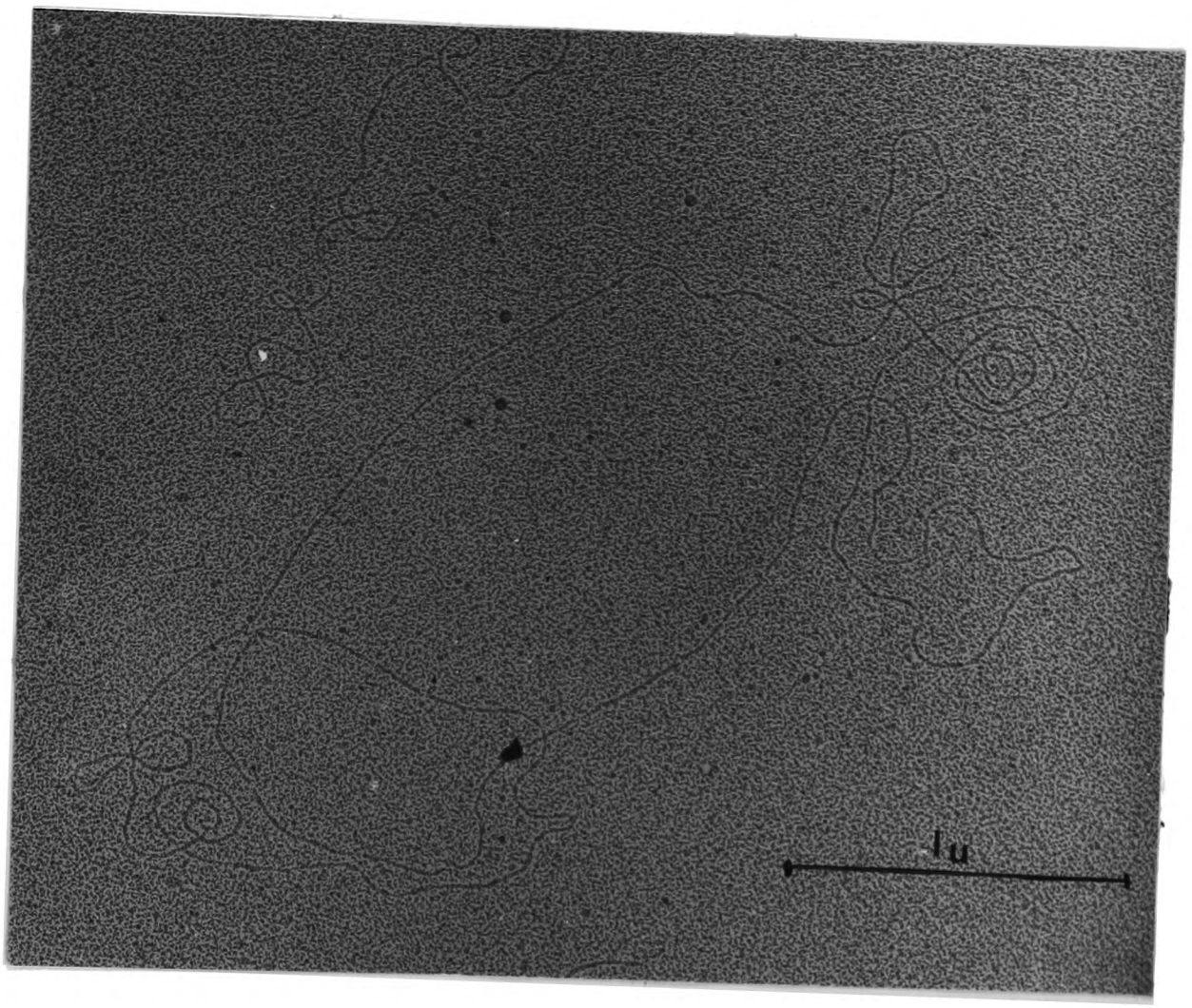


Fig. E 5.6. Circular mitochondrial DNA molecule from A.palestinensis.

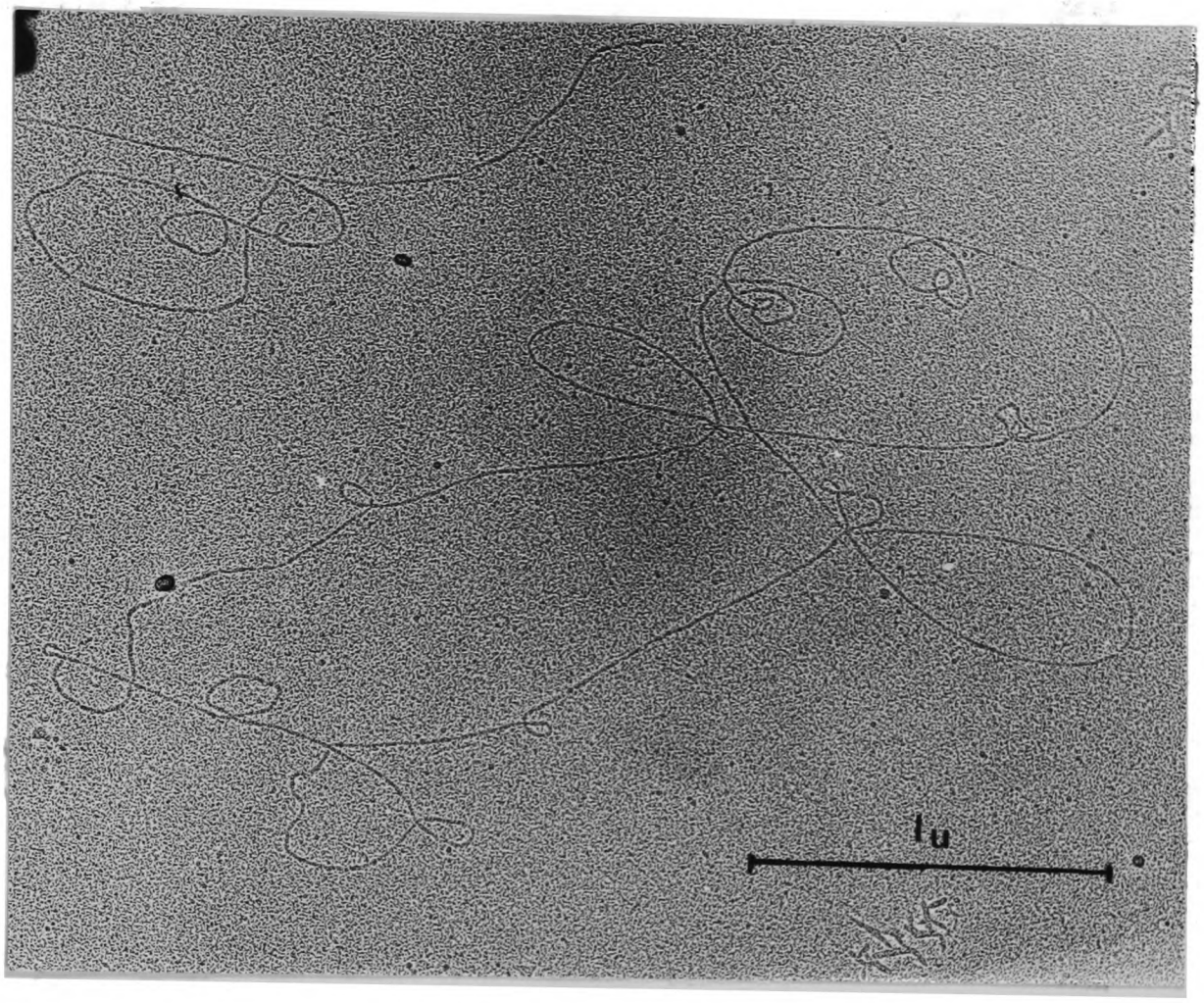


Fig. E 5.7. Circular mitochondrial DNA molecule from A.castellanii (W1).

DISCUSSION

A. Methods

The object of this study was to compare strains and species of Acanthamoeba by characterisation and hybridisation of their DNAs. This approach to the taxonomy of micro-organisms has been widely used to study relationships among bacteria (Silvestri and Hill 1965, Kingsbury 1967, and Heberlein, DeLey and Tijgat, 1967) and the data presented in the Introduction suggest that studies of DNA will also be useful in the classification of protozoa. Comparisons of protozoan DNA have been largely restricted to measurements of base composition and since there is little published information on techniques for the hybridisation of protozoan DNA as an aid to taxonomy, much of the work in this thesis became a study of methods.

I. DNA Preparation

An efficient method for the preparation of pure DNA in relatively large amounts was essential for studies on DNA hybridisation. Fortunately, Acanthamoeba can be grown axenically and large quantities of pure amoebae were readily obtainable. Despite this advantage, however, the isolation of DNA from Acanthamoeba was difficult (Blewett 1969, Kirtikar, Jensen and Meyers, 1967). Although a variety of methods have been described for the preparation of DNA from protozoa the technique of Adam et al (1969), referred to as Method I, was the only method which was known to be effective for the preparation of Acanthamoeba DNA. This technique was adequate for the production of DNA for characterisation and for the initial studies on its renaturation, but the two 60 hour periods of preparative ultracentrifugation which were required to purify the DNA made it unsuitable for the routine preparation of larger quantities.

In Method I the density gradients were prepared by dissolving sufficient

CsCl in a solution of DNA to raise its density to equal the buoyant density of the DNA. This solution was then centrifuged for 60 hours at 35,000 rpm, during which time the CsCl molecules were redistributed to form a density gradient. Brunk and Leick (1969) developed a method for producing rapidly equilibrating density gradients, in which the first lengthy stage in the formation of the gradient (i.e. the redistribution of CsCl in the liquid column) was simulated by establishing a high CsCl concentration in the lower half of the centrifuge tube prior to centrifugation. To do this they layered dilute CsCl solution containing the DNA sample over an equal volume of saturated CsCl solution. Gradients prepared in this way equilibrated rapidly because the amount of CsCl which had to be redistributed, and the distance through which it had to move were reduced, and, because the DNA was dissolved in a solution with a lower density than its own, it sedimented rapidly on to the interface between the two CsCl solutions.

The rapidly equilibrating CsCl density gradient technique was tested using purified Acanthamoeba DNA and equilibrium was reached in between 15 and 24 hours. This was longer than the 12 hours which Brunk and Leick had found to be sufficient but a 24 hour period of centrifugation represented such an improvement over the original 60 hours, that it was thought unnecessary to experiment with shorter centrifugation times. When this technique was used as a means of purifying crude DNA extracts, however, very poor yields of DNA were recovered from the gradients. It was concluded that DNA was lost by being trapped in the protein which was precipitated from the crude DNA solution when the saturated CsCl solution was added to it. Experiments were therefore carried out to develop a method for purifying the DNA before it was fractionated on CsCl gradients. Since the object of the experiments was to render DNA preparations suitable for centrifugation by the new technique, the effectiveness of the different procedures was assayed

by comparing the absorbance profiles obtained after centrifugation.

Contamination with protein was thought to be the most likely cause of the loss of DNA. All the purification treatments were therefore preceded by pronase digestion of the crude DNA extract. Pronase digestion followed by phenol or chloroform deproteinisation resulted in a very high background absorbance throughout the gradients. The removal of residual phenol by ether extraction and dialysis did not reduce the background absorbance. RNAase digestion and dialysis following deproteinisation also had little effect, indicating that the material of high absorbance was probably a mixture of degradation products from the enzymic treatments. The additional treatments of pronase digestion, phenol deproteinisation and dialysis following RNAase digestion successfully removed the contaminants which had produced the high background absorbance on the CsCl gradients. The absorbance profile obtained after the centrifugation of DNA samples which had been subjected to the complete purification procedure (Method II showed two absorbance peaks in the same relative positions as the bands described by Adam et al (1969). The width of the nuclear DNA band was slightly greater than that found for DNA prepared by Method I, suggesting that the increased handling of the DNA had caused some reduction in its molecular weight.

The omission of phenolphthalein diphosphate from the extraction solution was a more trivial modification. Either of the reagents sodium diethyldithiocarbamate or phenolphthalein diphosphate was effective in releasing DNA from Acanthamoeba (Blewett 1969). Phenolphthalein diphosphate is expensive and since it was not essential to the release of DNA from the amoebae, it was omitted from the extraction solution. In its final form Method II greatly simplified the preparation of DNA from Acanthamoeba by substituting chemical purification in place of repeated CsCl centrifugation. In fact the CsCl centrifugation step

was only required as a means of separating nuclear from mitochondrial DNAs and this operation could possibly be performed by the use of HAP columns since the mitochondrial DNA has a lower thermal denaturation temperature and could be selectively eluted from the column.

II. Isotopic labelling of DNA.

DNA hybrids are generally recognised using radio-active tracer techniques and the sensitivity of detection of hybrids depends almost entirely on the specific activity of the labelled DNA. The radio isotopes commonly used to label DNA are the beta emitters tritium, carbon-14 and phosphorus-32. Of these, phosphorus-32 was chosen as the most suitable isotope for labelling Acanthamoeba DNA because it can be detected in aqueous solution by the Cerenkov counting technique and it is relatively inexpensive. It was known that amoebae grown in peptone medium containing 5 - 10μCi ^{32}P / ml incorporated the isotope into their DNA but the specific activity of DNA labelled in this way was low. Much higher specific activities could be obtained by simply increasing the concentration of ^{32}P in the medium, but to make this technique practicable it was necessary to develop a method for obtaining high concentrations of amoebae in small volumes of culture.

Past experience had shown that the amoebae did not grow well in small stirred cultures, nor did they grow to a high concentration in stationary cultures. Experiments were carried out to test the growth of amoebae in small shaken cultures. The results of these experiments showed that A.castellanii Neff grew as readily in small shaken cultures as it did in the larger stirred cultures and reached a high concentration without the formation of cysts. An unusual feature of the shaken cultures was the formation of large clumps of amoebae containing several hundred cells. This clumping of the amoebae appeared to have no adverse effects and may even have been beneficial in that the clumps

formed a substrate on which the amoebae could complete cytokinesis, for the amoebae grown in shaken cultures did not become multinucleate (Band and Machemer 1963).

The DNA from amoebae grown in shaken cultures containing 1mCi of ^{32}P /30ml of medium had a high specific activity. The yield of amoebae from a single culture was small but it was possible to extract the DNA from as little as 0.1ml of packed cells using Method I. The problems of purifying the radio-active DNA were approached in the same way as those of purifying unlabelled DNA, and the procedure finally adopted was a simplified form of extraction Method II. The criteria used to evaluate the effectiveness of the purification treatments were the same as those employed in developing the modified extraction method. The assumption that the radio-active material which banded on the CsCl density gradients was DNA was based on the facts that,

i) during preparation the addition of ethanol produced fibrous precipitates typical of the normal DNA preparations

ii) the activity profiles obtained from CsCl gradients were almost identical to the absorbance profiles obtained with unlabelled DNA

iii) when samples of the high activity bands from a preparative CsCl gradient were heated on HAP columns, they produced thermal chromatograms typical of the nuclear and mitochondrial DNA of Acanthamoeba.

III. Hydroxyapatite fractionation.

The technique of hydroxyapatite chromatography has been used to study the renaturation and hybridisation of DNA. Under certain conditions single stranded DNA may be eluted from HAP while native DNA remains bound. By manipulation of the phosphate buffer concentration it is possible to elute selectively single stranded DNA from a mixture of native and denatured DNA.

Using the same property of HAP, native DNA may be bound to a column of HAP and eluted with phosphate buffer at a constant concentration but at increasing temperatures until the thermal denaturation temperature is reached at which point the DNA will become single stranded and may be eluted from the column. It was intended to use HAP chromatography for both the fractionation of hybridisation mixtures and for thermal denaturation and tests were carried out to determine the conditions needed to elute single stranded DNA from the HAP.

Hydroxapatite was prepared and used following the methods of Miyazawa and Thomas (1965). The radio-active DNA was treated with ultrasound before use because high molecular weight DNA does not flow readily through HAP columns. Acanthamoeba DNA has a high GC content and hence a high thermal denaturation temperature. To ensure that the test DNA was fully denatured it was heated for 10 minutes at 100 °C in 0.06M buffer then quenched at 0 °C before being absorbed on to HAP. A phosphate buffer concentration of 0.15 M was found to be effective for eluting single stranded DNA from HAP, but a proportion of the DNA was not eluted until buffer concentrations reached 0.18M and 0.36M. The fact that some of the DNA eluted at higher buffer concentrations than expected was explained in the light of the initial renaturation experiments as evidence for the presence of a rapidly renaturing fraction in the DNA of Acanthamoeba. It is more likely however that in these early experiments the volume of eluant used to wash the columns was insufficient. In subsequent experiments HAP fractionation was used to separate single stranded DNA from hybridisation mixtures and the ratio of buffer to HAP was much higher. In these experiments very little of the DNA in the zero incubation time samples remained bound after washing with 0.15M buffer.

DNA was thermally denatured on HAP columns at a buffer concentration of 0.12M. Acanthamoeba nuclear DNA has a base composition of 61% GC and a thermal

denaturation temperature of 97°C in SSC (0.15M NaCl). Because the thermal denaturation temperature of a given DNA increases with the ionic strength of the solution, it was important to use the lowest buffer concentration capable of eluting single stranded DNA from HAP, in order to obtain the best thermal denaturation curves. Even with the use of 0.12M buffer rather than the chosen optimum concentration of 0.15M the melting curves obtained from samples of nuclear DNA did not follow the complete sigmoid curve expected and the value for Tm was 90°C, some 5°C below the expected value. The low thermal denaturation temperature was at first ascribed to difficulties encountered in heating the column above 95°C with a water circulating system. In later experiments, when an oil circulating system was used to heat the column, a more satisfactory denaturation curve was obtained and column temperatures of up to 99°C were achieved, but the value for Tm was still lower than expected. The apparently low Tm was not due to a failure to denature the DNA, because a final elution at 95 - 100°C with 0.36M buffer did not remove a large amount of radio-activity from the column.

IV. Renaturation of Acanthamoeba DNA.

The rate at which DNA reassociates depends upon the frequency with which complementary sequences collide. The greater the concentration of a particular sequence the greater the rate at which it will reassociate. DNA from an organism with a small genome comprising few sequences will, at a given concentration, contain many more copies of its few sequences than will the DNA from an organism with a large genome made up of very many different sequences. This relationship between the complexity of DNA (in terms of the number of different sequences and genome size) and thermal renaturation was demonstrated by Marmur and Doty (1961) through comparisons of the extent to which different DNAs renatured. Subsequently Britten and Kohne (1966) and Wetmur and Davidson

(1968) derived quantitative relationships between renaturation rate and complexity of DNA and showed that the DNA of eucaryotes contained fractions of differing renaturation rates. These fractions are described in terms of their renaturation rates as fast, intermediate and slow. The existence of these fractions stems from the frequency with which identical or similar sequences are repeated in the genome. The fast fraction consists of sequences that are repeated up to a million times or more (Waring and Britten, 1966, Britten and Kohne, 1966), while at the other extreme, the slow fraction is made up of sequences which are unique or at best repeated a few times only. The intermediate fraction contains sequences which are repeated perhaps a few hundred times, or of groups of sequences that, though not identical, are sufficiently similar to reassociate.

The fact that the DNA of eucaryotes does not renature as a homogeneous population of sequences has a profound effect on the design of hybridisation experiments. Before attempting to compare base sequences in Acanthamoeba by hybridisation it was important to establish how the amoebal genome was organised into renaturing fractions and to determine the conditions under which a high proportion of the DNA would renature. The method of Wetmur and Davidson (1968) was used for the preliminary investigation because the spectrophotometric technique is the simplest and most direct method of following the course of DNA renaturation. Also, when the data are plotted to show the second order reaction rate the different renaturing fractions can be readily detected.

From the results of the spectrophotometric determinations of the renaturation rate of Acanthamoeba DNA it was concluded that most of the DNA was too complex to renature at the low concentrations used. Incubating the high GC content DNA of Acanthamoeba castellanii at 70 °C rather than 60 °C would

have increased the renaturation rate, but because it was intended to use in hybridisation studies DNA from other species which had a lower GC content it was decided to use a standard incubation temperature of 60 °C throughout. Increasing the ionic strength of the solution to 1M NaCl would also have increased the renaturation rate but duplex formed under these conditions might prove unstable when returned to the solutions of low ionic strength required for HAP fractionation.

Britten and Kohne(1966) introduced the term Cot for the product of DNA concentration multiplied by time of incubation and showed that the Cot value required for 50% renaturation was dependent on the complexity of the DNA. To obtain a higher percentage renaturation of Acanthamoeba DNA it was therefore necessary to incubate samples of DNA to a high Cot value, that is, to incubate concentrated solutions of DNA for long periods. Acanthamoebae are not a particularly rich source of DNA and although the modified extraction procedure greatly simplified the preparation of DNA the amounts available were not large. To achieve high concentrations of DNA it was therefore essential to reduce the volume of the solution to a minimum. The renaturation of Acanthamoeba DNA introduced several technical problems. Small volumes of solution had to be incubated for long periods without excessive evaporative losses; samples of the solution had to be collected at intervals to follow the progress of renaturation and the samples had also to be assayed to determine the extent of renaturation.

The problems of sampling the renaturation mixture were solved by dividing the solution into aliquots before incubation rather than repeatedly removing samples from a single sealed container. This, of course, reduced still further the volume of the solutions to be incubated but because these volumes were so small the samples could be sealed into individual capillary tubes thereby

preventing evaporation. The capillary tube technique made it possible to reduce the volume of the DNA solutions to 1ml, making it easier to obtain DNA concentrations of up to 1mg/ml. Further difficulties were encountered in handling such small volumes of DNA solution during preparation for renaturation; these difficulties were overcome by:

i) embedding the sonication tube in ice to prevent overheating the solution

ii) denaturing the DNA solution in a modified pasteur pipette to reduce evaporation

iii) treating all the glassware with a silicone coating to improve the recovery of solutions.

The techniques for manipulating small volumes of DNA solution proved to be successful and an almost quantitative recovery of the solutions was achieved. The method for analysing the reaction mixture was less satisfactory. The most direct method appeared to be the accurate dilution of the samples followed by the measurement of their absorbance at 60°C, from which the hypochromicity of the solution could be calculated. In practice this method did not give consistent results, presumably because the dilution and absorbance measurements were not sufficiently accurate. Estimations of the percentage renaturation based on the restoration of hyperchromicity were more consistent, probably because the measurements were relative, not absolute, and were less susceptible to errors in dilution. Since the dilution technique was not reliable it was not possible to determine accurately the original absorbance for the native DNA in each sample and calculations of the percentage renaturation were accordingly based on an assumed hyperchromicity factor of 1.36 for native DNA (Wetmur and Davidson 1968). When Acanthamoeba nuclear DNA was denatured in the preliminary renaturation experiments the hyperchromic rise was very close to a value of 1.36. Errors arising from the use of the 1.36 hyperchromicity factor were probably less

significant than errors in the measurement of the small changes in absorbance during thermal denaturation of the diluted samples. Variations in absorbance were magnified when calculated out as estimates of percentage renaturation, the plots of renaturation rates of amoebal DNAs show that although the points are clustered about a straight line there is a considerable scatter. Despite the inaccuracies in the assay method the capillary tube renaturation technique provided the required information on the conditions necessary to obtain a high percentage renaturation of Acanthamoeba DNA without using either very large amounts of DNA or relaxed conditions of renaturation.

V. Hybridisation of Acanthamoeba DNA.

Having developed a technique for obtaining a high percentage renaturation of homologous DNA it was possible to apply this technique to the production of hybrid DNAs. Hybrid DNAs are prepared by renaturing together the DNA from different organisms and hybridisation techniques differ in the methods used to distinguish the hybrid DNA from the homologous reaction. The use of isotopes is common to all hybridisation techniques; Schildkraut et al (1961) used the mass isotope N15 to label bacterial DNA and detected hybrid DNA by analytical ultracentrifugation. Radio-isotopes are more commonly used to label DNA for hybridisation studies; in the agar (Bolton and McCarthy 1962) and nitrocellulose filter (Denhardt 1966) techniques the hybrid DNA is detacted by measuring the amount of radio-activity which is bound to physically immobilised DNA.

The agar and filter techniques have been used to study base sequence homologies among many groups of organisms. (Kingsbury 1967, Heberlein, DeLey and Tijgat 1967, Hoyer, McCarthy and Bolton, 1964, Gibson 1966). The application of these techniques to the hybridisation of procaryote DNA has

provided useful information on taxonomic relationships; the same hybridisation techniques have proved less successful, however, when applied to eucaryote DNAs. The agar technique provides an estimate of the extent of sequence homology between two DNAs based on the ratio between the homologous and the heterologous reactions. Whereas the simple procaryote DNAs may renature to a high final level under the conditions used in hybridisation experiments, the more complex eucaryote DNA may be only partly renatured. Estimates of the sequence homology between eucaryote DNAs may therefore represent comparisons of only a part of the two genomes. The presence in eucaryote genomes of DNA fractions of differing renaturation rate may also influence the results of hybridisation studies. The intermediate DNA fraction consists of similar but not identical sequences, the so called families of sequences of Britten and Kohne(1966); it is thought that closely related organisms share a large number of families of sequences. Hybridisation of eucaryote DNAs may reveal homologies among these families of sequences at the expense of the more slowly renaturing fractions of the two genomes.

A second factor which complicates the interpretation of hybridisation studies is the fact that the heterologous renaturation rate is lower than that of the homologous reaction (Sutton and MacCallum, 1971). This effect is attributed to the obstruction of complementary sequences by the formation of temporary unstable duplex between non-complementary strands. If estimates of sequence homology are to be based on the ratio between the homologous and the heterologous reactions it is important that this difference in renaturation rates is taken into account.

The reassociation of DNA is not an all or nothing phenomenon and the degree of sequence homology necessary before two DNA strands will renature is greatly influenced by the conditions of incubation, especially by the

temperature and the ionic concentration. These factors are also important if the hybridisation technique adopted depends upon HAP chromatography for the separation of the hybrid DNA. Hydroxyapatite columns can distinguish between double and single stranded DNA and this property has been used to separate hybrid DNA from hybridisation mixtures. But since partially renatured DNA elutes from HAP at a lower buffer concentration than well formed duplex, the buffer concentrations used for HAP fractionation effectively define the hybrid DNA. The quality of the hybrid DNA is important and should be considered in all hybridisation studies. Laird et al (1969) showed that the thermal stability of renatured DNA was dependent on the goodness of fit of the complementary strands. A depression of $1^{o}C$ in the thermal denaturation temperature was found to indicate that approximately 1.5% of the base pairs were mismatched.

Before attempting to use hybridisation techniques to explore sequence homologies between amoebae it was necessary to ensure that -

i) the greatest possible proportion of the test genomes were being compared, i.e., the hybridisation mixture must be incubated under conditions such that the homologous reaction nears completion.

ii) the quality of the hybrid was tested rather than the quantity.

The hybridisation technique adopted for comparing sequence homologies between Acanthamoeba DNAs depended upon the capillary tube renaturation technique for achieving a high percentage renaturation of the homologous DNA, and used HAP fractionation to select the hybrid molecules. The first hybridisation experiments (E 4.1) were carried out primarily to determine the amount of hybrid DNA formed compared to the homologous reaction. The results of this experiment showed that HAP fractionation of the reaction mixture

permitted estimates of the percentage hybridisation but introduced problems of defining duplex molecules. This problem was avoided in the first experiment by considering all DNA that eluted above 0.15M buffer as duplex, since the DNA in the 0.18M fraction was sufficiently well renatured to undergo thermal denaturation on heating on HAP. The difficulties of selecting hybrid molecules on HAP might have been reduced by treating the renatured DNA with nucleases to remove single stranded free ends from the renatured complementary sequences. Nevertheless, the definition of what was and was not good duplex was critical to the HAP fractionation technique for assaying the formation of hybrid DNA. Under the fractionation conditions used in experiment E 4.1 it was found that the final levels of hybrid produced were very similar for the three heterologous amoebal DNAs tested, though the rate of hybrid formation appeared to decrease with increasing differences between the amoebae.

To determine the thermal stability of the hybrid DNA the capillary tube renaturation technique was modified so that the whole DNA sample was concentrated into a single tube. The test DNA was sonicated and denatured as usual, then dialysed against distilled water and finally dehydrated. The dried DNA was redissolved in 0.05ml of buffer containing radio-active DNA; by this means very high DNA concentrations were achieved and consequently very high Cot values. The effect of dehydration on the DNA was not tested, but Britten and Kohne (1966) used dehydrated, commercially prepared DNA for renaturation experiments and Gelderman et al (1971a) used a similar technique of dehydration and re-solution for the renaturation of Entamoeba DNA.

The final level of the homologous reactions in the second series of hybridisation experiments (E 4.2) was high and indicated that the hybrids were formed after a large proportion of the sequences in the genomes being tested

had been free to react. Because the homologous reaction was continued to a very high Cot value and reached a high final level it was reasonable to assume that even if the heterologous reaction rate were depressed, as suggested by Sutton and MacCallum (1971), the amount of hybrid formed would be close to the true final level. The reaction mixture was analysed by absorbing all the DNA on to HAP at low temperature and eluting at a constant buffer concentration with increasing temperature. In this way the quality of the hybrid formed could be estimated. In its final form the technique adopted fulfilled the requirements listed above in that -

i) the homologous reaction reached a high final level, indicating that a large proportion of the genomes had been compared, and

ii) the comparisons of the two DNAs were based on the quality of hybrid formed rather than on the final levels of arbitarily defined "good" duplex.

B. Results.

I. The DNA of Acanthamoeba

The DNA of Acanthamoeba contains two components, the minor component was identified as mitochondrial DNA by its renaturation rate (Adam et al, 1969). Further evidence for the mitochondrial origin of the minor DNA was obtained in this study from the presence of enhanced amounts of this DNA in isolated mitochondria and from the observation that some of the DNA in this component is in the form of closed circles. The circularity of mitochondrial DNA was first described by Nass (1966) and since then the mitochondrial DNA of a large number of chordates has been found to contain circular molecules. Among the

metazoa, the circumference of the circular mitochondrial DNA molecules is remarkably uniform; contour lengths of about 5μ have been reported for the mitochondrial DNA of mammals and birds (Sinclair et al, 1967), amphibia (Wolstenholme and Dawid, 1967) and arthropods (Polan et al, 1973, Tanguay and Chaudhary, 1972).

Mitochondrial DNA of micro-organisms is more varied in size. Agsteribbe Kroon and Van Bruggen (1972) obtained circular DNA with a contour length of 19μ from Neurospora crassa. The kinetoplast DNA of the haemoflagellates contains complexes of minicircles ranging in circumference from 0.3 - 0.8μ (Renger and Wolstenholme, 1970, Simpson and Da Silva, 1971) as well as longer linear molecules. Suyama and Miura (1968) were unable to find circular molecule in the mitochondrial DNA of Tetrehymena and reported the presence of linear molecules 17μ long. From renaturation studies Flavel and Jones (1970, 1971) estimated a molecular weight for Tetrahymena mitochondrial DNA of 30 million, a value very close to the expected molecular weight of a 17μ long molecule.

The mitochondrial DNA of Acanthamoeba was found to contain circular molecules with a circumference of 14 - 15μ. The number of circular molecules found was low, but in view of the difficulties experienced in developing a DNA extraction method for Acanthamoeba (Blewett 1969, Kirtikar et al 1967) the paucity of circles may have been due to degradation of the DNA during extraction. Apart from the unique kinetoplast DNA of the haemoflagellates, circular mitochondrial DNA has not been described for any other species of protozoa.

The major component of Acanthamoeba DNA must be nuclear DNA, although this could not be demonstrated by extracting DNA from isolated nuclei. Measurements of the base composition of both components of the DNA of different Acanthamoeba spp. show a surprising uniformity. Of 11 strains of species

tested 7 have the same DNA base composition as the Neff strain of A.castellanii, despite the fact that the Singh, H and W1 strains were isolated in Britain while the Neff, P27, A30 and A35 strains were isolated in the USA. It is possible that the procedure used to isolate amoebae from soil or water, exerts a strong selective pressure in favour of A.castellanii, though the abundance of amoebae in isolates from water or soil suggests that this is unlikely. The DNA has been characterised from too few isolates to determine whether or not the base composition of acanthamoebae varies broadly throughout the range for the genus but if Acanthamoeba is truly asexual there is no reason why the DNA base composition of the amoebae should not vary more widely.

II Renaturation of Acanthamoeba DNA.

The capillary tube renaturation technique was developed to study the renaturation of amoebal DNA as a preliminary to the hybridisation experiments. The results of the renaturation experiments indicated that almost the whole genome of Acanthamoeba was made up of slowly renaturing DNA. Britten and Kohne(1966) showed that a Cot curve (percentage renaturation plotted against log Cot) of a single renaturing fraction of DNA follows a sigmoid curve similar to a thermal denaturation curve. The mid portion of the curve follows a straight line, which if extrapolated to the axis, shows a hundred-fold increase in Cot between 0% and 100% renaturation. An increase much greater than 100 indicates that the DNA sample is not homogeneous and contains fractions of differing renaturation rates. The Cot curves for Acanthamoeba DNA suggest that the DNA was not a single homogeneous renaturing fraction, but since the technique used to estimate percentage renaturation was slow the Cot curves were drawn through few points and it was not possible to recognise fractions of slightly different renaturation rates. The Cot values for the

DNAs tested fell within the range 50 - 100, values consistent with the whole genome forming a slowly renaturing fraction. A.castellanii (Neff was estimated to contain 0.5 picograms of DNA (Blewett 1969) while Byers, Rudick and Rudick (1969) reported DNA contents of 2.3 picograms in log phase cells and 1.1 - 1.3 picograms in early stationary phase cells of Acanthamoeba. Byers et al grew the amoebae in stirred cultures in which a large proportion of the amoebae become multinucleate (James and Byers 1967, Kjellstrand 1968). If an allowance is made for the fact that the amoebae become multinucleate, then the genome of Acanthamoeba may be estimated to contain between 0.5 and 1.0 picogram of DNA, corresponding to about 10^8 base pairs. On the basis of this estimate Acanthamoeba DNA is intermediate in complexity between bacterial and mammalian DNA, similarly the $Cot\frac{1}{2}$ of Acanthamoeba DNA as measured by the capillary tube renaturation technique was intermediate between those of E.coli DNA ($Cot\ \frac{1}{2} = 10$) and calf thymus slow fraction DNA ($Cot\ \frac{1}{2} = 3{,}000$) (Britten and Kohne 1966).

The $Cot\frac{1}{2}$ for Acanthamoeba DNA is within the range that has been found for the DNAs of other species of protozoa, as shown in Table D1. The values for $Cot\frac{1}{2}$ of the few protozoan DNAs which have been tested range from 34.6 in E.histolytica (Laredo) to 380 in P.aurelia, and 400 in E.moshkovski. The results obtained by Allen and Gibson (1972) with Paramecium and Tetrahymena DNAs gave values for Cot $\frac{1}{2}$ which were more than double those obtained by Flavel and Jones (1971) and Soldo and Godoy (1972). There was also a considerable difference in the estimates of the proportion of repeated sequences in the DNA of these ciliates. Allen and Gibson (1972) used an HAP fractionation technique to measure renaturation of ciliate DNA, whereas Flavel and Jones (1971) and Soldo and Godoy (1972) measured renaturation rates in 1M NaCl using Wetmur and Davidson's (1968) technique. It is possible that the discrepancies between

Table D1. Renaturation rates of protozoan DNA.

Ref.	Organism	$Cot_{\frac{1}{2}}$	Kinetic complexity x 10^{11}	% repeated fraction
1.	P. aurelial/540	272	1.84	15
	1/90	280	1.89	15
	2/71	290	1.96	15
	8/138	380	2.57	15
	8/229	380	2.57	15
	8/299	380	2.57	15
	T. pyriformis mating type I			
	1/8	215	1.45	20
	1/D	230	1.55	25
	1/D/1	255	1.72	20
	7/UC-651	240	1.62	30
	1/7	100	0.68	20
	1/C	130	0.88	15
2.	T. pyriformis 1/A	-	0.43	5
	T	-	0.40	5
3.	P. aurelia 299	-	0.64	4
4.	C. reinhardi	-	0.46	0
5.	E. histolytica (Laredo)	34.6	-	-
	E. histolytica HK9	110	-	-
	E. moshkovski	400	-	-
-	Acanthamoeba spp.	50-100	-	7-10
-	T. pyriformis	100	-	-

References.

1. Allen & Gibson 1972
2. Flavel and Jones 1971
3. Soldo and Godoy 1972
4. Bastia et al 1970
5. Gelderman et al 1971a

the results of renaturation rate studies on ciliate DNAs are due to differences in technique.

III Hybridisation of Acanthamoeba DNAs

The results of the hybridisation experiments using the thermal stability technique showed that this technique could distinguish between Acanthamoeba DNAs. DNA from the H, Singh and Wl strains of A.castellanii was hybridised with A.castellanii Neff strain DNA. The final levels of the hybridisation reactions were almost the same as those of the homologous reactions. The thermal denaturation curves for the hybrid DNAs were parallel to those of renatured Neff strain DNA, and the observed depression of the thermal denaturation temperature was 2.5°C, which according to Laird et al (1969) represents approximately 4% mismatching between the base sequences of the strains tested. These results were as expected, the DNAs from the four strains of amoebae were very similar in base sequence to the Neff strain DNA.

The amount of hybrid DNA produced between Neff strain DNA and those of A.palestinensis and A.polyphaga was about two thirds that of the homologous reaction. The thermal denaturation curves of the interspecific hybrid DNAs differed qualitatively from the denaturation curves of the intraspecific hybrids. Up to 30% of the genomes of A.polyphaga and A.palestinensis are made up of sequences which are not present in the genome of A.castellanii Neff strain. The sequences that are shared between the species and are sufficiently similar to renature formed less stable hybrids than those formed between strains of A.castellanii. Using the relationship of Laird et al (1969) the Neff-polyphaga hybrid was estimated to be mismatched by 7.5% and the Neff-palestinensis hybrid by 9%. The base composition of A.astronyxis DNA is 50% GC, 11% lower than that of A.castellanii and as expected there was no

detectable sequence homology between these DNAs.

The DNA from six different Acanthamoebae was compared to A.castellanii Neff strain DNA using the hybridisation technique developed. The results of the hybridisation experiments were as expected; the DNA of the H, Singh and W1 strains was almost identical to that of the Neff strain while the two species A.polyphaga and A.palestinensis showed greater differences and A.astronyxis DNA was distinctly different in base sequence.

The present system for the classification of the naked amoebae was founded by Schaeffer (1926) who proposed a system based on characters such as size and form in locomotion, nuclear properties and cytoplasmic inclusions. Unfortunately Schaeffer did not attempt to classify parasitic amoebae or free living soil amoebae, but Bovee (1953) claimed that Schaeffer's system was suitable for the classification of small amoebae. Subsequently Bovee and Jahn (1965) and Jahn and Bovee (1965) divided the Sarcodina into two classes on the basis of the mechanism of movement. Singh (1952, 1972) and Singh and Das (1970) maintained that morphological characters such as shape, pseudopodial form and size are too unstable to be used as generic criteria and proposed instead that the Amoebida should be divided into three families on the basis of the mode of nuclear division. In view of the acknowledged difficulties in the classification of naked amoebae and the contrasting taxonomic systems that have been proposed by different workers, it is not surprising that there is some confusion over the identity of amoebae in the genus Acanthamoeba. The classification of the ameobae which have been studied in this thesis follows that proposed by Page (1967), who adopted an approach similar to that of Schaeffer (1926) and defined several genera of small free living amoebae (Page 1967, 1967a).

Comparative studies of Acanthamoeba by Adam (1964) and Adam et al (1969)

indicated that A.castellanii, A.palestinensis, A.polyphaga and A.astronyxis are distinct species, and that the isolates tested were strains of A.castellanii. These findings, based upon biochemical and serological evidence, are in agreement with the conclusions of Page (1967) drawn from studies of the morphology of the amoebae. The results obtained in this thesis are consistent with present information on the taxonomic status of the amoebae tested. The characterisation of amoeba DNA showed that out of six strains tested, five were similar in base composition to A.castellanii. Of these five strains three were assigned to the species A.castellanii by Page (1967), the remaining two strains may be tentatively included in the same species. Isolate Al, which differed in base composition was shown by Adam (1964) to be serologically distinct from the Neff, Singh and Wl strains of A.castellanii.

A knowledge of the base composition of amoeba DNAs has been useful in distinguishing between species of Acanthamoeba. Of the eleven amoebae which have been tested to date, seven possessed "castellanii type" DNA; three amoebae differed markedly from the "castellanii type" in the base composition of either or both DNA components and only one ameeba (A.palestinensis) showed a small difference in base composition from the "castellanii type". The DNA has been characterised from too few isolates of Acanthamoeba to be able to predict whether or not the genus will exhibit a broad spectrum of base composition values or whether the amoebae will fall into discrete groups of characteristic base ratios. Analysis of DNA base composition has, so far, been sufficient to distinguish between known strains and species of Acanthamoeba. It is not certain that this method would continue to be sufficient if it were to be applied to the classification of larger numbers of isolates of Acanthamoeba. The value of base composition measurements in taxonomy is limited by the fact that identity in base ratios does not imply conspecificity, and by difficulties

in assessing the significance of small differences in base composition. The hybridisation technique which has been developed helps to reduce these limitations by making it possible to test for sequence homology in DNAs of identical base composition and by determining the extent to which sequences differ between DNAs of similar base composition.

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APPENDIX Yields of DNA obtained using the original and modified preparation procedures.

Wt. packed amoebae (grams)	yield of DNA (ug)*	ug DNA/gram packed amoebae
i) Original procedures		
20	1,104	55
10	342	34
10	600	60
15	784	52
ii) Original extraction method and modified CsCl technique		
5	114	23
5	116	23
iii) Modified extraction and CsCl techniques		
10	371	37
16	538	34
13	461	34
15	300	20
12	319	26
10	225	22
7	184	26
14	300	21

* Amount of DNA recovered from the nuclear DNA band after the first centrifugation on a preparative CsCl gradient.

APPENDIX. Thermal denaturation data for Acanthamoeba hybrid DNAs.

Thermal denaturation data for Neff-W1 hybrid DNA.

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
30.5	1171	5.5
35.0	445	7.7
41,0	424	9.7
46.0	417	11.6
51.0	489	13.9
55.0	291	15.3
60.5	273	16.6
66.5	324	18.1
71.5	631	21.1
75.5	1095	26.2
80.0	2283	36.9
82,5	2418	48.3
85.0	2022	57.8
88.0	2721	70.6
90.0	2331	81.5
92.5	1825	90.1
95.0	1125	95.4
0.36M PB.	966	100.0

Thermal denaturation data for Neff-H hybrid DNA.

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
25.0	441	2.9
30.0	132	3.8
35.0	97	4.5
40.0	89	5.1
45.0	193	6.4
51.0	299	8.4
55.5	377	11.0
61.0	335	13.2
66.0	455	16.3
71.0	811	21.8
76.2	1295	30.5
81.0	1678	41.9
83.0	1173	49.8
85.5	1808	62.1
88.5	1609	72.9
90.5	1860	85.5
93.5	878	91.5
96.5	955	98.3
0.36M PB.	250	99.9

Thermal denaturation data for Neff-Singh hybrid DNA.

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted
30.0	401	3.0
35.5	275	4.9
40.5	282	6.9
44.0	295	9.1
50.0	369	11.8
55.0	341	14.3
60.5	319	16.5
66.5	470	20.2
71.5	779	25.9
76.5	1092	33.8
81.0	1512	44.6
82.5	997	51.9
85.5	1649	64.3
88.0	1702	77.1
90.0	1106	85.2
92.5	812	90.9
95.5	478	94.2
98.0	295	96.1
0.36M PB.	506	99.5

Thermal denaturation data for Neff-*A.palestinensis* hybrid DNA

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
25.0	569	4.1
31.0	193	5.5
35.0	114	6.3
40.5	174	7.5
45.7	262	9.4
51.0	453	12.7
56.0	629	17.3
61.0	581	21.6
66.0	704	25.9
71.2	1250	35.1
77.0	2477	53.5
82.0	1317	63.1
84.5	785	68.9
87.5	892	75.5
90.0	1048	83.2
92.5	1116	91.4
95.2	638	96.1
98.2	230	97.8
0.36M PB.	205	99.3

Thermal denaturation data for Neff-A.polyphaga hybrid DNA

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
20.0	755	4.1
27.0	846	8.7
31.0	534	11.6
35.0	447	14.0
39.0	459	16.5
45.0	680	20.2
50.0	781	24.5
56.0	785	28.8
60.5	505	31.6
65.5	537	34.5
70.0	630	37.9
75.0	1168	44.3
80.0	2192	56.3
82.5	1930	66.8
85.0	1898	77.2
87.5	1396	84.8
90.0	1083	90.7
95.0	972	96.0
0.36M PB.	631	99.4

Thermal denaturation data for Neff-A.astronyxis hybrid DNA.

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
31.0	572	3.8
36.0	314	5.9
42.0	359	8.3
47.0	629	12.5
52.0	1153	20.2
55.0	1263	28.7
60.5	1614	39.6
66.0	3750	65.0
70.0	1132	72.6
75.0	869	78.4
77.2	466	81.5
80.5	373	84.0
82.7	315	86.1
85.0	377	88.6
88.0	388	91.2
90.5	367	93.6
93.0	390	96.2
96.5	316	98.3
0.36M PB.	161	99.4

Thermal denaturation data for Neff-Tetrahymena hybrid DNA.

Temp. C.	P32 counts/10 min.	Cumulative % P32 eluted.
35.0	1316	9.1
40.0	626	13.6
45.0	535	17.3
50.0	880	23.4
55.0	1597	34.5
60.0	2235	50.0
65.2	2068	64.4
70.0	1734	76.4
75.2	932	82.9
80.0	479	86.2
82.0	266	88.0
85.0	207	89.4
87.0	231	91.0
90.0	208	92.4
92.0	272	94.3
95.0	271	96.1
97.0	170	97.2
0.36M PB.	329	99.5