ISOLATION AND CHARACTERIZATION OF "NUCLEOPROTEIN" FROM *BACILLUS SUBTILIS*

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The regulation of enzyme synthesis is believed to occur through the mediation of a repressor molecule which in a few cases, has been characterized genetically and physiologically. However, to elucidate its biochemical details, the repressor molecule itself must be isolated and its interaction with the various elements of the regulated system studied[1]. There is some evidence that the repressor may be a protein[2]. It is hypothesized that protein may be combined with genetic material, DNA, and the protein may be involved in the regulation of gene expression[3]*.

On the other hand nuclear DNA has been observed cytochemically together with protein and RNA in a particle in cells of bacteria such as Bacillus megaterium [4]. Attempts have been made to isolate these particles, so called "Nuclear fraction", from B. megaterium[5], Escherichia coli[6], as well as Bacillus subtilis[7], but in most of these cases characters of these nuclear fraction have not studied extensively.

In our laboratory, a series of attempts have been made to dlucidate the molecular repression mechanism in protein synthesis using B. subtilis[8,9]. To investigate

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* Recently, the biochemical character of a repressor has been elucidated[26] and even the repressor protein molecule was isolated in the other case[28].

the regulation of protein synthesis at the level of gene expression, we have tried to isolate an entity of gene expression and to investigate its character and biological role. We are able to isolate a nuclein acid - protein complex (designated as "nuclear fraction") which contains the majority of cell's DNA and which has constant composition of DNA, RNA, and protein in the ratio of 1 : 0.2 - 0.3 : 0.8 - 0.9.

Properties and biological roles of the protein in the complex have been studied in relation to DNA. It seems likely that the protein molecules are bound to DNA in a specific manner. Its biological role, however, is not yet clear. In this paper we mainly report the isolation method of the protein from the nuclear fraction and its properties and also discuss its possible biological meaning.

EXPERIMENTAL

Bacterial Strain --- B. subtilis marburg strain (SB 15) was a gift from Dr. Yukinori Hirota, Faculty of Science, Osaka University and was used throughout these experiments.
Growth condition --- Bacteria were cultured aerobically at 37°C in the following medium: 1% Bacto-peptone (Difco Lab. certified grade USA), 1 % sodium lactate, 0.24 % Tris,

0.3 % NaCl, 0.005 % $MnSO_4$, 0.03 % $MgSO_4 . 7H_2O$, 0.01 % $CaCl_2 . 2H_2O$. Bactopeptone contained varing amounts of phosphate depending upon the batch used. Before use, phosphate was removed from the medium as a precipitate of magnesium phosphate and the final inorganic phosphate ion concentration of the medium was adjusted to 0.25 mM by adding potassium phosphate.

After inoculation with 5 % (v/v) of an overnight culture, the bacteria were incubated for 3 to 3.5 hours at 37°C. When the cells reached OD_{650} = 0.8, they were chilled with ice and were harvested by centrifugation. One liter of the culture medium yielded about 1.5 g. cells (wet weight).

Buffers used in preparation procedure: --- Buffer A; 10^{-2} M Tris-acetate pH 7.3 containing 10^{-2} M magnesium acetate, 10^{-3} M ethylenediaminetetraacetic acid (EDTA), and 10^{-2} M β-mercaptoethanol.

Buffer B; 10^{-2} M Tris-acetate pH 7.3 containing 10^{-4} M magnesium acetate, 10^{-5} M EDTA and 10^{-3} M β-mercaptoethanol.

Buffer C; 10^{-2} M Tris-acetate pH 7.3 containing 10^{-2} M magnesium acetate, 10^{-4} M EDTA and 10^{-2} M β-mercaptoethanol.

Isolation of nuclear fraction: --- Experiments were carried out at 2 - 4°C throughout the isolation

procedure. The principle of the procedure was based on Butler's procedure[5] used for B. megaterium. The summary of the procedure is schematically illustrated in Fig.1.

Step a. Preparation of protoplasts and lysate: --- One gr. of wet cells was suspended in 10 ml of 0.3 M sucrose in Buffer C and 2 mg of egg white lysozyme (Sigma Chemical Corp., USA. 3 x crystallized) was added. The cell suspension was incubated in the cold room overnight, resulting in the complete conversion of the cells into protoplasts, which were observed under a phase contrast microscope. In order to lyse the protoplasts, EDTA and Lubrol W (donated from the Imperial Chemical Industries LTD., England) were added to the suspension at final concentrations of 10^{-3} M and 0.5 % respectively. After one hour, by which time most of protoplasts lysed, the lysate was subjected to the next preparation step.

Step b. Sucrose density layered centrifugation: --- Eight ml aliquots of 63 % sucrose and 45 % sucrose in Buffer A and 16 ml of 28 % sucrose in Buffer A were layered successively in a SW 25.2 tube with the most dense solution at the bottom. Twenty ml of the lysate of protoplasts were overlayered at the top, and the tube was centrifuged at 21,000 rev./min. (54,000 x g.)for 30 min. in the SW 25.2 rotor of a Spinco Model L. After centrifugation, the 45 - 60 % sucrose interphase was collected

with a pipette. Cell membrane fragments and most of the DNA were found in this fraction. This fraction was then diluted six-fold with 0.1 M sucrose in Buffer A and was centrifuged (78,000 x g.) for 40 min. to pellet the membrane fraction containing the DNA.

Step c. Separating membrane fragments from DNA-containing fraction: --- In order to detach the DNA-containing fraction from the cell membrane, 1 g of the resultant pellet was homogenized with a Potter homogenizer in 2 ml of 0.2 M sucrose in Buffer B. The suspension was centrifuged at 122,000 x g. for 30 minutes. The homogenization and centrifugation of the pellet were repeated twice. The fraction which sedimented mainly contained cell membranes. The supernatant collected from these three centrifugations was layered on a sucrose density gradient as follows.

Step d. Sucrose density gradient centrifugation: --- Five ml of the supernatant collected from the three centrifugations was layered on 44 ml of sucrose gradient (10 - 20 % in Buffer B) and centrifuged in the Spinco SW 25.2 roter at 24,000 rev./min. (70,000 x g.) for 16 hours. After the centrifugation, 15 drop fractions (about 2 ml/tube) were collected by puncturing the bottom of the tube. The optical density at 260 mμ and concentration of DNA, RNA, as well as protein in each fraction was measured.

As shown in Fig. 2, the main UV absorbing peak contain most of DNA, some protein, and RNA. The peak fraction was pelleted by a centrifugation (96,600 x g. 14 hr.) in order to remove soluble protein contaminated in it. The pellet was designated "nuclear fraction".

Enzyme assay of polymerases: --- RNA-polymerase (RNA nucleotidyl transferase: 2.7.7.6): Chamberlin & Berg's[10] assay system for RNA-polymerase was modified as follows: 3 μ moles Tris-HCl (pH 7.6), 2 μ moles magnesium acetate, 20 mμ moles EDTA, 1.6 μ moles $MnSO_4$, 0.4 μ moles β-mercaptoethanol, 10 μg DNA prepared from B. subtilis, 17 mμ moles each GTP, UTP and CTP and 17 mμ moles $C^{14}ATP$ (10^4 cpm) in a total volume of 0.1 ml. Enzyme samples were added to 0.1ml of the above assay system and were incubated at 37°C for 20 minutes. Blank samples minus enzyme were run concomitantly. The reaction was stopped by the addition of 1 mg bovine serum albumin (Armour Lab., USA.Fraction V) and 0.1 ml of 7 % perchloric acid. The precipitate was washed two times with the perchloric acid solution and was filtered on a type HA Millipore filter (Millipore Filter Corp., USA). The dried filters were counted with a liquid scintillation counter (Nuclear Chicago Corp. USA).

DNA-polymerase (DNA nucleotidyltransferase: 2.7.7.7): the assay system for DNA-polymerase of Richardson et at.[11]

was modified as follows: 10 μ moles Tris-HCl (pH 9.0), 0.8 μ moles magnesium acetate 0.2 μ moles EDTA, 0.4 μ moles -mercaptoethanol, 10 μg DNA of B. subtilis 5.8 mμ moles each d-GTP, d-CTP and d-TTP and 4 mμ moles C^{14}d-ATP (10^3 cpm) in total volume of 0.1 ml. Enzyme samples (0.1 ml) was added to 0.1 ml of assay system and incubated at 37°C for 20 minutes. Blank samples minus enzyme were run concomitantly. The reaction was stopped with perchloric acid (the same concentration as used for RNA-polymerase assay), was filtered on Millipore filter (HA type), and was counted.

Preparation of DNA --- From cells DNA was extracted by the method of Saito and Miura[12], and from the nuclear fraction DNA was also extracted by the same method except for lysozyme treatment.

CsCl density gradient centrifugation --- About 4 g of crystalline CsCl (Mitsuwa's pure chemicals K.K., Osaka, Japan) was added to 3.4 ml of a sample solution in Buffer B.

The refractive index of this solution was adjusted to 1.394 at 25°C with an Abbe Refractometer (Atago, Tokyo, Japan Model 302) and placed in a Spinco SW 39 tube. The tube was centrifuged at 38,000 rev./min. (118,000 x g.) for 48 hours in the SW 39 rotor at 2°C.

Chemical analysis of DNA, RNA and protein --- Samples were precipitated with 5 % perchloric acid, and washed twice with 5 % perchloric acid and once with 90 % ethanol.

The precipitates were dissolved in 2 ml of 1 N NaOH. Aliquots of this solution were used for the following analyses. DNA was measured by the diphenylamine reaction of Burton[13] using calf thymus DNA (Worthington Biochemical Corp., USA) as a reference standard. The blue colour was developed overnight at 37°C and the optical density of this colour was measured at 625 mμ . Alkaline labile RNA was measured by the orcinol method[14] using yeast RNA (Toyo Boseki Corp., Lot. G.) as standard. Protein was measured by the Lowry's method[15] using crystalline bovine serum albumin (Armour Lab., USA, FractionV) for standardization.

The amino acid composition of nuclear protein was analyzed using an automatic amino acid analyzer (Beckman Spinco Automatic Amino Acid Analyzer 120 B, USA) according to the manuscript of the analyzer. The protein hydrolysate was prepared by the method of Tsugita and Fraenkel-Conrat[16].

Electron microscopy of the nuclear fraction:--- The nuclear fraction was dialysed against water entirely, then fixed, and stained with 2 % phosphotungstic acid (pH 7) on a mesh. After dried the mesh, carbon particles were fixed on it and the nuclear fraction was observed by a electronmicroscope (Nihon Denki Kogaku, JEM-5, Japan).

RESULTS

Properties of nuclear fraction --- The nuclear fraction obtained as described in the experimental section contained DNA, RNA and protein in the ratio of 1 : 0.2 - 0.3 : 0.8 - 0.9 by weight (Table 2). This fraction accounts for 40 % of the total cellular DNA, 2 % of the cellular RNA, and 1 % of the cellular protein. This nuclear fraction was examined for homogeneity by sucrose gradient centrifugation. The resultant profile showed essentially a single peak and no other significant amount of UV absorbing material (Fig. 3). When the peak fractions in the repeated sucrose density centrifugations of the nuclear fraction were analyzed for DNA, and protein content, the weight ratio of these components was always constant within an experimental error. The peak fractions from the second and the third sucrose gradient centrifugation were both composed of DNA, RNA and protein in the ratio of 1 : 0.2 - 0.3 : 0.8 - 0.9. The ratio was approximately identical with that of the nuclear fraction obtained by centrifugation after the first sucrose gradient centrifugation. The nuclear fraction was also found in a single peak in Sephadex (G-75) chromatography as shown in Fig. 4. The conditions are shown in the legend to Figure 4. The UV absorption spectrum of the

nuclear fraction (Fig. 5) is similar to that of DNA with an maximum absorption at 258 mμ, and absorption minimum at 238 mμ: however the ratio of $OD_{260\ m\mu}/OD_{280\ m\mu}$ was 1.81.

The sedimentation property of the nuclear fraction was compared with that of DNA isolated directly from the cells by a phenol method described in the experimental section. The nuclear fraction and P^{32} labelled DNA were mixed together and subjected to a sucrose density gradient centrifugation. The sedimentation behavior of the nuclear fraction detected by absorption at 260 mμ and of P^{32}-DNA was similar, indicating that the "molecular size" of DNA and of the nuclear fraction are nearly the same (see Fig. 6). The sedimentation coefficient (Sw_{20}) of the nuclear fraction was measured as about 12S in 0.7 mM phosphate buffer, pH 6.9 (at $OD_{260\ m\mu}$= 1.0). This Sw_{20} value was smaller than that of DNA prepared from the cells (Sw_{20} = 20). This discrepancy may be explained by concentration dependency of a sedimentation constant.

The RNA- and DNA-polymerase activity were measured in each preparation step. As shown in Table 1, about a half of DNA-polymerase activity was present in supernatant fraction of the sucrose layered centrifugation and the other half in the fraction of cell membrane-DNA mixture. When the fraction of cell membrane fragments and DNA was

pelleted by the following centrifugation, almost all DNA-polymerase activity accompanied with it remained in the soluble fraction. RNA-polymerase, on the other hand, was found in the fractions containing the nuclear fraction throughout its preparation steps. The enzyme activity was recovered in good yield (80 - 90 % in each step) up to the sucrose layer centrifugation. During the repeated extraction of nuclear fraction into 0.1 M sucrose solution (cf. the step c in preparation of the nuclear fraction), inactivation of the enzyme took place, and only 30 to 40 % of the original activity was recovered in association with the nuclear fraction. This inactivation of the enzyme does not occur if the extraction was carried out in high sucrose concentration.

The nuclear fraction was also observed electron microscopically which was shown in Fig. 7a, b. Electron dense discs were mainly observed: their diameter was 0.2 - 0.9 μ and the surface of the discs seemed to be covered with globular protein like particles (Fig. 7A).

Isolation of the components of the nuclear fraction --- The purified nuclear fraction was treated in the following manner in order to effect separation of its components. The nuclear fraction was suspended in CsCl (0.91 g/ml.) solution in Buffer B and centrifuged as described in experimental part. The profiles are shown in Fig. 8.

The precipitated fraction was composed only of RNA and DNA was found in a peak located at density 1.70. This DNA fraction contained less than 10 % of RNA and less than 2 % of protein by weight. Most of protein was found at the top of the centrifuge tube, 30 % in a soluble form and 70 % in an insoluble film on the surface of the solution. This protein band also contained RNA as shown in Table 2.

In order to isolate DNA the nuclear fraction was shaken with an equal volume of 90 % phenol aqueous solution for 15 min. at room temperature. The water layer was centrifuged at 2,000 x g. for 15 minutes. Most of the DNA was recovered in this soluble aqueous layer and RNA was found both in the phenol layer and in the precipitated fraction from the aqueous layer. The precipitate contained RNA and protein but not DNA. The details of the analysis are shown in Table 3.

Protein was isolated by two different methods. 1) One volume of the nuclear fraction was added to two volumes of glacial acetic acid, yielding precipitate after 60 minutes at 4°C. The solution was clarified by centrifugation and the clear solution was then dialyzed against Buffer A. The protein precipitated upon completion of dialysis and was collected by centrifugation. 2) To a suspension of the nuclear fraction (150γ/ml) in Buffer B

were added 5×10^{-3} M magnesium acetate, deoxyribonuclease I (5γ/ml) (Worthington Biochemical Corp., USA) and snake venom phosphodiesterase (5γ/ml) (Worthington Biochemical Corp., USA). The mixture was incubated for 16 hours at 37°C, resulting in a white precipitate. The solution was dialyzed against Buffer B centrifuged at 7,000 x g. for 20 minutes. This precipitation was composed mainly of protein. After the digestion of the nuclear fraction, the resulting mixture was sometimes purified further by the acetic acid treatment described above.

Characterization of DNA isolated from the nuclear fraction --- The nuclear fraction DNA was centrifuged in a sucrose gradient and essentially a single peak was observed. Incubation of this peak fraction with ribonuclease I (100γ /ml) did not cause any significant change but incubation with deoxyribonuclease I (5γ/ml) made disappearance of 98 % of the non-dialysable optically dense material. This material contained RNA and partially digested DNA as shown in Fig. 13.

The DNA obtained by the CsCl density gradient centrifugation and DNA extracted from P^{32} labelled whole cell with phenol were centrifuged together in a sucrose gradient tube (Fig. 9). The peak of nuclear fraction DNA which was measurable by optical density at 260 mμ and peak of P^{32} were found in the same position,

indicating similar distribution in size of DNA chain obtained by the two different isolation procedure of DNA.

Characterization of RNA isolated from the nuclear fraction --- After fractionation of the nuclear fraction by the CsCl density gradient centrifugation, RNA was found mainly in the bottom of centrifugation tube (RNA I 41 %) and near the surface of CsCl solution together (RNA II 38 %) with protein, where protein also was located (Table 3). A minor part of RNA (RNA III 21 %) was also found with DNA peak (Fig. 8). The three fractions pooled from the CsCl density gradient and containing RNA I, II, and III (Fig. 8) were then recentrifuged separately on sucrose density gradient centrifugations. The resultant profiles of these centrifugations are shown in Fig. 10 a and b. The estimated Sw_{20} values are 10S RNA I and 3S for RNA II, taking the value for DNA as 20S. Judging from their density which was shown by their location on the CsCl gradient centrifugation, RNA I seems to be free RNA and RNA II may be combined with protein. However, the protein peak and RNA peak in Fig. 10 b are distinguishable, indicating that both the mixture of protein and RNA II-protein complex exist.

The following questions were then asked as to the minor component of RNA (RNA III): (a) does the DNA fraction contain rapidly labelled RNA ? (b) are ribosomes

or ribosomal RNA in this fraction ?

(a) The question (a) was attacked by a rapidly labelling RNA with P^{32} as follws. The cells were grown in the medium described in the experimental section (except phosphate concentration was reduced to 0.15 mM) and were harvested at an earlier growth stage ($OD_{650\ m\mu}$ = 0.7) in the previous experiment. The cells were converted into protoplasts as described in Experimental.

One gram of the protoplast were suspended in 10 ml of 0.3 M sucrose in Buffer C and to the suspension was added 0.6 mC(specific activity: 55.2 Ci/mg. P) of ortho-phosphate and incubated at 30°C. After 1 minute the solution was cooled and 10^{-3} M EDTA and 0.5 % of Lubrol W were added. The protoplasts were lysed by these procedures. The nuclear fraction (P^{32} labelled) thus obtained (Fig.11) was centrifuged on CsCl gradients. The centrifugation profile is shown Fig. 12. In this figure the rapidly labelled RNA was located in the peak of DNA (RNA III). However, the rapidly labelled RNA was found mainly in the membrane fraction (Fr. (8) in the preparation process of the nuclear fraction ; see Fig. 1) and this distribution was not altered by changing the magnesium concentration from 1×10^{-3} M to 5×10^{-3} M. According to these results the nuclear fraction seems to contain a part of the rapidly labelled RNA.

(b) With regard to the question (b), it can be assumed that free ribosomal subunits are not present in DNA-containing fraction (Fr.2 in Fig.8). Because under our experimental condition of CsCl density gradient centrifugation ribosomal subunits of B. subtilis sediment near the bottom of a centrifuge tube. Although ribosomes of E.coli[7] are located at a density of about 1.6 which is nearly coincident with density of DNA. However, one may wonder if the DNA-bound ribosomes exist in the DNA peak fraction. To answer this question, the P^{32}-labelled fraction (Fr.2 in Fig.8) was digested with deoxyribonuclease I (10γ/ml) for 30 min. at 30°C. Before and after the digestion, the fractions were centrifuged in a sucrose gradient and the result are shown in Fig.13.

After adding 5 % trichloroacetic acid (TCA) into each tube, radioactivity in the TCA precipitates was counted. Peak I in Fig. 13 was revealed to be RNA, peak II undigested DNA, and peak III partially degraded oligodeoxyribonucleotides. The remaining radioactivity in TCA precipitates was 2 % of the radioactivity before the digestion in peak I, 4 % in peak II, and 6 % in peak III, showing that about 94 % of total radioactivity present originally was digested and 88 % was removed as TCA soluble digests.

The peak I in Fig. 13 coincides with the 30S

ribosomal peak which is shown by a separate sucrose gradient centrifugation of ribosomes prepared directly from B.subtilis and which is indicated by an arrow in the Fig. 13.

Characterization of protein isolated from the nuclear fraction --- The two isolation procedures (descrived above) recovered 30 - 90 % of the total protein contained in the nuclear fraction. Analyses were made of the amino acid composition of the protein which was isolated from the various preparations of the nuclear fraction using the various isolation procedures described above. The analyses given in Fig. 14 show a good agreement among a variety of samples. This protein, which may be named "nuclear protein" (NP) contained more acidic amino acids than basic amino acids, and neutral or acidic protein.

The nuclear protein was insoluble in neutral and acidic salt solution and soluble in 66 % acetic acid, 0.1 N sodium hydroxide, 0.5 % sodium lauryl sulfate in 10mM Tris (pH7.0) or 7 M urea solution in Buffer B. The protein was chromatographed on G-75 Sephadex and a single peak was observed. This peak contained orcinol reaction positive material.

The gradient centrifugation in CsCl of the nuclear protein resulted in two fractions of protein. One (NP I) was insoluble in the CsCl solution and floated on the surface, and the other (NP II) was soluble and located near

the top of the tube (see Table 3). The RNA II was present together with NP I as mentioned above. When this fraction was treated with phenol, this RNA was found only in a phenol phase including an interphase together with the protein byt in an aqueous phse in which RNA is found usually. This fact indicates that the RNA II is bound to the protein structure. Neither physical state nor molecular size of the RNA II & NP I complex were investigated.

The protein in the nuclear fraction was further studied as to its physical role toward DNA, that is, its ability to combine with DNA. A preliminary study was made by a spot test. One tenth mg of the nuclear protein and 0.1 mg DNA isolated by the phenol method were suspended in 0.1 ml of Buffer B. The mixture was allowed to stand for 17 hours at 6°C and was then spooted on the same filter paper (Toyoroshi No. 51). As a control, unincubated DNA and nuclear protein were also spotted on the same filter paper. After drying the papers at room temperature, they were sprayed with a solution of 2 % bromophenol blue in 10 % acetic acid, followed by spraying with 10 % aqueous ammonia solution. A difference in color was found between the nuclear protein and the incubation mixture of the protein and DNA. Free protein stained strongly in contrast to the weak staining by the mixture; no colour developed on the DNA spot. This observation

indicates that a change in the tertiary confomation of the protein occurs upon incubation with the DNA. It has been also observed that denatured DNA did not react as well with the dye as did the native DNA when the same spot test was made with the mixture of the heat denatured DNA and the protein.

A more detailed study was undertaken to comfirm the above observations. To dissociate DNA and protein at first, the nuclear fraction (DNA : Protein $\simeq$ 1 : 0.9) was centrifuged on CsCl density gradient in the same way as indicated in Fig. 8. The separated DNA and the protein fraction I and II (Fractions(2), (4), (5) in Table 3) were then mixed. To the mixture urea was added to the final concentration of 7 M and it was left at 4°C for 17 hours. The mixture was then dialyzed against Buffer B to remove urea. In parallel, the nuclear protein alone, without addition of DNA, was treated the same way as above. Under these condition DNA,did not show any change in its ultraviolet absorption spectrum. The protein remained dissolved as long as it is in 7 M urea in Buffer B. If urea is removed from the protein solution without DNA by dialysis, almost all protein will be recovered in precipitates. However, when the nuclear protein - DNA mixture was dialyzed against Buffer B no precipitation appeared, suggesting that recombination may have occurred

between the protein and the DNA ("deoxyribonucleo-protein", DNP).

In order to characterize the DNP the following experiments were performed. The DNP was centrifuged on a sucrose gradient in the same condition as described in legend to Fig. 3. The peak of DNP which was measured by $OD_{260\ m\mu}$ and Lowry's method, corresponds to the starting nuclear fraction. No significant amount of other UV-absorbing components nor a Foline positive peak were detected.

The DNP was subjected to chromatography on a Sephadex G-75 column. The mixture of the protein and DNA in a 7 M urea solution was placed on the column after removing urea by dialysis against 10 mM Tris-acetate buffer pH 7.4. Elution was made with the buffer and the effluent in each fraction tube was analyzed for protein (by Foline test) and DNA (by Diphenylamine test). As shown in Fig. 15 A, both protein and DNA are eluted together. If free nuclear protein is chromatographed through the Sephadex G-75 column under the same condition as above, it will be eluted in the position indicated with an arrow in the figure.
A Sephadex G-75 chromatography was also applied for the mixture without removing urea. Essentialy the same result as the previous experiment was obtained (Fig. 15 B). These experimental results indicate that

DNA-protein complex is formed in the urea solution.

The melting points (Tm) of DNA, DNP and the nuclear fraction were studied. In each experiment, Tm of DNP or the nuclear fraction was measured in parallel with Tm of DNA. An example of the experiment was shown in Fig. 16, which shows the Tm values of DNA and the nuclear fraction are 69°C and 71°C, respectively. The absolute Tm values fractuate (ca. ± 2°C) from experiment to experiment, it is, therefore, difficult to compare absolute Tm values of DNP and the nuclear fraction. However, from the result of several experiments it was clear that Tm of both DNP and the nuclear fraction is always higher than that of DNA which is measured in parallel. This fact is also an evidence that the protein is bound to DNA in a way similar to its original form in the nuclear fraction.

The composition of the DNP isolated was analyzed. The ratio between DNA and protein in the recombined DNP was 1 : 0.9 as the same as that of the starting nuclear fraction (1 : 0.9 in this preparation)(Table 5).
The same recombination phenomenon was also observed in mixture of DNA and the nuclear protein obtained as precipitate by deoxyribonuclease digestion followed by acetic acid extraction (descrived above).

A recombination experiment was also carried out

between the nuclear protein in form of suspension and DNA in solution. If the recombination reaction had taken place, the resultant DNP should be found in solution. The result was that only part of the protein combined with DNA (see Table 5). When various amounts of nuclear protein and DNA were mixed in the presence of urea, the DNP with various compositions of DNA and nuclear protein was obtained (Table 5). A composition of resultant DNP reflects the ratio of DNA and protein in a reaction mixture, indicating that DNA present in the nuclear fraction has more binding capacity to protin.

DISCUSSION

The fraction containing a genetic material, DNA, and the materials which presumablly exist in combination with it in intact bacterial cells was isolated from B. subtilis. This fraction, named the nuclear fraction, may be said to be "pure" in the sense that it consists of nucleoprotein of constant sedimentation properties as shown by repeated sucrose gradient centrifugations (Fig. 3) and constant composition of DNA, RNA, and protein as shown by chemical analyses (Table 2). The ratio of the components of the nuclear fraction (DNA : RNA : protein $\pm$ 1 : 0.2 - 0.3 : 0.8 - 0.9) is similar to

that of the nuclear material of B. megaterium isolated by Butler et al.[5], and that of the deoxyribonucleoprotein of B. subtilis by Bhagavan et al.[21]

DNA-polymerase has been shown to exist bound to DNA in bacterial cells[19]. However, in our experiments, the DNA-polymerase activity was found in the soluble layer separated from DNA in the sucrose layered centrifugation after treating protoplasts with Lubrol W as shown in Table 1. This observation suggest either that the enzyme is not bound to DNA in this cell or that the Lubrol W treatment destroys the linkage between the enzyme and DNA.

The RNA-polymerase associated with the membrane fraction containing most of the cellular DNA (see Table 1) up to the step of the sucrose layered centrifugation. The inactivation of the enzyme was, however, occured after that step because the membrane fraction was placed in the solution of low sucrose concentration. If the isolation procedure of the nuclear fraction is improved, RNA-polymerase will be prepared in an active form with DNA as reported in the several articles[7,19,20].

The three kinds of RNA were obtained from the nuclear fraction (RNA I, II, and III) when it was subjected to a CsCl density gradient centrifugation. RNA I appered to be free RNA having a sedimentation constant of 10S.

RNA II exists tightly associated with protein in a form inseparable by SDS-phenol treatment. This fact indicates that the RNA is bound to protein covalently of hindered in a rigid protein structure. Although the nature of this RNA has not yet been investigated, it may have a role in binding of protein with DNA, as suggested in the researches by Huang et al.[22] and Benjamin et al.[23] The RNA III which is present together with DNA in the CsCl density gradient becomes free from DNA after deoxyribonuclease treatment. The sedimentation coefficient of this RNA is approzimatry 30S. Further research is needed to determin whether or not this RNA is hybridized with DNA, but at present the RNA is likely to be a nascent RNA made on the DNA template, on the ground that rapidly labeled RNA also exists in the nuclear fraction.

Efforts have been made to dlucidate the role of the nuclear protein in connection with gene activity. In case of animal cells there are observations (e.g.24) suggesting that protein, for example histone, represses gene action by making a complex with DNA. The regulation of protein synthesis in bacteria has been hapothesized to oparate directry on gene by the mediation of a repressor[1], probably a protein molecule[26]. Another hypothsis indicates that the regulation may operate though the translational process which possibly takes

place coordinately with the transcription[27]). At any rate it seems likely that protein has an important role in gene regulation.

The nuclear proteins isolated as precipitates by three independent methods (acetic acid extraction, deoxyribonuclease treatment and increasing ionic strength) gave roughly the same amino acid composition (Fig. 14). The protein detached from DNA when treated with CsCl and recombined again with DNA when they were mixed together in a urea solution. The recombination occurred in cold and the resultant complex had the same sedimentation property as the original nuclear fraction. The recombination complex was stable in a neutral buffer with low magnesium concentration and was eluted at a speed different from the free nuclear protein through a Sephadex G-75 column. These experimental results show clearly the presence of protein bound to DNA in a special way.

A preliminary experiment showed that the nuclear protein is composed of many electrophoreticaly different species. It is conceivable that many kinds of protein molecules exist bound to DNA. The DNA isolated from the nuclear fraction has more capacity to combine with the nuclear protein than had possessed originaly. This may mean that the capacity of DNA to combine with protein is not always saturated when it is present in a cell

depending upon the conditions of the cell.

Haung *et al.*[24] have made a study of reconstitution of calf thymus nucleohistone and of its biological activities. They have shown that the protein inhibites RNA-polymerase activity. Johns *et al.*[25], however, made a comment that the reconstituted nucleohistone was unstable in a physiological condition. In contrast with the nucleohistone, the deoxyribonucleoprotein recombined in our experiments is stable in a physiological condition.

The fact that the nuclear protein makes a stable complex with DNA strongly suggests that it has a special role in gene expression. In order to answer the question of how the protein in the nuclear fraction works during gene expression, we are trying to construct a *in vitro* system in which a special enzyme is translated passing through the transcriptional process.

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SUMMARY

The nuclear fraction was isolated from B. subtilis. It is composed of DNA, RNA, and protein in the ratio of 1 : 0.2 - 0.3 : 0.8 - 0.9. The physicochemical properties of the nuclear fraction were also studied.

The nuclear fraction was dissociated by a CsCl density gradient centrifugation into DNA with a portion of RNA, free RNA, protein with another portion of RNA, and free protein. The three RNA fractions thus separated had different sedimentation coefficients.

Amino acid composition of protein in the nuclear fraction was analyzed. From the analysis, the protein was found to be either neutral or acidic protein, different from nuclear protein in animal cells, histon, which is basic.

When the nuclear protein was mixed with the nuclear DNA, a protein - DNA complex is formed. The recombined complex behaved in the same manner as the original nuclear fraction in a centrifugational field and through a Sephadex column.

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LEGEND TO FIGURES AND TABLES

Table 1: DISTRIBUTION OF DNA, RNA, AND PROTEIN AND DNA-, RNA-POLYMERASE ACTIVITIES IN EACH FRACTION INDICATED IN FIG. 1.

Foot Note (F.T.): Chemical composition was measured in some fraction obtained from first three centrifugation procedures in Fig. 1. The values are indicated in present of the amount of the sample applied in each centrifugation.

Table 2: YIELDS OF DNA, RNA AND PROTEIN IN EACH FRACTION FOR I GR. CELL.

(F.T.): *1) This fraction contains cell membranes and the nuclear fraction. *2) This fraction contains the nuclear fraction. *3) In the nuclear fraction, the ratio of DNA : RNA : protein was usually 1 : 0.2 - 0.3 : 0.8 - 0.9.

Table 3: SUBFRACTIONATION OF THE NUCLEAR FRACTION BY CsCl DENSITY GRADIENT CENTRIFUGATION.

(F.T.): Fractions (1 - 6) in Fig. 8 were analyzed for the content of DNA, RNA and protein. Fraction 5,6 are surface and bottom fraction of the CsCl solution.

Table 4: PHENOL TREATMENT OF THE NUCLEAR FRACTION.

(F.T.): The nuclear fraction in an aqueous solution was treated with phenol. The upper aqueous phase is named as the aqueous phase I. To the phenol layer added an equal volume of water and shaked. The resultant aqueous phase was treated with ether x 3 to remove phenol and centrifuged. The supernatant is called aqueous layer II.

Table 5: RECOMBINATION OF DEOXYRIBONUCLEO-PROTEIN.
*1) The ratio of protein to DNA was 0.9 in this preparation. *2) no urea treatment. *3) Fract. (2) and (5) was used (Fig.5). *4) Fract.(2), (4) and (5) was used (Fig.6).

Fig. 1; ISOLATION OF THE NUCLEAR FRACTION
(F.T.): The isolation procedure of the nuclear fraction is summarized schematically. Numbers in parenthese indicate fractions.

Fig. 2: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF DNA-CONTAINING FRACTION (7)
(F.T.): Five ml of Fraction(7) in Fig. 1 was centrifuged in a 10 % - 20 % sucrose density gradient in the Buffer B at 24,000 rpm. for 16 hr. by Spinco SW25.2 rotor. About 2 ml of each fraction was collected and analyzed for DNA, RNA, and protein in each tube.

Fig.3: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE NUCLEAR FRACTION

(F.T): Two ml of suspension of the nuclear fraction was centrifuged in a 5 - 20 % of sucrose density gradient in the Buffer B at 24,000 rpm . for 17 hr. by SPINCO SW25.1 rotor. About 2 ml of each fraction was collected and analyzed for DNA, RNA, and protein in each tube.

Fig. 4: COLUMN CHROMATOGRAPHT OF THE NUCLEAR FRACTION.

(F.T.): This figure showes Sephadex G-75 column (1 x 75 cm) chromatography of the nuclear fraction. The elution was made with the Buffer B containing 7 M urea. Two ml of each fraction was collected.

Fig. 5: UV ABSORPTION SPECTRA OF THE NUCLEAR FRACTION AND DNA OF B.SUBTILIS.

(F.T.): Optical density of DNA and the nuclear fraction in the Buffer B was measured at room temperature by Beckman Spectrophotometer DU in wave length ranging from 232 mμ to 300 mμ.

Fig. 6: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE NUCLEAR FRACTION AND DNA OF B.SUBTILIS.

(F.T.): Both the nuclear fraction from the unlabelled cells and DNA from the P^{32} labelled cells were centrifuged in a 3 - 20 % sucrose density gradient in the Buffer B at 39,000 rpm

for 3.5 hr. by SPINCO SW 39 rotor at 2°C.

Fig. 7: ELECTRON MICROGRAPHY OF THE NUCLEAR FRACTION.

(F.T.): The nuclear fraction was subjected to negative staining with 2 % phosphotungstic acid, and observed by an electron microscope (Nihon Denki Kogaku, JEM-5, Japan). Magnifications are a); x 30,000 and b); x 40,000.

Fig. 8: CsCl DENSITY GRADIENT CENTRIFUGATION OF THE NUCLEAR FRACTION.

(F.T.): The nuclear fraction was centrifuged in CsCl density gradient solution at 38,000 rpm for 47 hr. using a SPINCO SW39 rotor at 2°C. After centrifugation, fraction(1) - (6) were pooled.

Fig. 9: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF P^{32}-DNA ISOLATED FROM THE NUCLEAR FRACTION AND DNA FROM WHOLE CELL.

(F.T.): The DNA isolated from the P^{32} labelled nuclear fraction and from unlabelled cells were centrifuged in a 3 -20 % sucrose density gradient solution in the Buffer B, at 38,000 rpm for 4.3 hr. at 2°C by SPINCO SW 39 rotor.

Fig. 10: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF RNA I, RNA II, AND DNA.

(F.T.): RNA I, RNA II, and DNA (subfraction(1), (5) and (2), respectively, in Fig. 8) were centrifuged in a 3 - 20 % sucrose density gradient solution in 10 mM Tris-acetate (pH 7.3)

containing 0.5 % sodium lauryl sulfate (S,L.S.) at 38,000 rpm for 2 hr. at 2°C by SPINCO SW39 rotor. 10a: DNA containing RNA III (Fr.(2) in Table 3) and RNA I (Fr.(1) in Table 3) were centrifuged in two different tubes and patterns of these are drawn in the same graph.

Fig. 11: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE NUCLEAR FRACTION PULSE LABELLED WITH P^{32}.
(F.T.): The nuclear fraction pulse labelled with P^{32} was centrifuged in a 3 - 20 % sucrose gradient solution in the Buffer B at 24,000 rpm for 15 hr. by SPINCO SW25.1 rotor.

Fig. 12: CSCl DENSITY GRADIENT CENTRIFUGATION OF THE P^{32} PULSE LABELLED NUCLEAR FRACTION.
(F.T.): The nuclear fraction pulse labelled with P^{32} was centrifuged in a CsCl density gradient at 38,000 rpm for 48 hr. at 2°C by SPINCO SW 39 rotor.

Fig. 13: SUCROSE DENSITY GRADIENT CENTRIFUGATION OF DNA.
(F.T.): The fraction containing DNA (Fr.(2) in Fig.8) was digested with DNase and centrifuged in a 3 - 20 % sucrose density gradient solution in the Buffer A at 38,000 rpm for 100 min. by SPINCO SW39 rotor. Acid insoluble P^{32} in each tube was counted.

Fig. 14: AMINO ACID COMPOSITION OF THE NUCLEAR PROTEIN.

(F.T.): Amino acid composition of the nuclear protein prepared by various methods, described as experimental section. The histograms drawn by the thin line and dotted line are data from each analysis. The histograms in bold line indicate the average values.

Fig. 15: THE COLUMN CHROMATOGRAPHY OF DEOXYRIBONUCLEOPROTEIN

(F.T.): The nuclear fraction was eluted with (a) 10 mM Tris-Acetate (pH 7.4) and (b) with 7 M urea solution in 10 mM Tris-Acetate (pH 7.4).

Fig. 16: MELTING PROFILE OF DNA ISOLATED FROM WHOLE CELL AND THE NUCLEAR FRACTION.

(F.T.): The nuclear fraction and DNA from whole cell were dialyzed against 0.7 mM potassium phosphate buffer (pH 7.0) for 8 hr. at 8°C. $OD_{260\ m\mu}$ of these solution was measured at each temperature by Ito's Spectrophotometer (Ito Chotanpa, Tokyo, Japan).

Fig. 17: MELTING PROFILE OF DNA ISOLATED FROM WHOLE CELL AND THE RECOMBINED DNP.

(F.T.): Tm was measured under the same condition as in Fig. 16.

Table 1: Distribution of DNA, RNA and Protein and DNA-Polymerase Activities in each Fraction.

Fraction	main Components	DNA content (%)[a]	RNA content (%)[b]	Protein content (%)[c]	Polymerase activities (%) DNA-	RNA-
(2) Lysate						
20,000 rpm. 30 min.						
(3-1) Sup.	Cytoplasm	8	88	66	49	23
(3-2) 28 % Layer	Cytoplasm	3	8	11	10	9
(3-3) 45 % Layer		5	0	1	1	1
(4) 45-63 % Interphase	Nuc. Fract.	81	4	21	35	63
3-4) 63 % Layer	intact Cell	3	0	0	4	3
30,000 rpm. 40 min.						
(5) Sup.		4	8	19	99	27
(6) Residue	Nuc. Fract.	96	92	81	1	73
45.000 rpm. 30 min.						
(7) Sup.	Nuc. Fract.	50	20	20	50	70
(8) Residue	Membran	50	80	80	50	30

Table 2. Yields of DNA, RNA and Protein.

FRACTION	DNA(mg)	RNA(mg)	PROTEIN(mg)
(2) Lysate	5	20	120
(6) *1)	4	0.8	20
(7) *2)	2	0.3	4
Nuclear Fraction *3)	2.0 ~ 1.6	0.4 ~ 0.3	1.5 ~ 1.2

Table 3: Subfractionation of the Nuclear Fraction by CsCl Density Gradient Centrifugation.

FRACTION	COMPONENT	DNA		RNA		PROTEIN	
		(γ)	(%)	(γ)	(%)	(γ)	(%)
(1)	RNA I	0	0	23	29	0	0
(2)	DNA, RNA III	342	87	9	11	6	2
(3)		33	8	8	10	0	0
(4)	PROTEIN II	19	5	4	5	86	24
(5)	PROTEIN I, RNA II	0	0	26	33	260	73
(6)	RNA I	0	0	10	12	3	1

Table 4: Phenol Treatment of the Nuclear Fraction.

FRACTION	DISTRIBUTION (%)			RATIO OF CONTENT (w/w)	
	DNA	RNA	PROTEIN	RNA/DNA	RNA/PROTEIN
AQUEOUS LAYER I	92	12	—	0.03	—
AQUEOUS LAYER II	8	8	—	0.03	—
PRECIPITATION	—	80	100	—	0.2

Table 5: Recombination of Deoxyribonucleo-protein.

EXPERIMENTS	COMPOSITION OF MIXTURE DNA : PROTEIN (w/w.)	COMPOSITION OF REC. DNP DNA : PROTEIN (w/w)
NATIVE DNP (NUCLEAR FRACTION)		1 : 0.9 *1)
RECOMBINED DNP		
1	1 : 0.9 *2) *3)	1 : 0.5
2	1 : 0.5 *3)	1 : 0.5
3	1 : 0.9 *4)	1 : 0.9
4	1 : 1.9 *5)	1 : 1.9

Fig. 1: Isolation of the Nuclear Fraction.

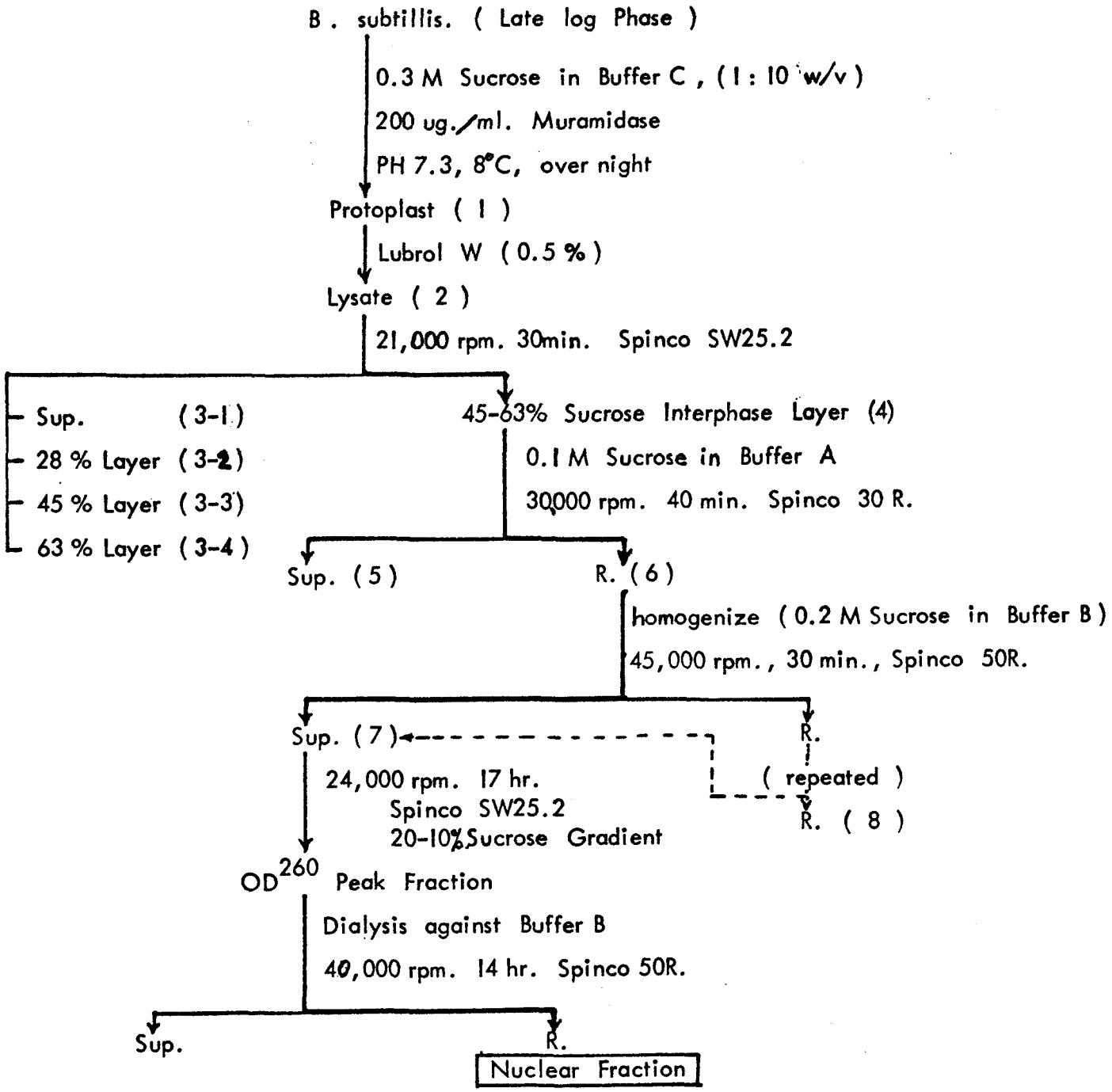


Fig. 2: Sucrose Density Gradient Centrifugation of Fraction(7) in Fig. 1.

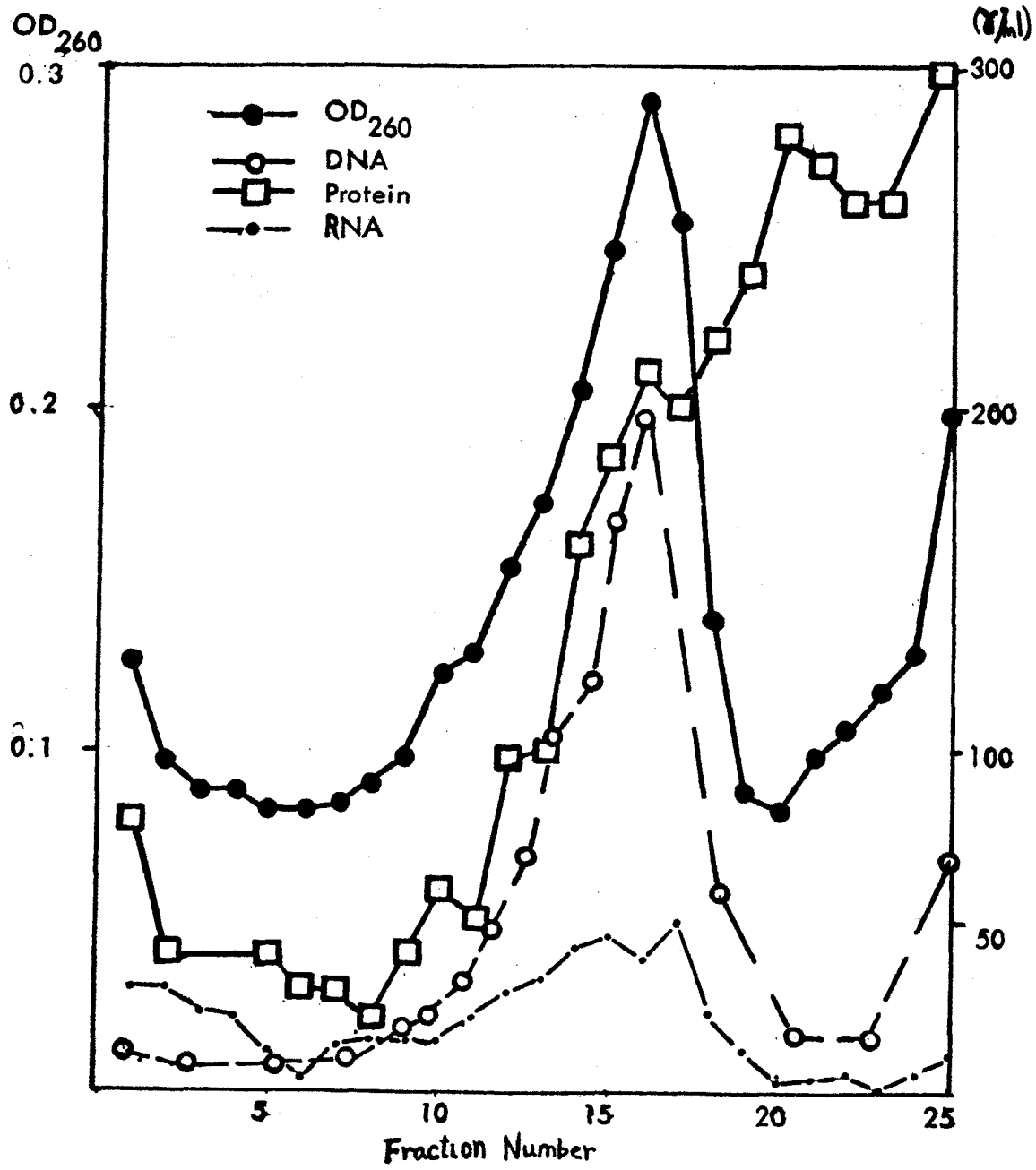


Fig. 3: Sucrose Density Gradient Centrifugation of the Nuclear Fraction.

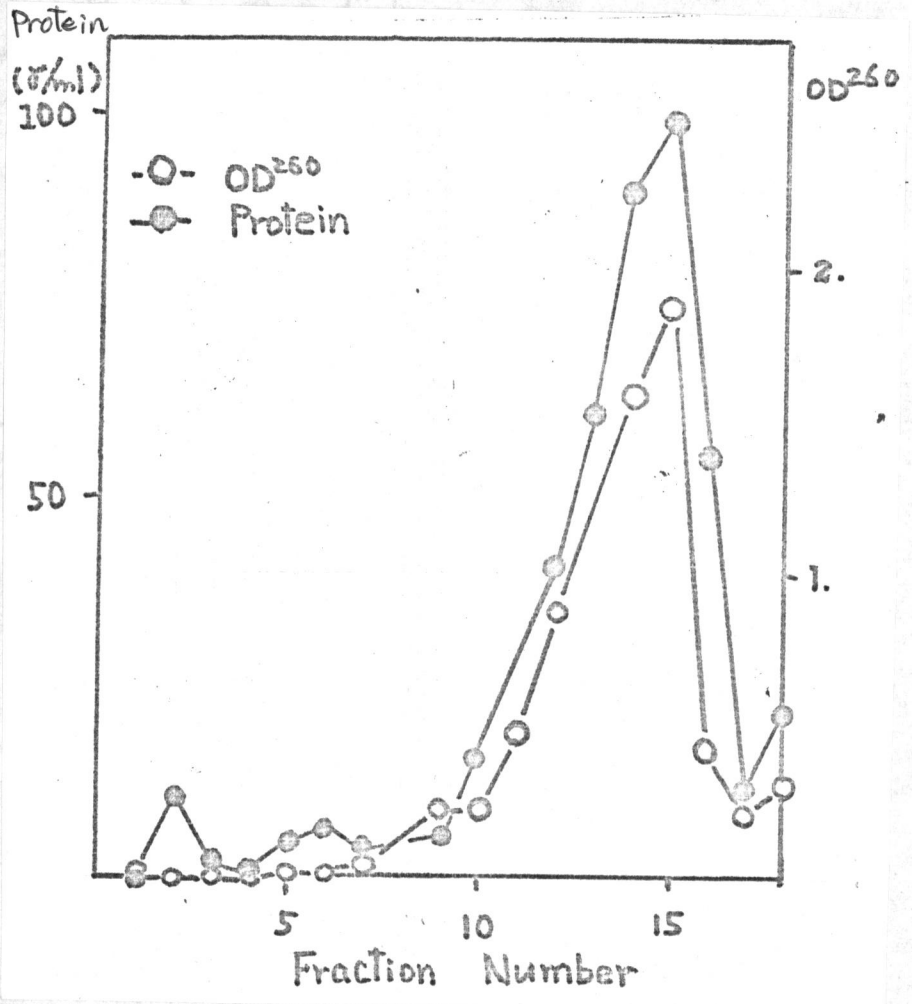


Fig. 4: Column Chromatography of the Nuclear Fraction .

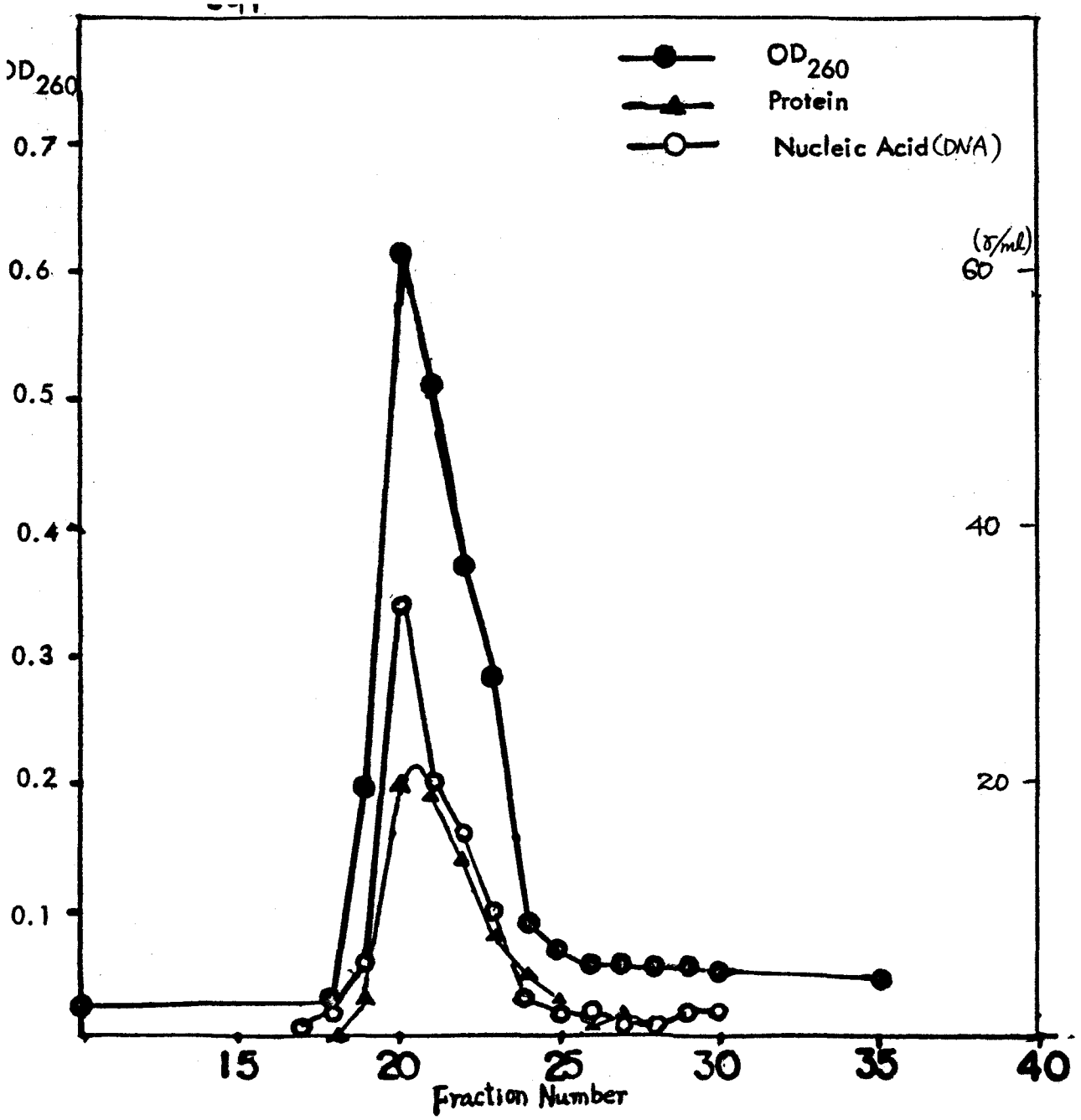


Fig. 5: UV Absorption Spectra of the Nuclear Fraction and DNA of B.subtilis.

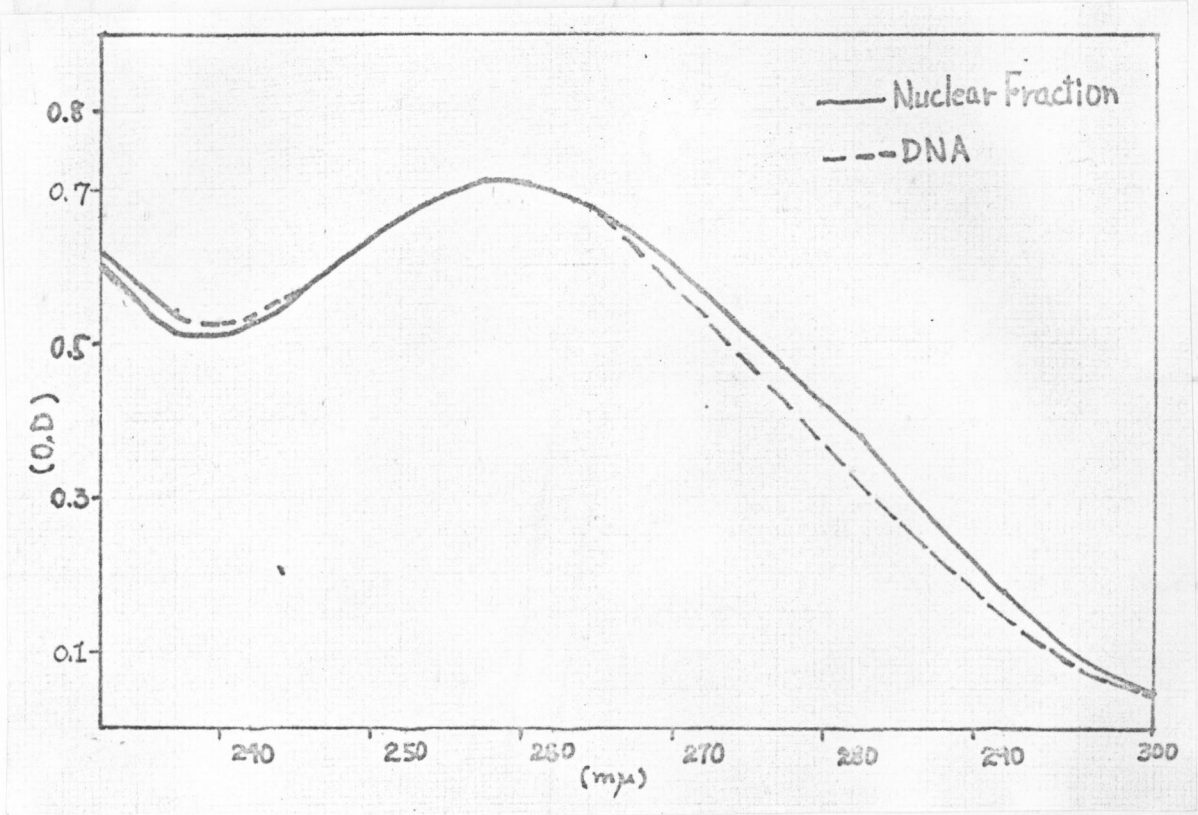


Fig. 6: Sucrose Density Gradient Centrifugation of the Nuclear Fraction and DNA of B. subtilis.

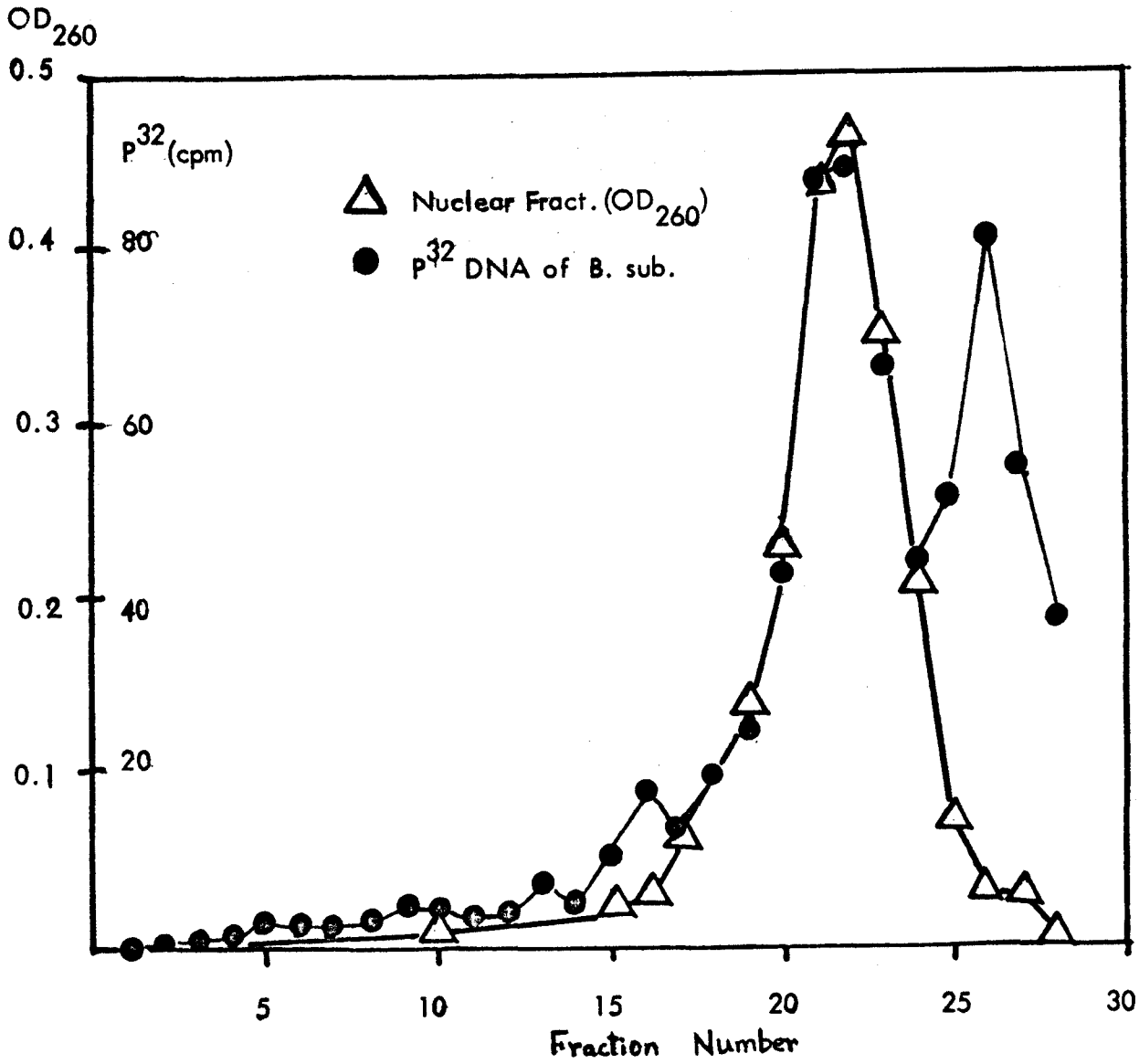
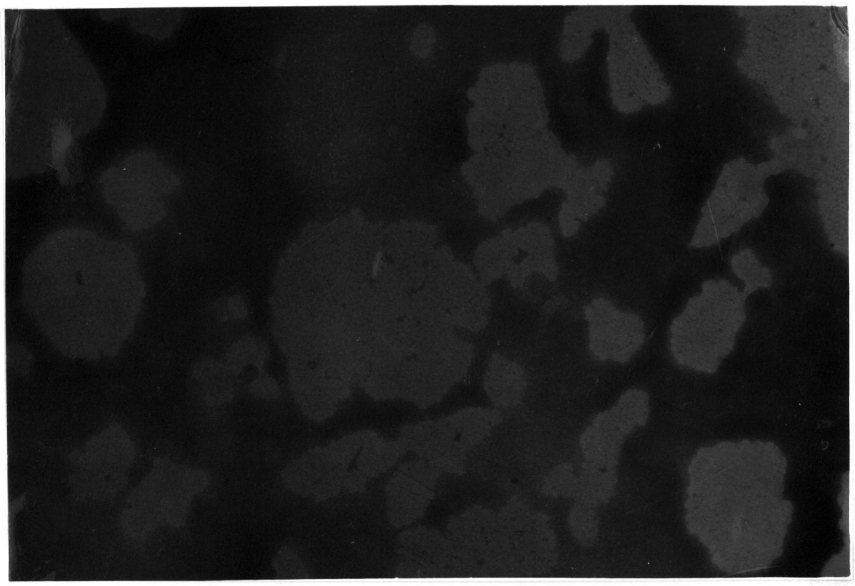


Fig. 7: Electron Micrography of the Nuclear Fraction.

a)



b)

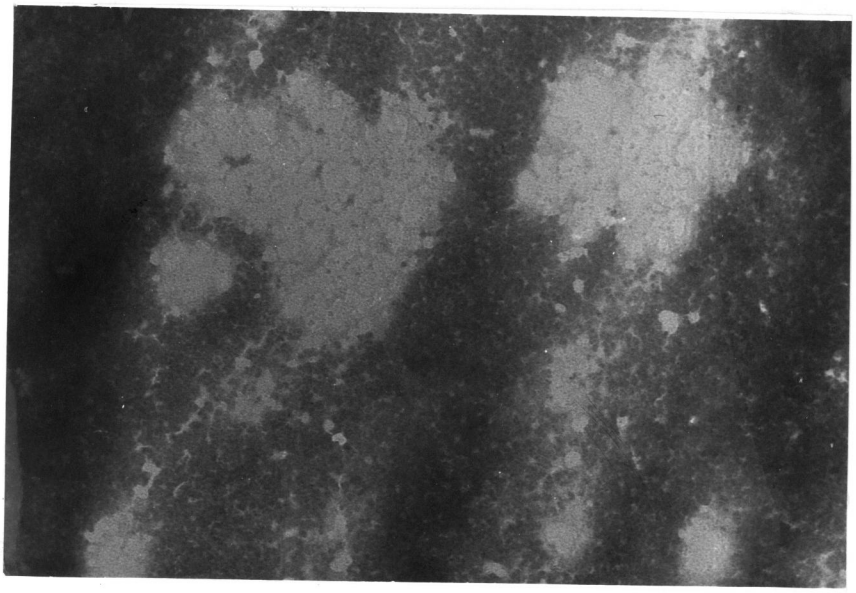


Fig 8: CsCl Density Gradient Centrifugation of the Nuclear Fraction.

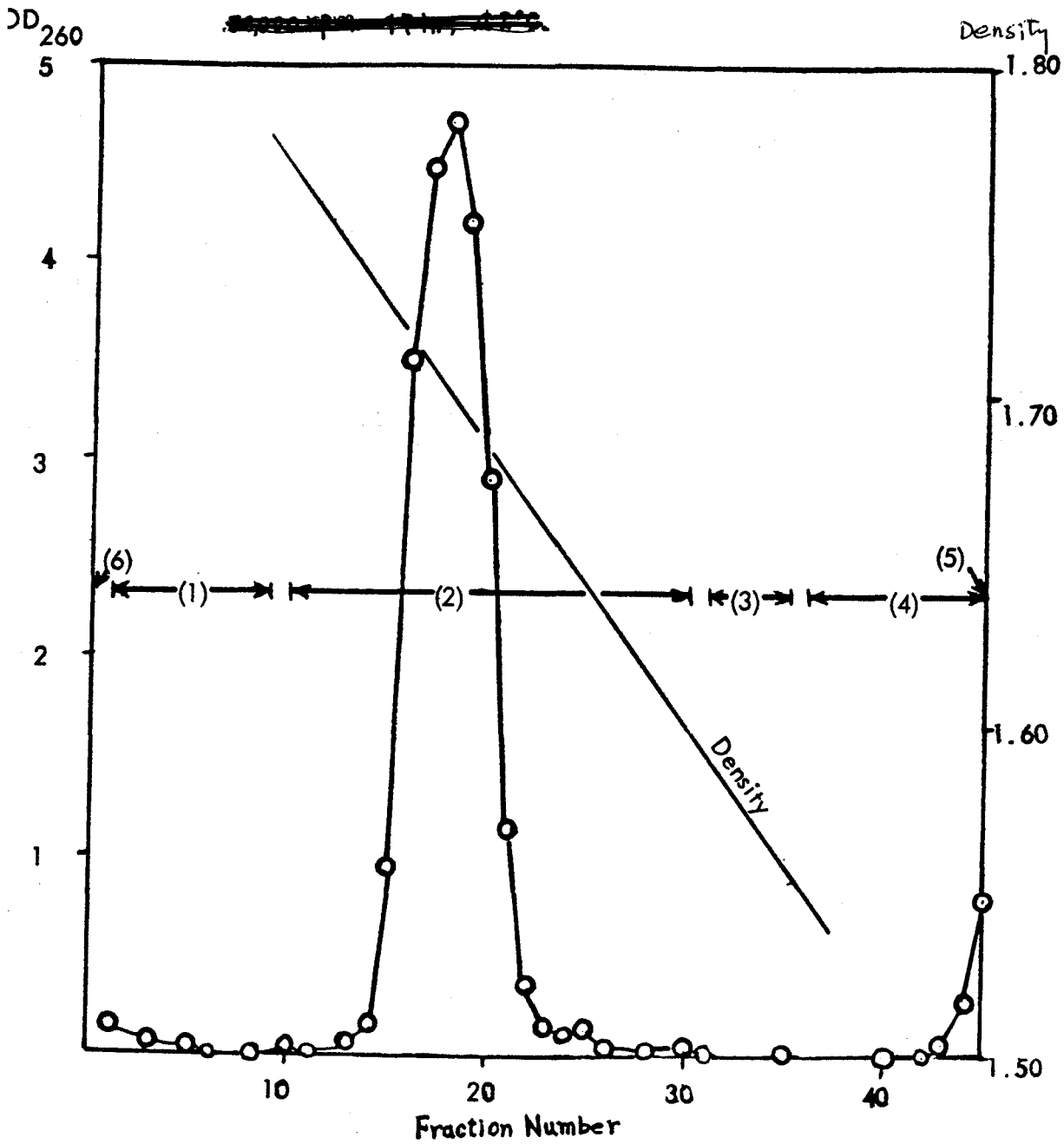


Fig. 9: Sucrose Density Gradient Centrifugation of P^{32}- DNA Isolated from the Nuclear Fraction and DNA from Whole cell.

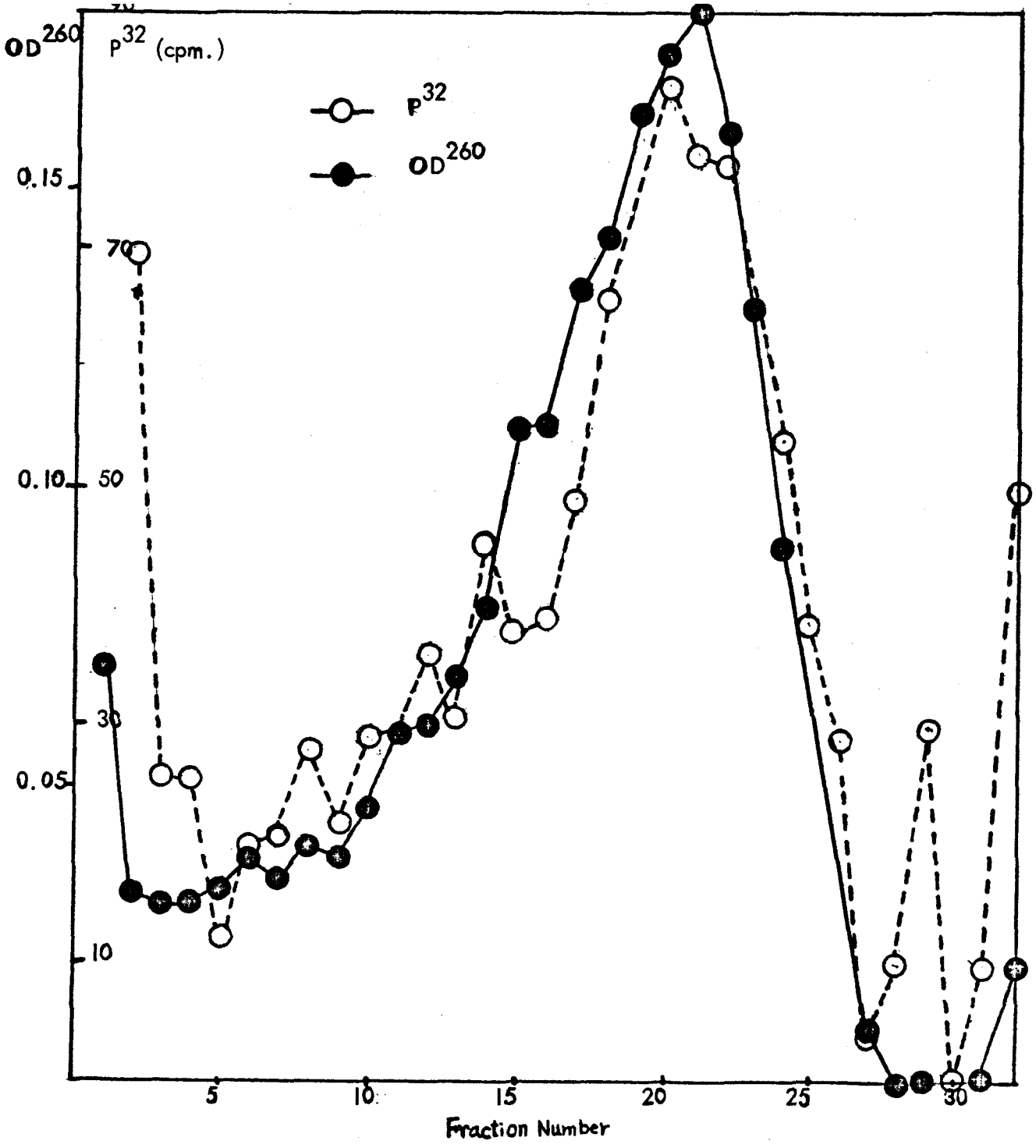


Fig. 10) Sucrose Density Gradient Centrifugation of RNA I, RNA II, and DNA.

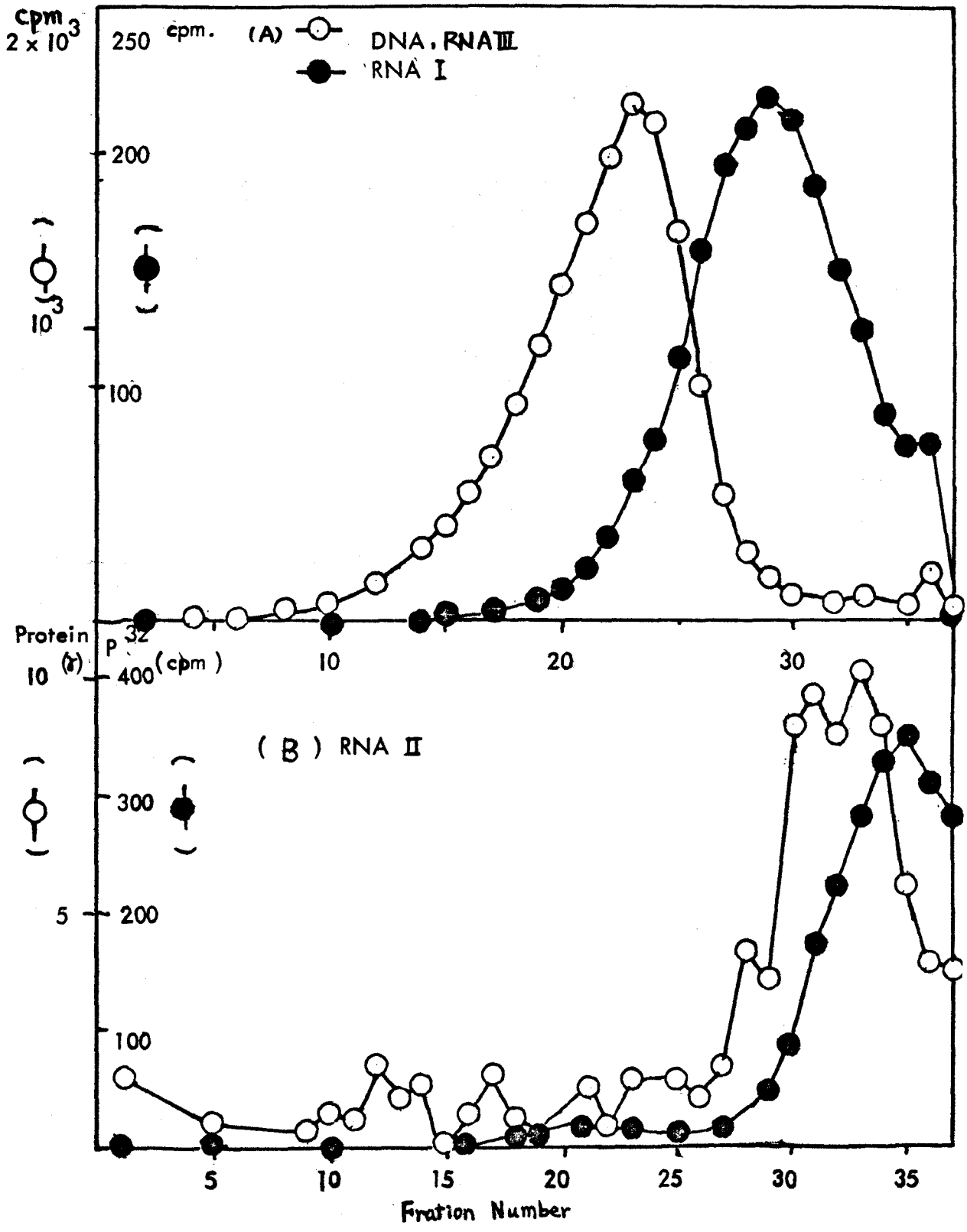


Fig. 11: Sucrose Density Gradient Centrifugation of the P^{32}-pulse labelled Nuclear Fraction

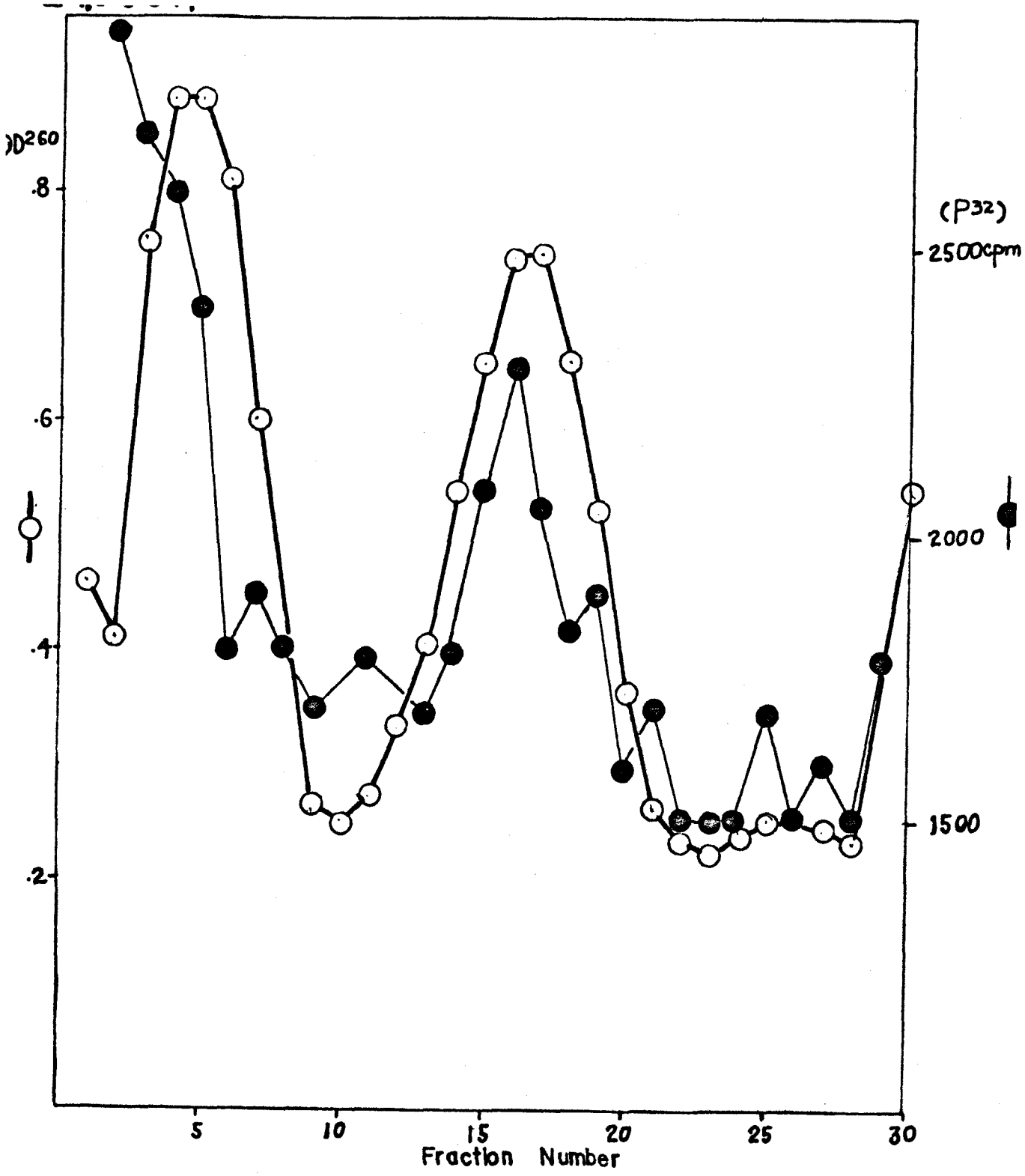


Fig. 12: CsCl Density Gradient Centrifugation of P^{32} Repulse Labelled Nuclear Fraction.

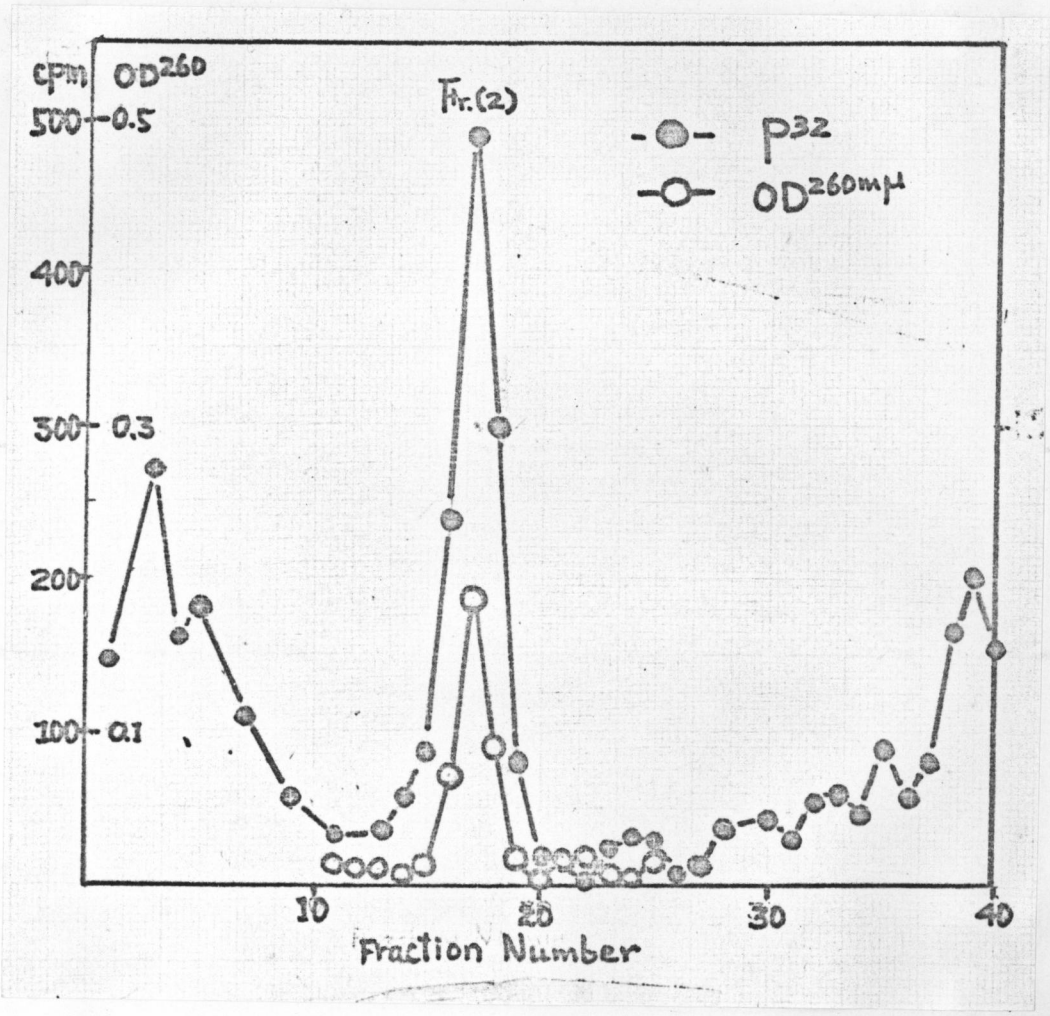


Fig. 13: Sucrose Density Gradient Centrifugation of DNA.

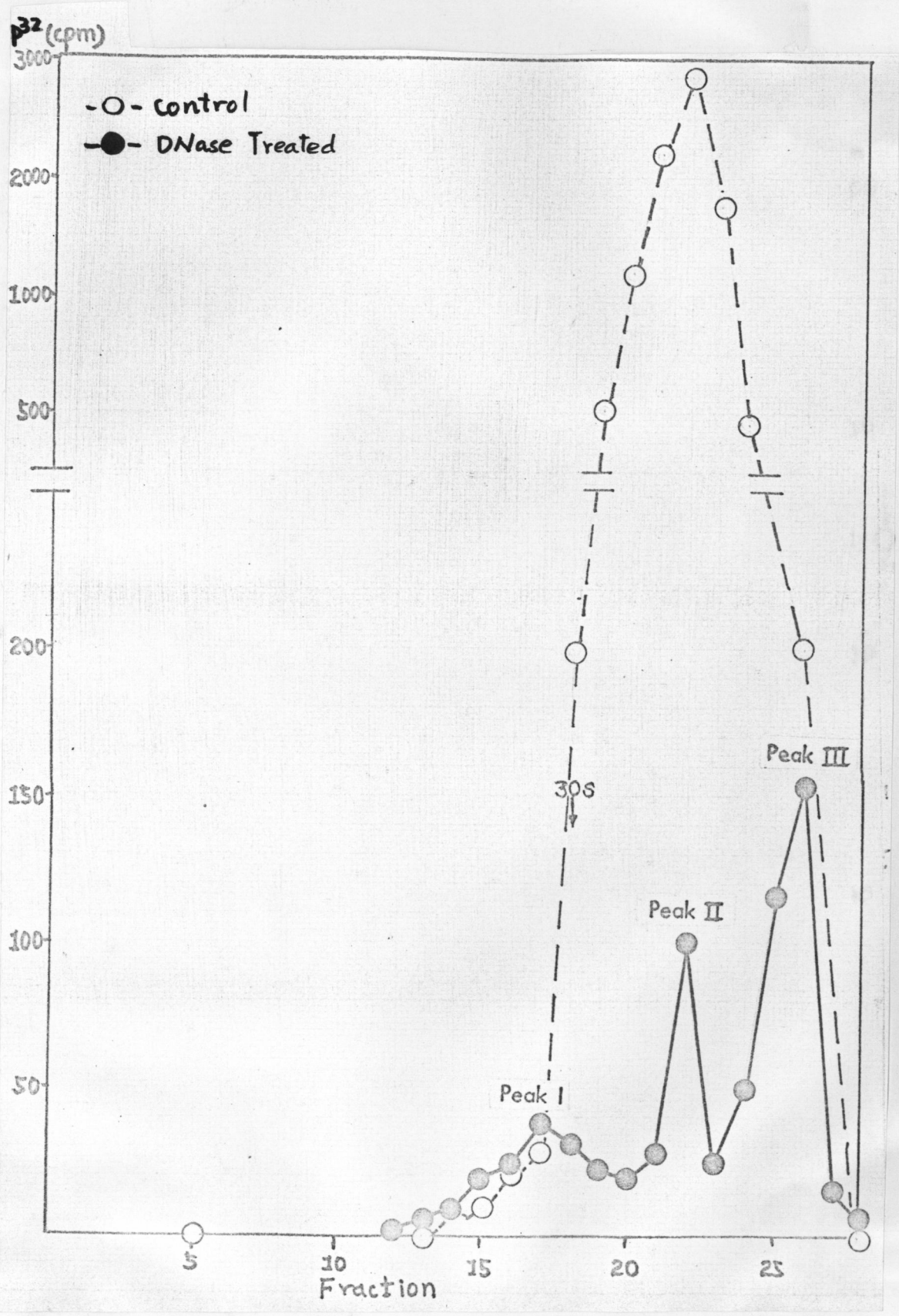


Fig. 14 AMINO ACID COMPOSITION OF The NUCLEAL ~~FRACTION~~ PROTEIN.

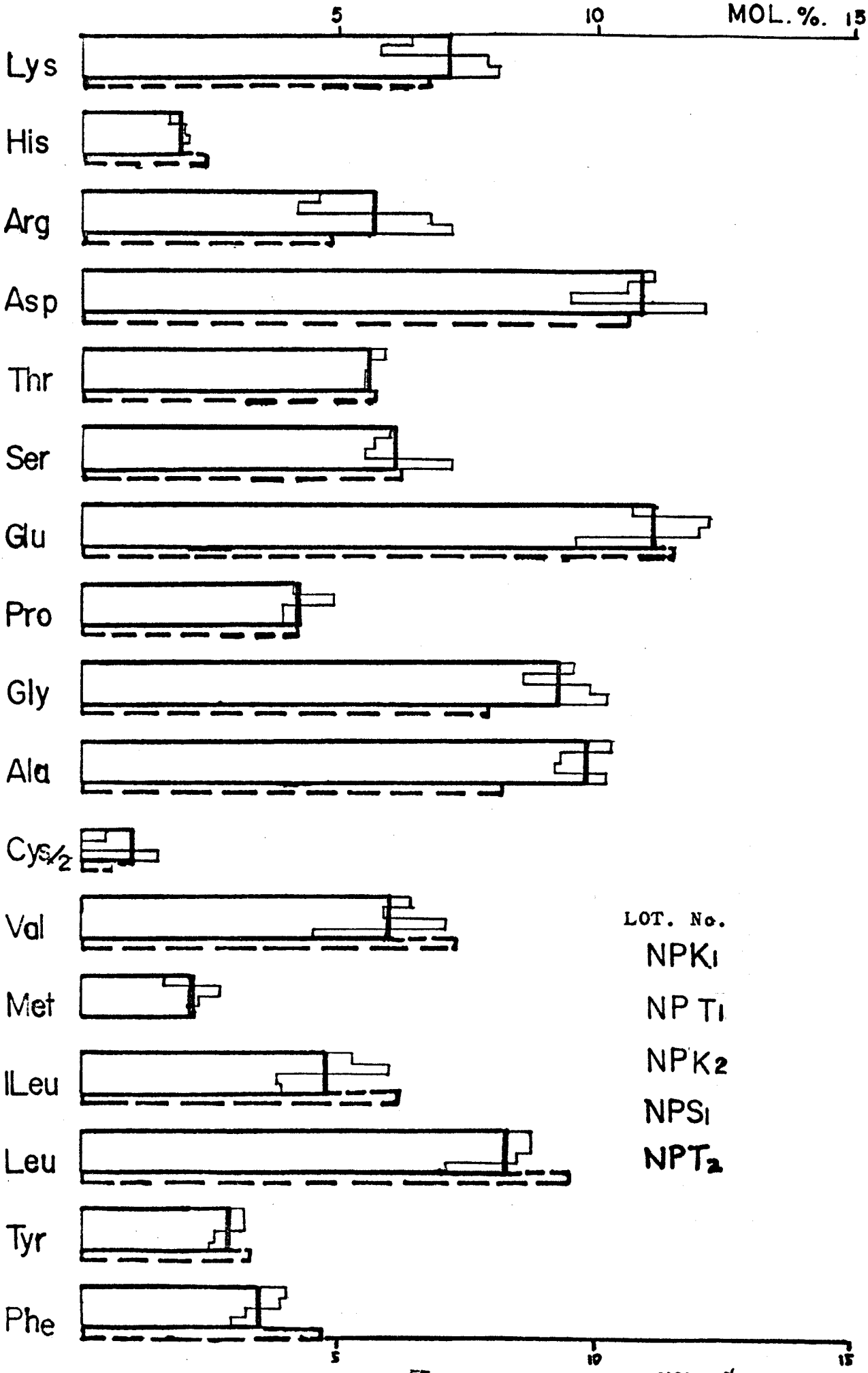


Fig. 15: The Column Chromatography of Deoxyribo-nuclepprotein.

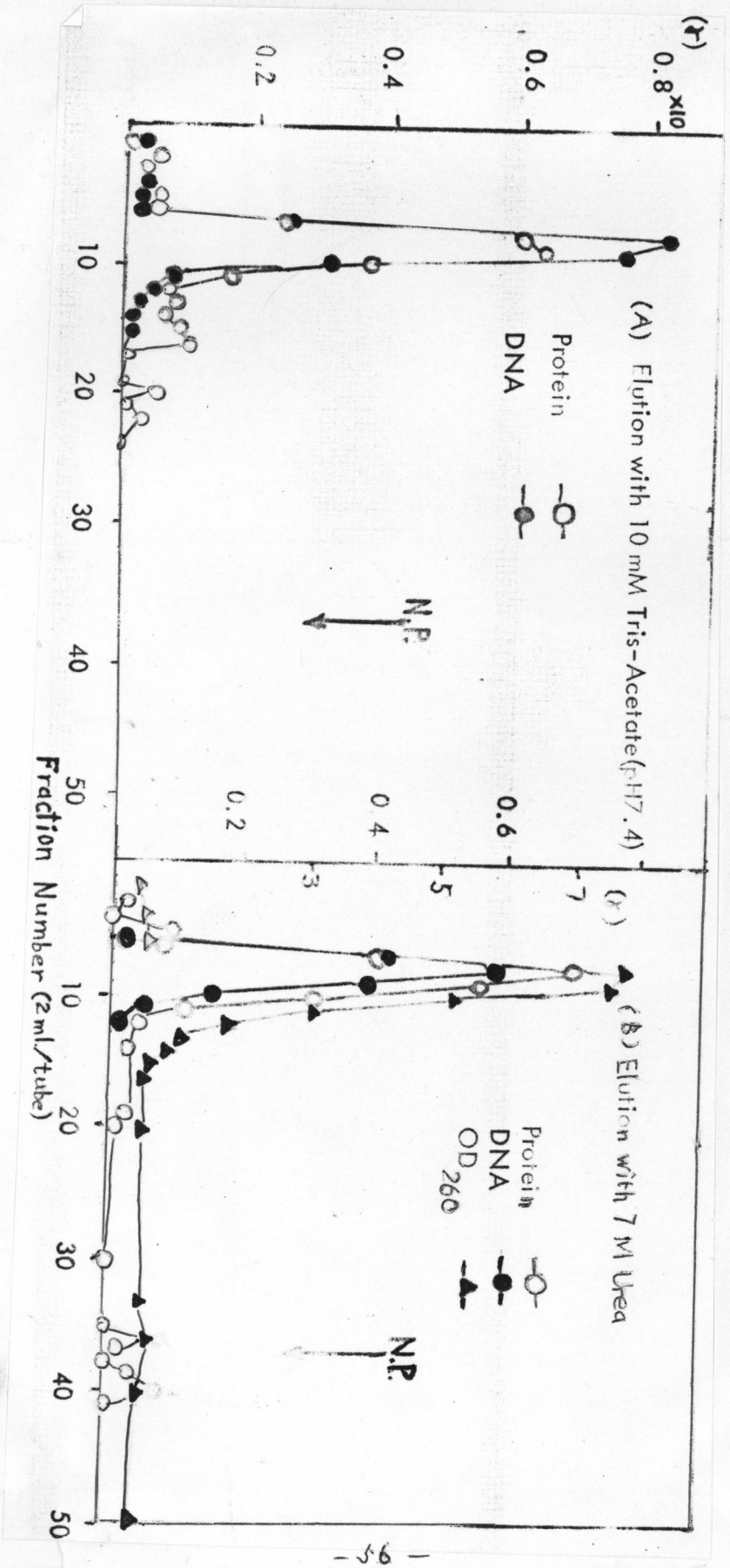


Fig. 16: Melting Profile of DNA from Whole Cell and the Nuclear Fraction.

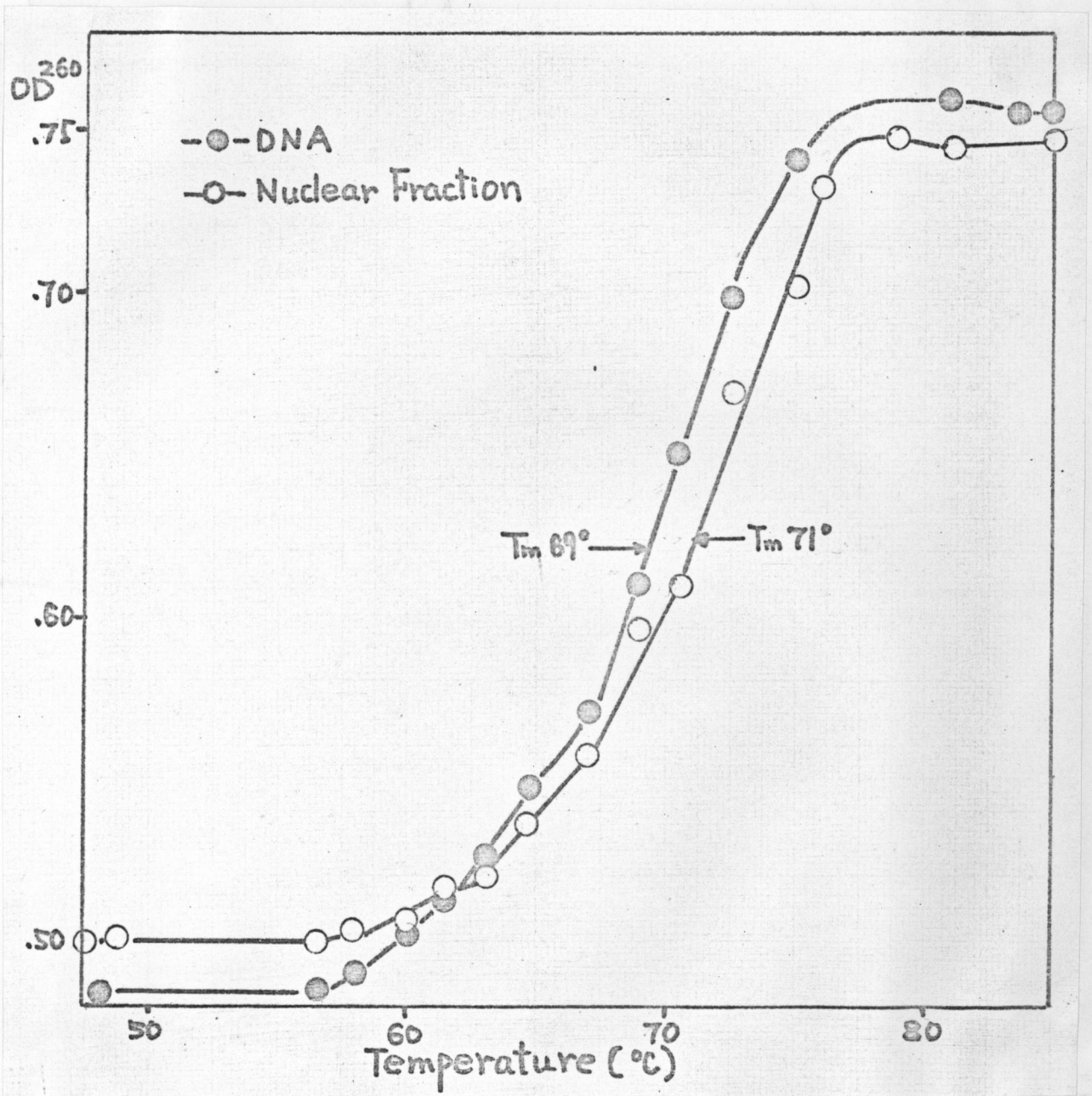


Fig. 17. Melting Profile of DNA and recombined DNP