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# ISOLATION AND CHARACTERIZATION OF "NUCLEOPROTEIN"

# FROM <u>BACILLUS</u> <u>SUBTILIS</u>

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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 <u>Bacillus megaterium</u>[4]. Attempts have been made to isolate these particles, so called "Nuclear fraction", from <u>B</u>. <u>megaterium</u>[5], <u>Escherichia coli</u>[5], as well as <u>Bacillus subtilis</u>[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 <u>B</u>. <u>subtilis</u>[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 % $MgSO_4 \cdot 7H_2O$, 0.01 %
$CaCl_2 \cdot 2H_2O$. 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 $37^\circ C$. 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 $\beta$-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 $\beta$-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 $\beta$-mercaptoethanol.

**Isolation of nuclear fraction:** --- Experiments were
carried out at $2 - 4^\circ C$ 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 proto- plasts 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 centrifu- gation, 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 rotor 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 $\mu$ moles Tris-HCl (pH 7.6), 2 $\mu$ moles magnesium acetate, 20 m$\mu$ moles EDTA, 1.6 $\mu$ moles MnSO$_4$, 0.4 $\mu$ moles $\beta$-mercaptoethanol, 10 $\mu$g DNA prepared from B. subtilis, 17 m$\mu$ moles each GTP, UTP and CTP and 17 m$\mu$ 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 perchlo-
ric 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 ana-
lyses. 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 standardiza-
tion.

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 centrifuga-
tion were both composed of DNA, RNA and protein in the
ratio of 1 : 0.2 - 0.3 : 0.8 - 0.9. The ratio was appro-
ximately 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
fracétion 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 centrifu-

gation 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 (150$\gamma$/ml) in Buffer B

were added $5 \times 10^{-3}$ M magnesium acetate, deoxyribonuclease I
($5\gamma$/ml) (Worthington Biochemical Corp., USA) and snake
venom phosphodiesterase ($5\gamma$/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
($100\gamma$/ml) did not cause any significant change but
incubation with deoxyribonuclease I ($5\gamma$/ml) made disappea-
rance 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 centri-
fugation 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 $250_{m\mu}$
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 ($OD_{650 \, m\mu}$ $\simeq$ 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 minufte 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 x $10^{-3}$ M to 5 x $10^{-3}$ 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 (10$\gamma$/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 oligo-deoxyribonucleotides. 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 frac-
tion --- 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 fracåtions 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 $\not\in$ 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 o.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 alonse,

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. $\pm$ 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 fracétion.

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 = 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 protoprasts 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
reserches 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 deoxyribo-
nuclease 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 proÁtein 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 saturÁated 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.


## ACKNOWLEDGEMENT

# 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 centri-

fugation.

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 CHROMATOGRAPHY 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 m$\mu$ to 300 m$\mu$.

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 obsefved 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 fract/ion was centrifuged

in CsCl density gradient solution at 38,000 rpm

for 47 hr. using a SPINCO SW39 rotor at 2°C.

After cen/trifugation, 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 GRADIEN/T 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 |

| FRACTION | COMPONENT | DNA | | RNA | | PROTEIN | |
|---|---|---|---|---|---|---|---|
| (1) | RNA I | $0^{(\gamma)}$ | $0^{(\%)}$ | $23^{(\gamma)}$ | $29^{(\%)}$ | $0^{(\gamma)}$ | $0^{(\%)}$ |
| (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 |

| 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 |

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| 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 |

Table 5: Recombination of Deoxyribonucleo-protein.

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**Fig. 1:  Isolation of the Nuclear Fraction.**

B . subtillis.  ( Late  log  Phase )

    0.3 M  Sucrose  in  Buffer C ,  ( 1 : 10 w/v )

    200 ug./ml.  Muramidase

    PH 7.3,  8°C,  over night

Protoplast  ( 1 )

    Lubrol  W  ( 0.5 % )

Lysate  ( 2 )

    21,000 rpm.  30min.   Spinco  SW25.2

- Sup.　　　　 ( 3-1 )　　　　　　45-63% Sucrose  Interphase  Layer  (4)
- 28 % Layer  ( 3-2 )　　　　　　　0.1 M  Sucrose  in  Buffer  A
- 45 % Layer  ( 3-3 )　　　　　　　30,000 rpm.  40 min.  Spinco  30 R.
- 63 % Layer  ( 3-4 )

Sup.  ( 5 )　　　　　　　R. ( 6 )

    homogenize  ( 0.2 M Sucrose  in  Buffer B )

    45,000 rpm. ,  30 min. ,  Spinco  50R.

Sup.  ( 7 )◄-----------------┐　　　　R.

    24,000 rpm.  17 hr.　　　　　　└__ ( repeated )

        Spinco  SW25.2　　　　　　　　　R. ( 8 )

        20-10% Sucrose  Gradient

$OD^{260}$  Peak  Fraction

    Dialysis  against  Buffer B

    40,000 rpm.  14 hr.  Spinco 50R.

Sup.　　　　　　　　　　R.

　　　　　　　　　Nuclear  Fraction

Fig. 2: Sucrose Density Gradient Centrifugation of Fraction(7) in Fig. 1.
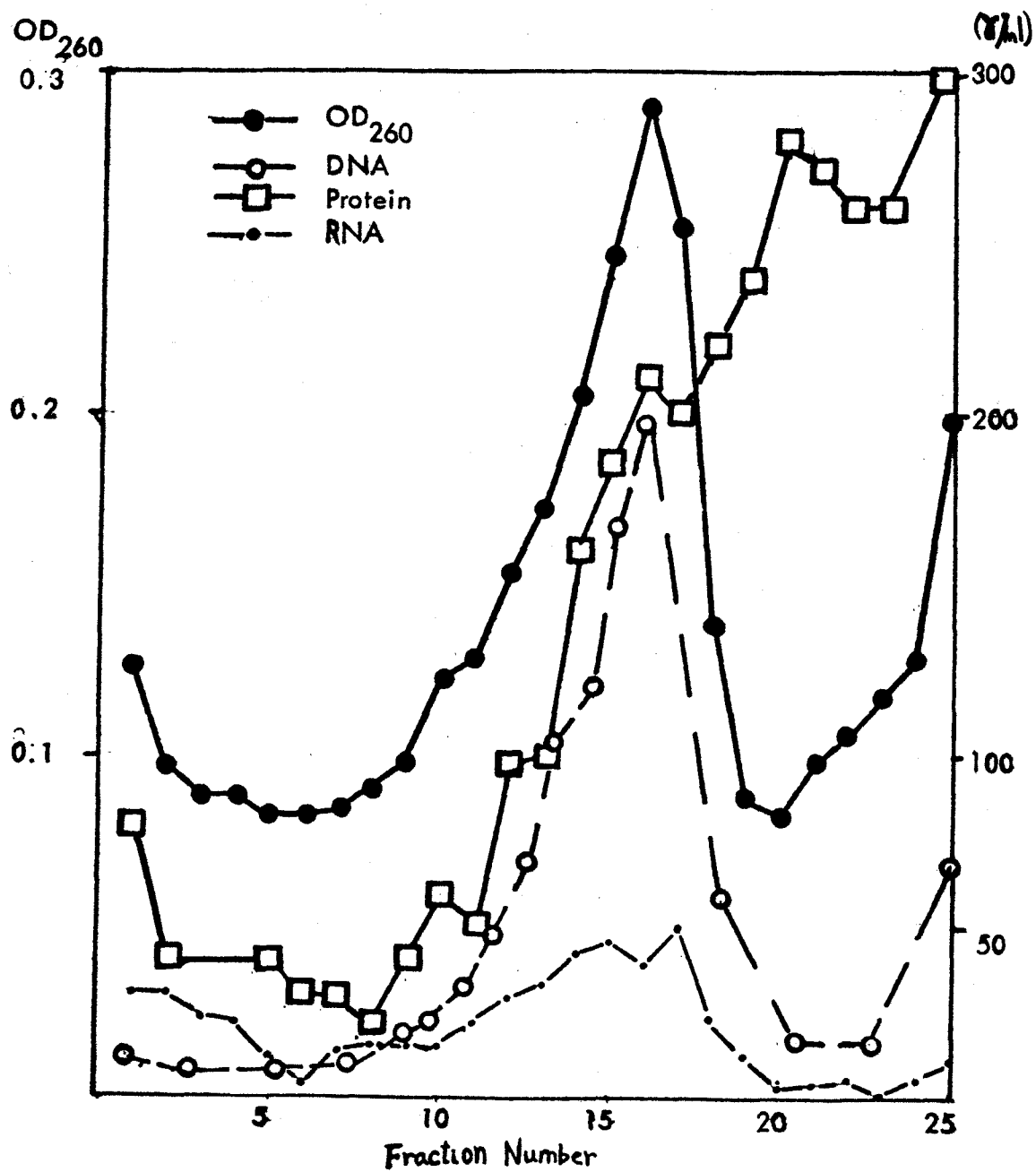
Fig. 3: Sucrose Density Gradient Recentrifugation of the
Nuclear Fraction.
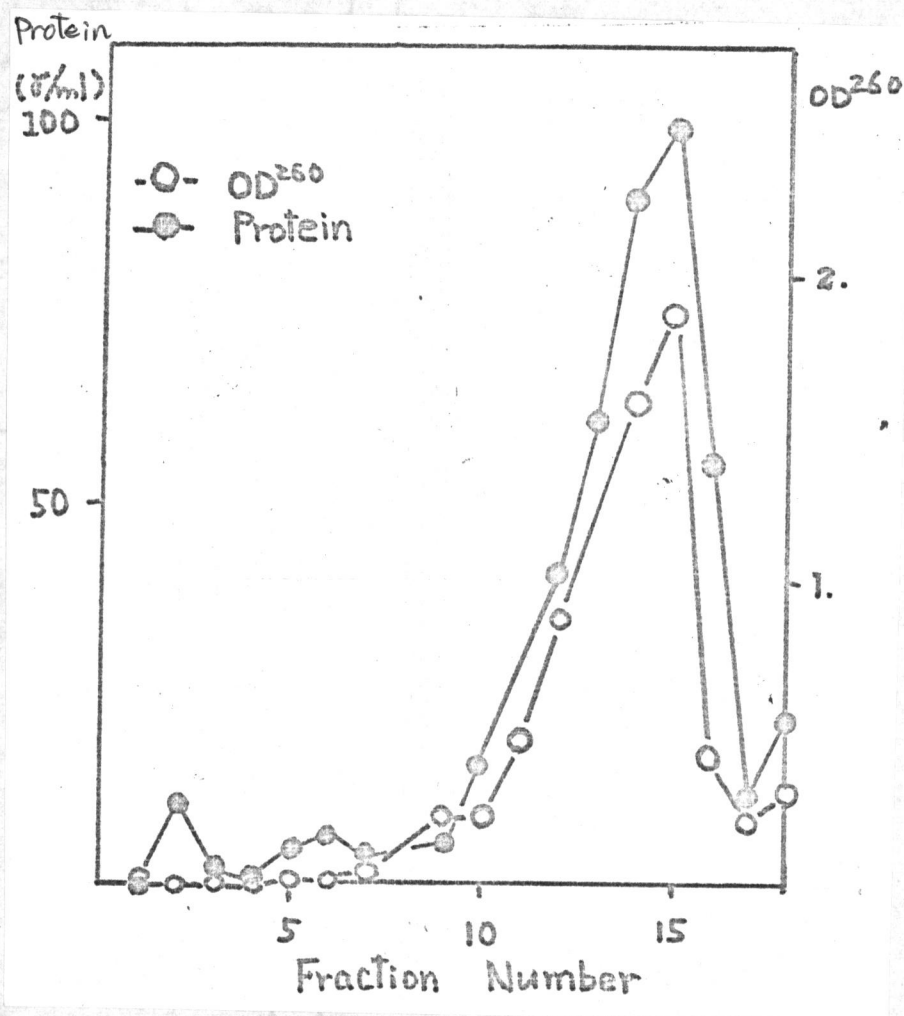
Fig. 4: Column Chromatography of the Nuclear Fraction .
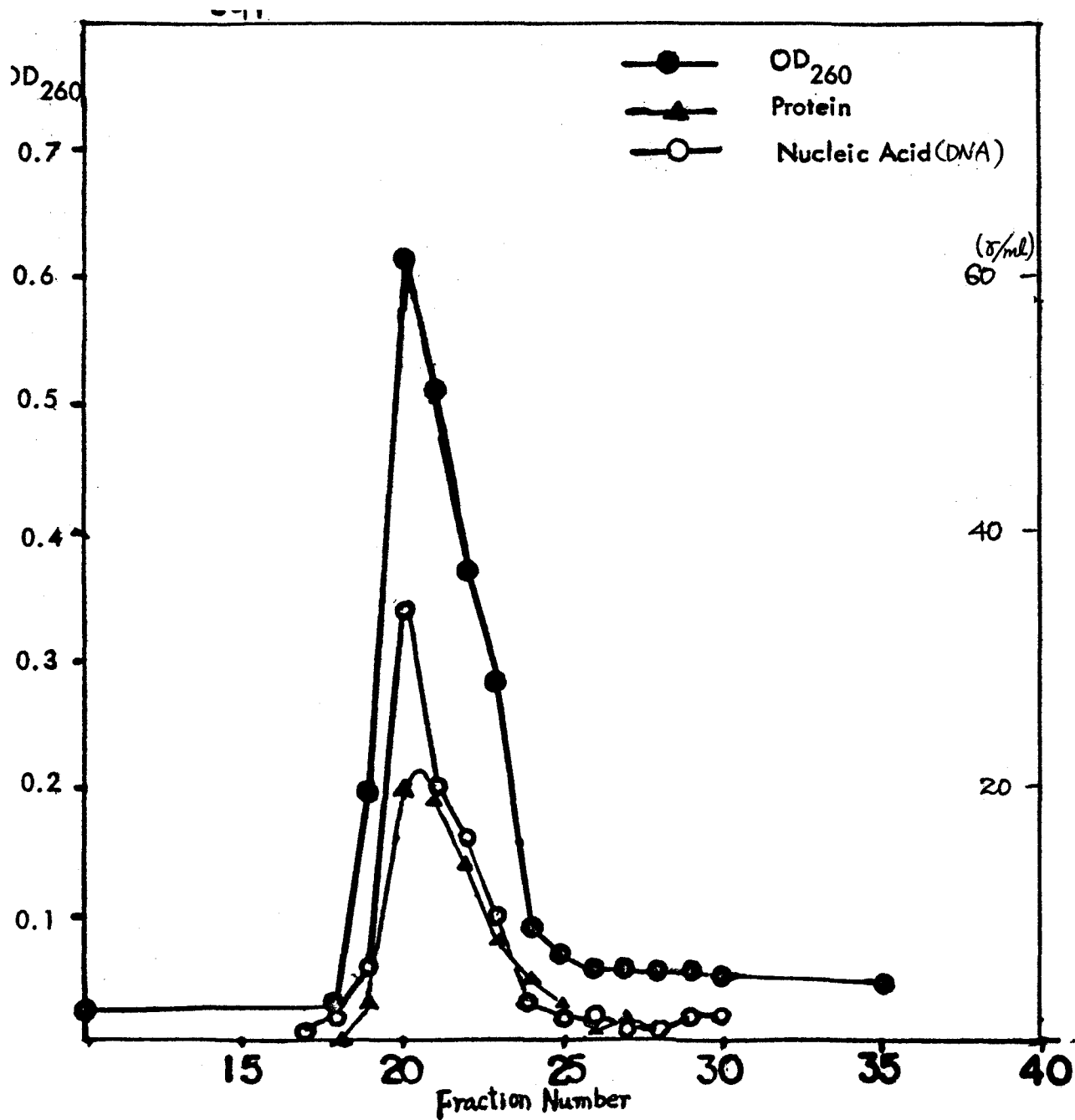
**Fig. 5:** UV Absorption Spectra of the Nuclear Fraction
and DNA of <u>B.subtilis</u>.
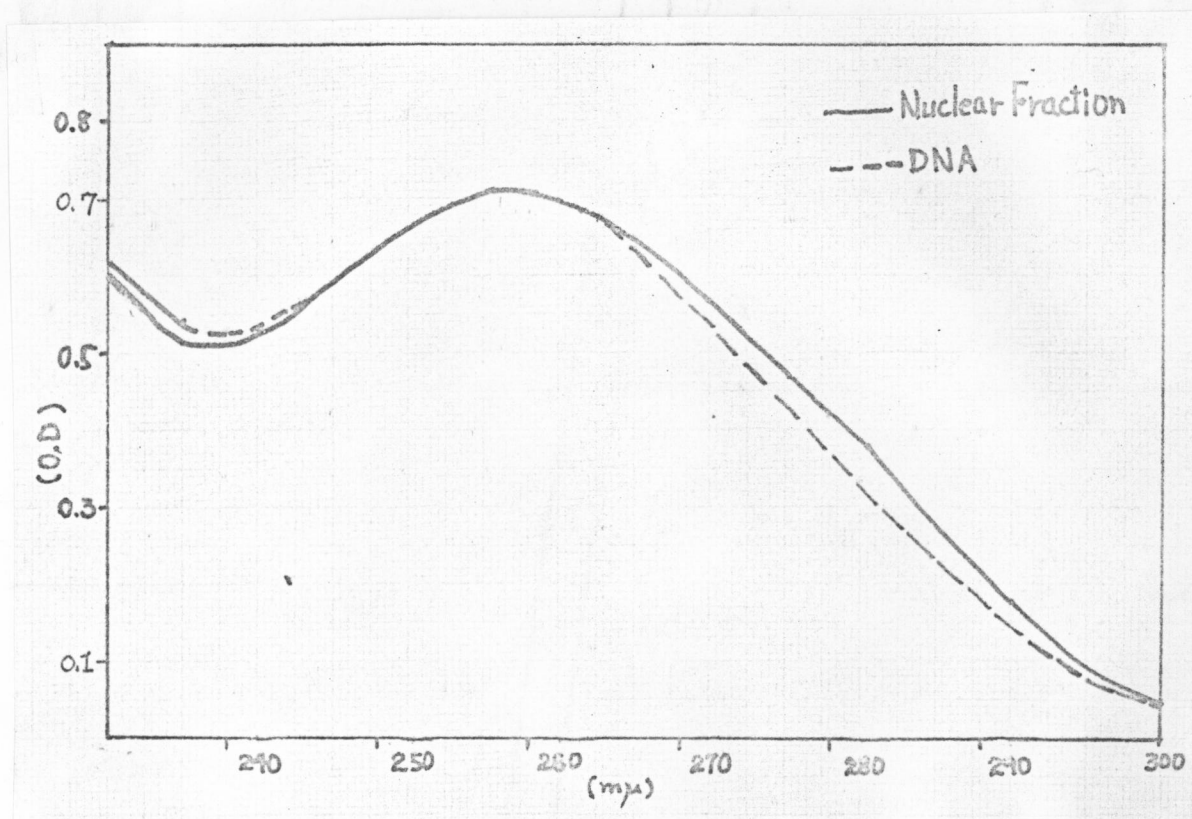
Fig. 6: Sucrose Density Gradient Centrifugation of the
Nuclear Fraction and DNA of B. subtilis.
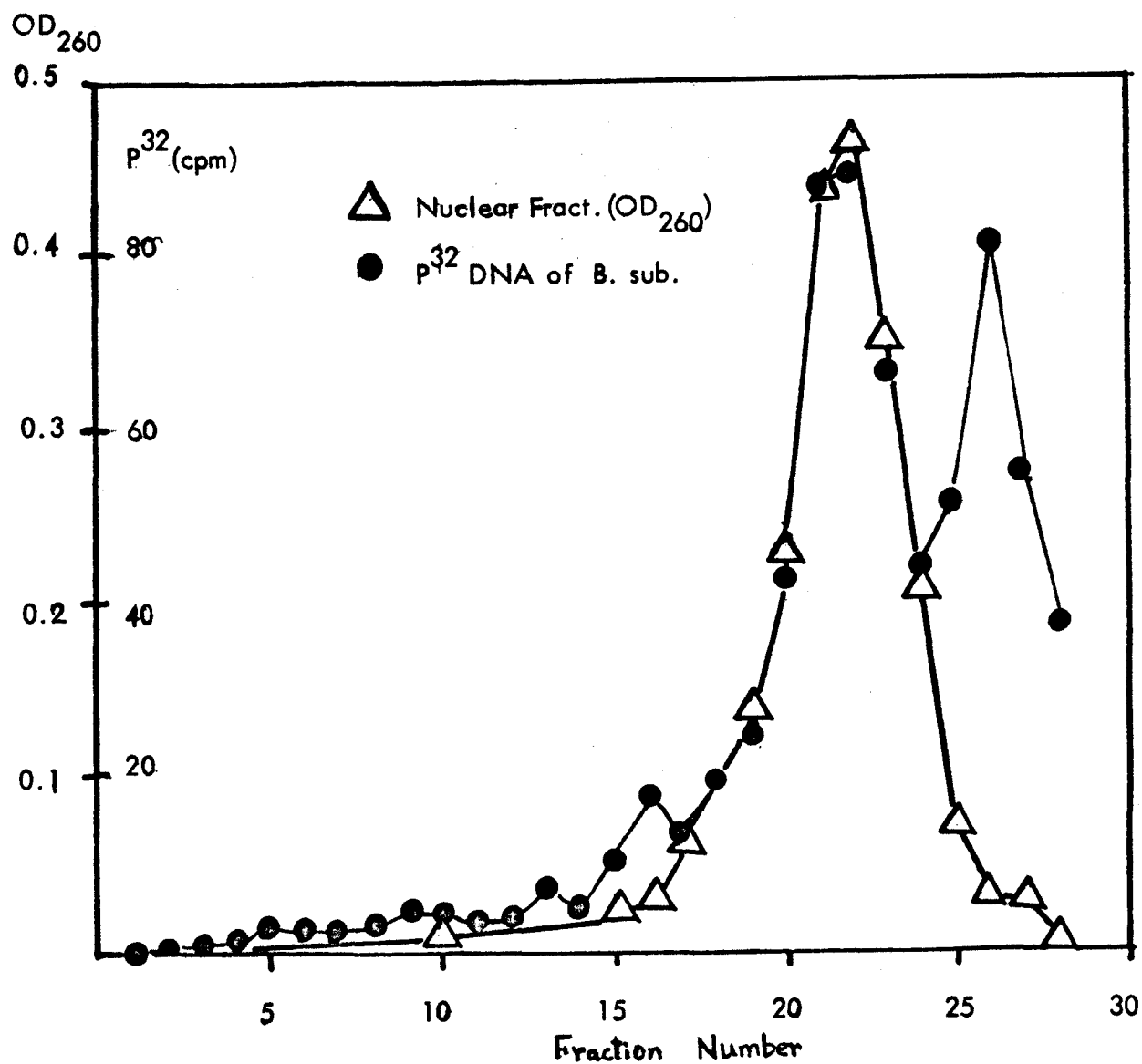
Fig. 7: Electron Micrography of the Nuclear Fraction.

a)
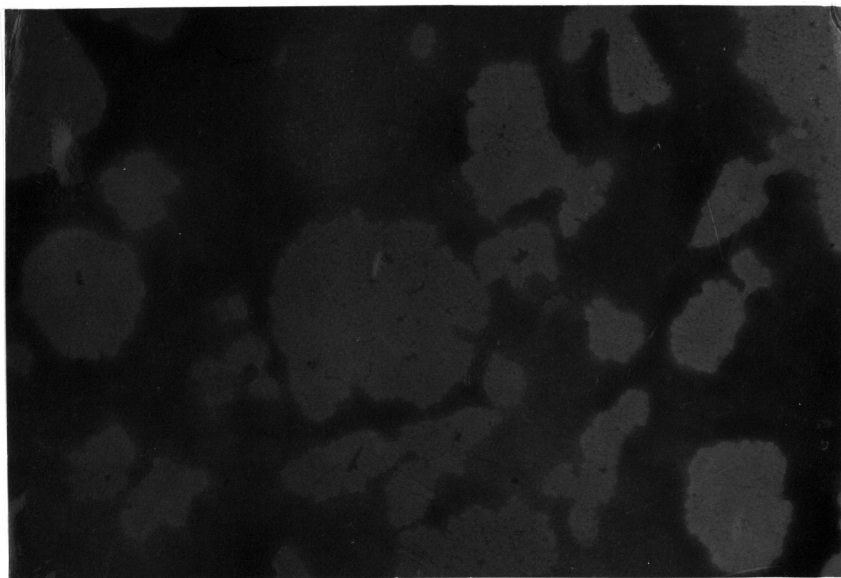


b)
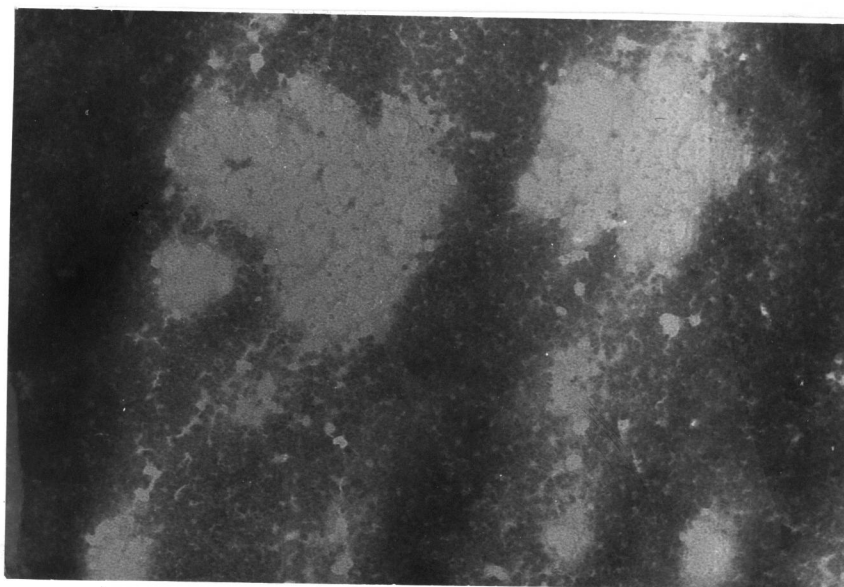
Fig 8:   CsCl Density Gradient Centrifugation of the Nuclear Fraction.
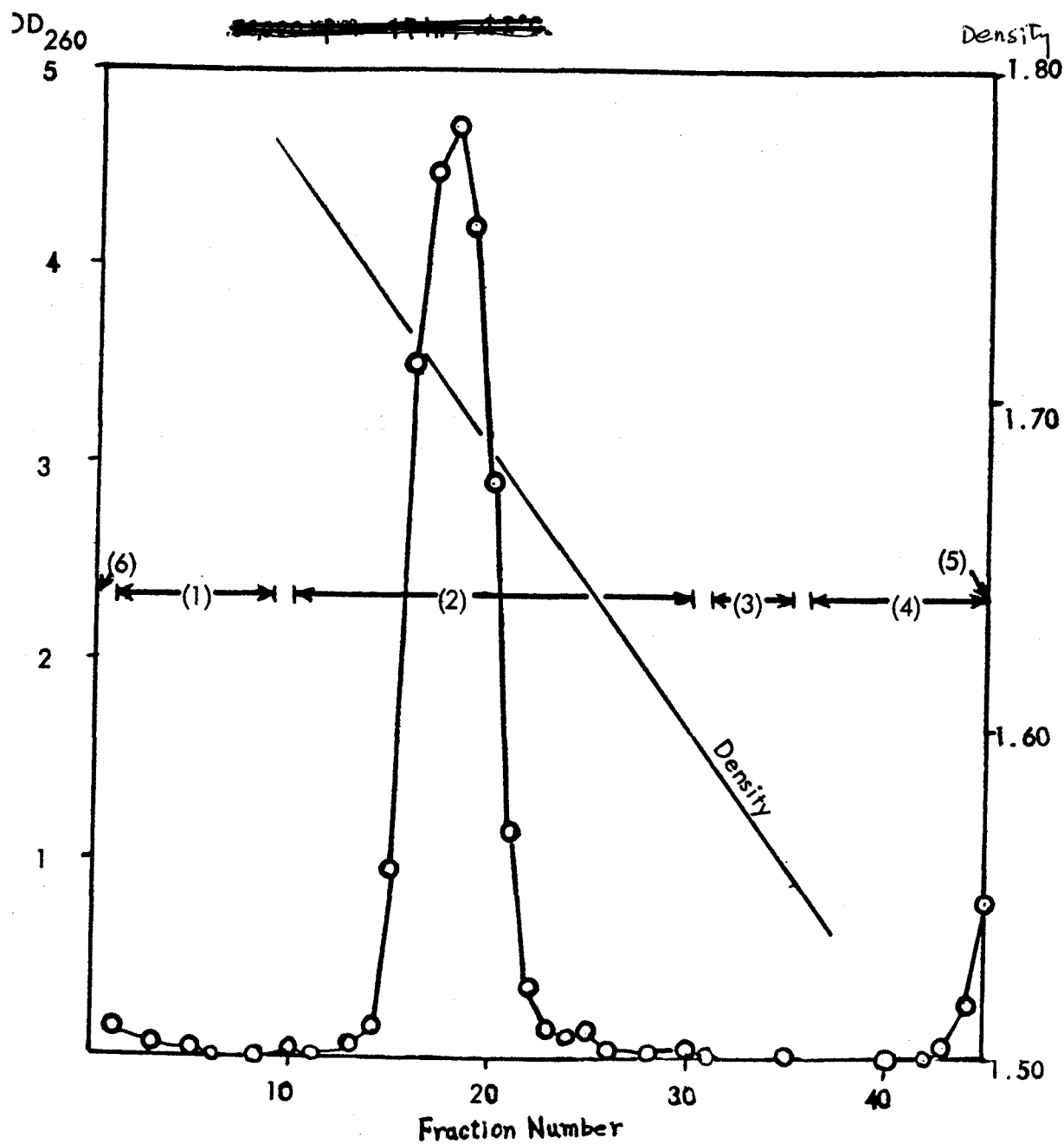
Fig. 9: Sucrose Density Gradient Centrifugation of
$P^{32}$- DNA Isolated from the Nuclear Fraction and
DNA from Whole cell.
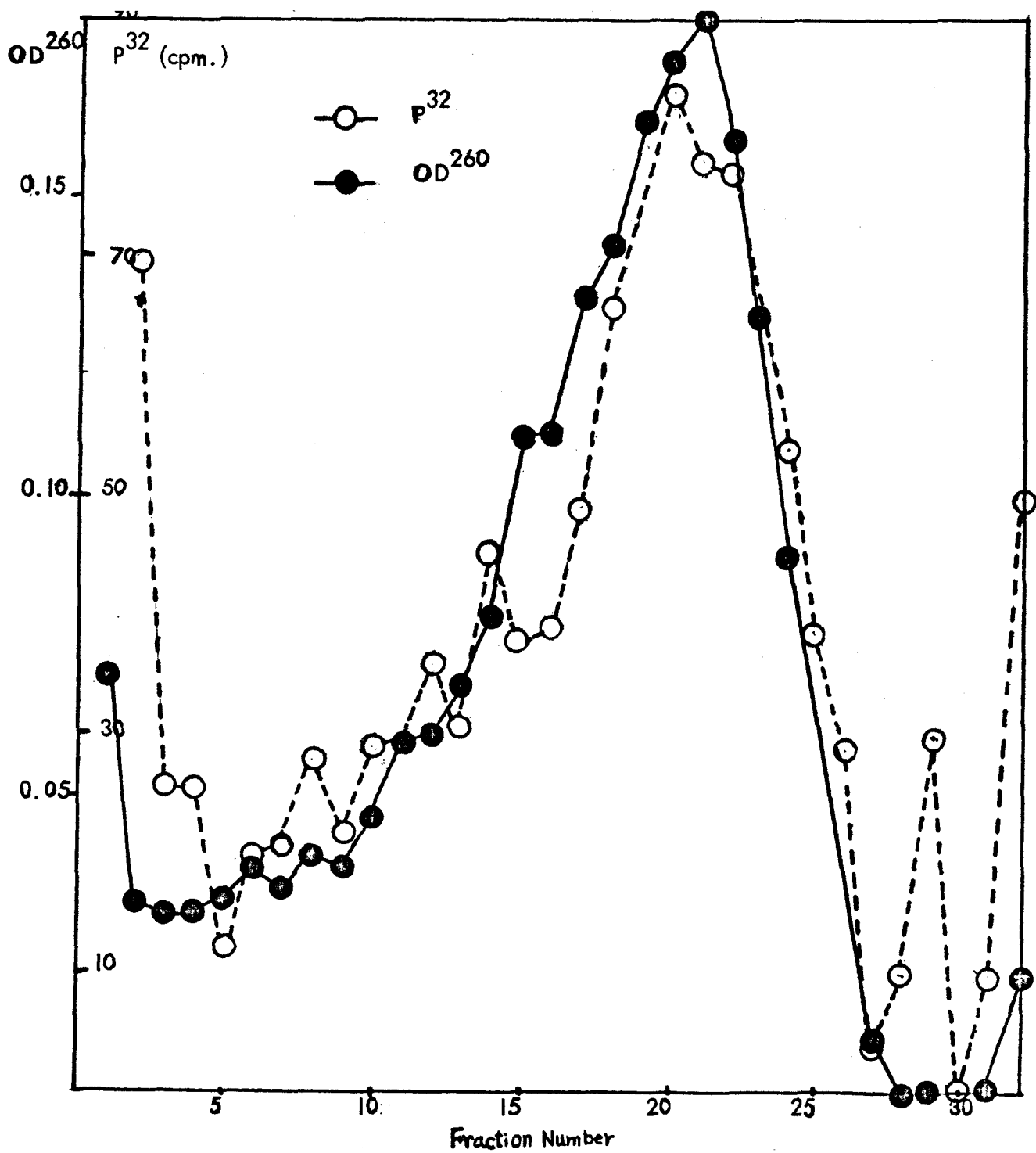
Fig. 10) Sucrose Density Gradient Centrifugation of
RNA I, RNA II, and DNA.
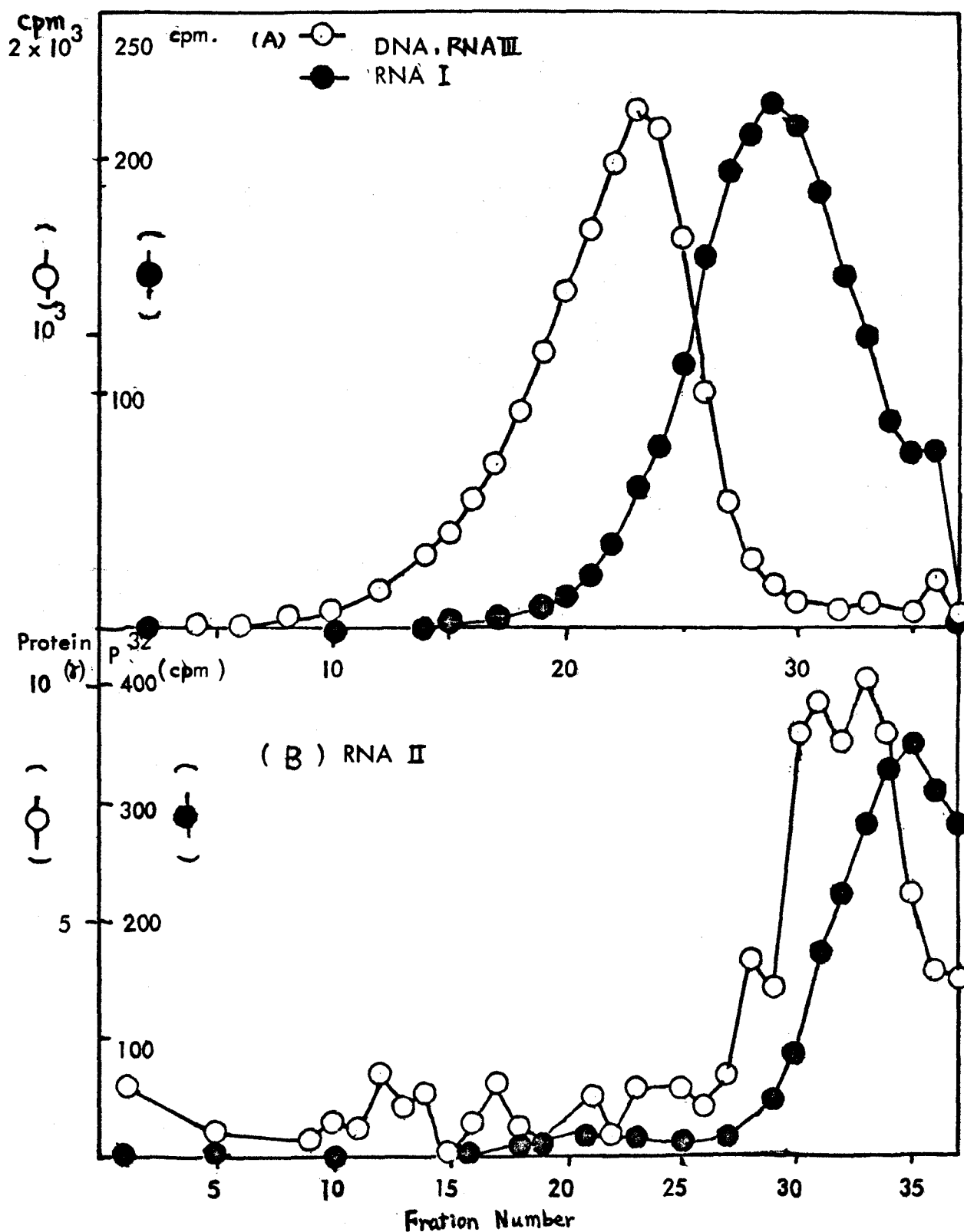
Fig. 11: Sucrose Density Gradient Centrifugation of
the P$^{32}$-pulse labelled Nuclear Fraction
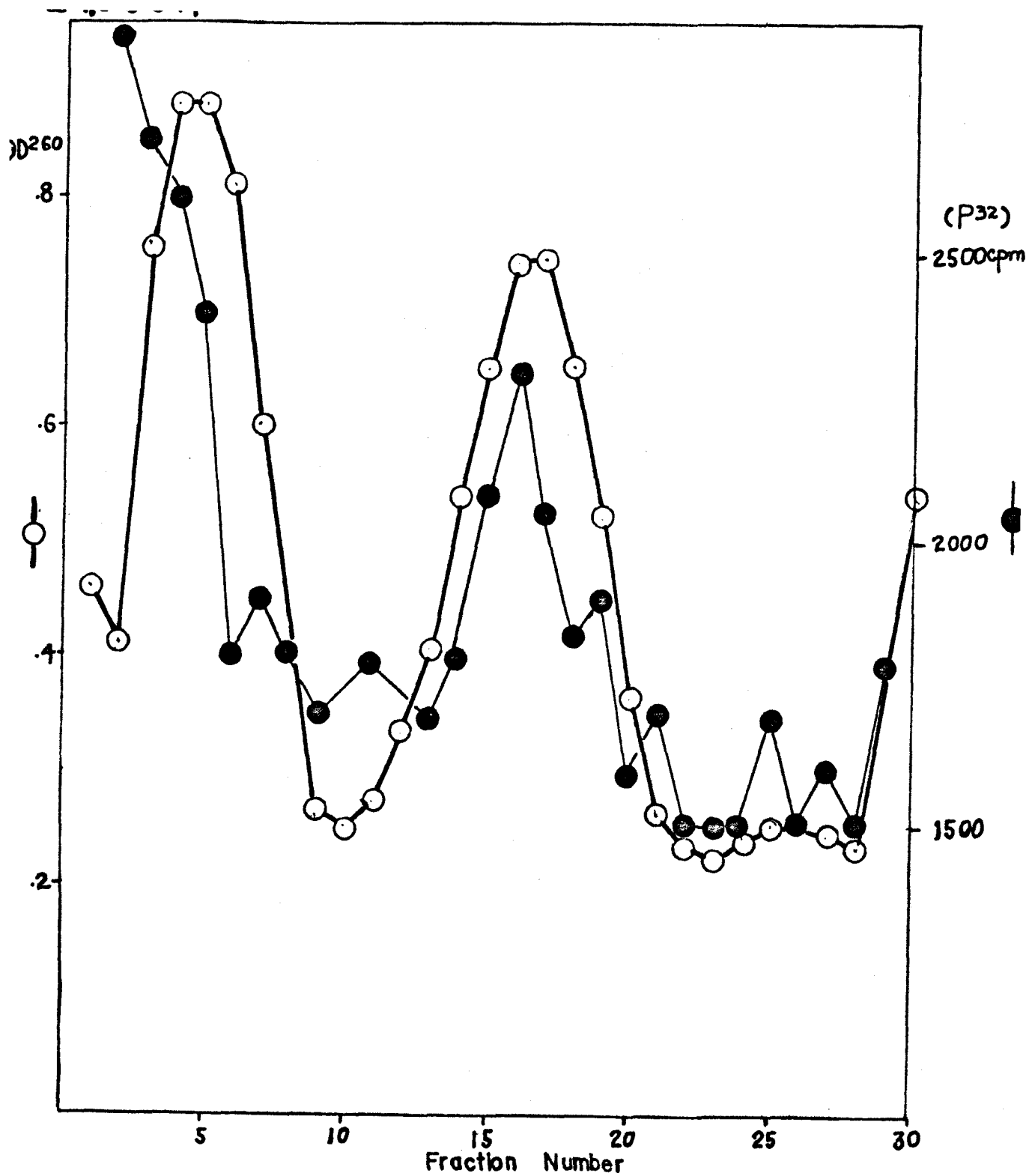
Fig. 12:  CsCl Density Gradient Centrifugation of P$^{32}$

Pulse Labelled Nuclear Fraction.
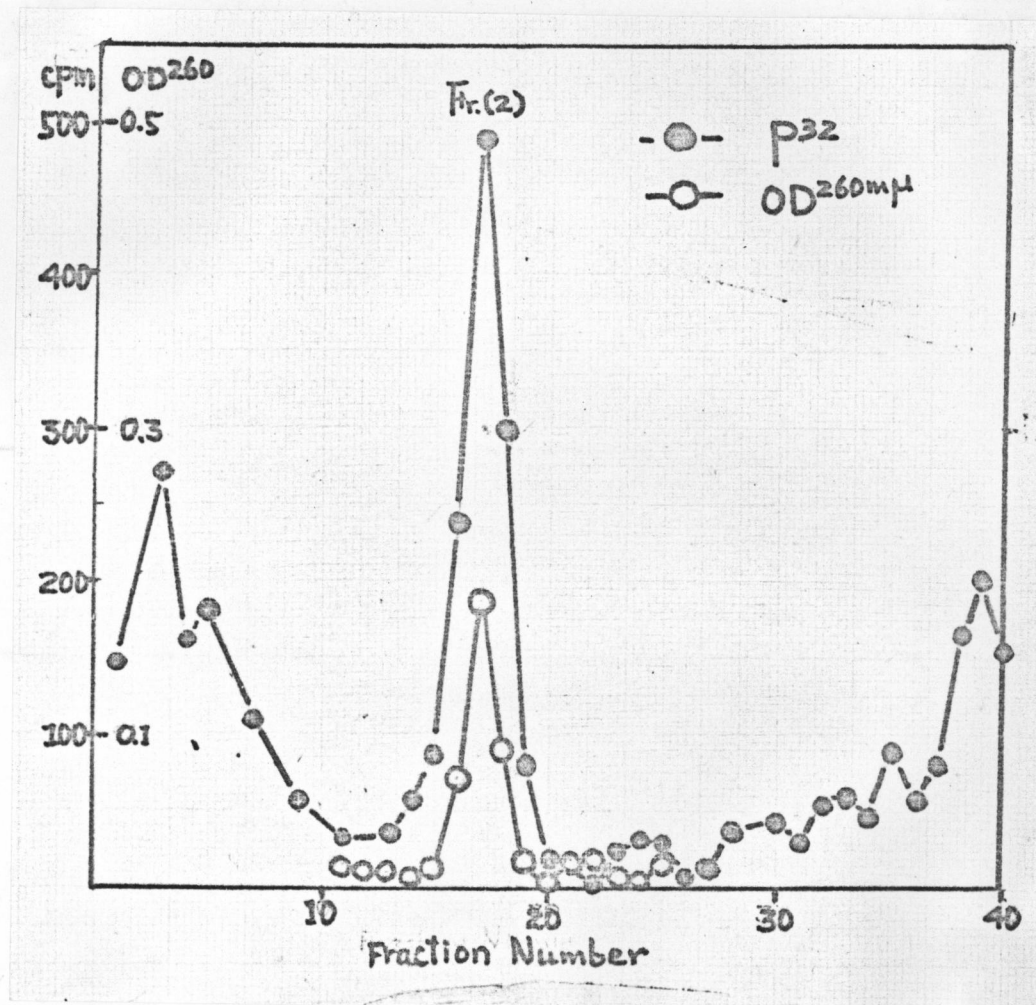
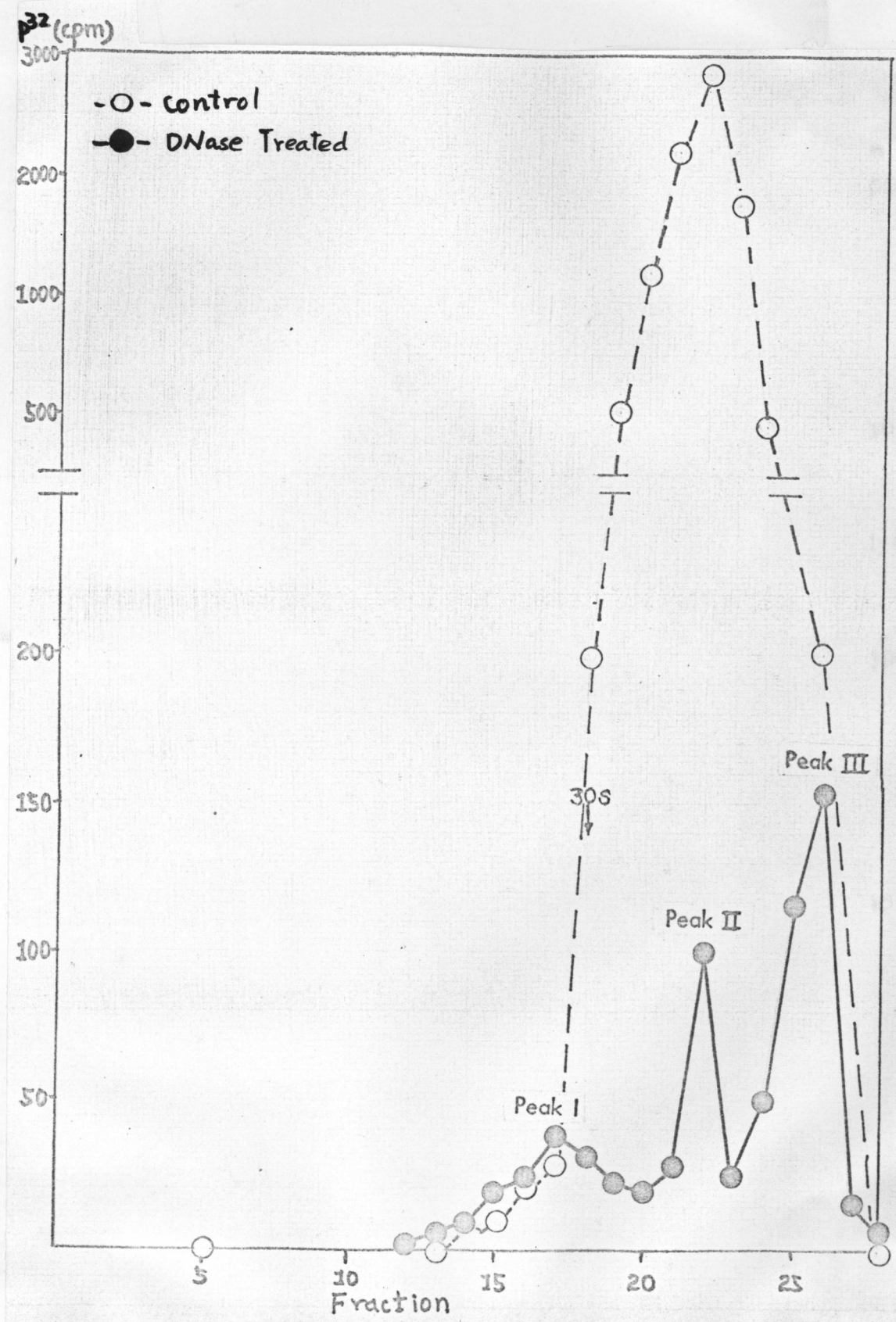Fig. 13: Sucrose Density Gradient Centrifugation of DNA.

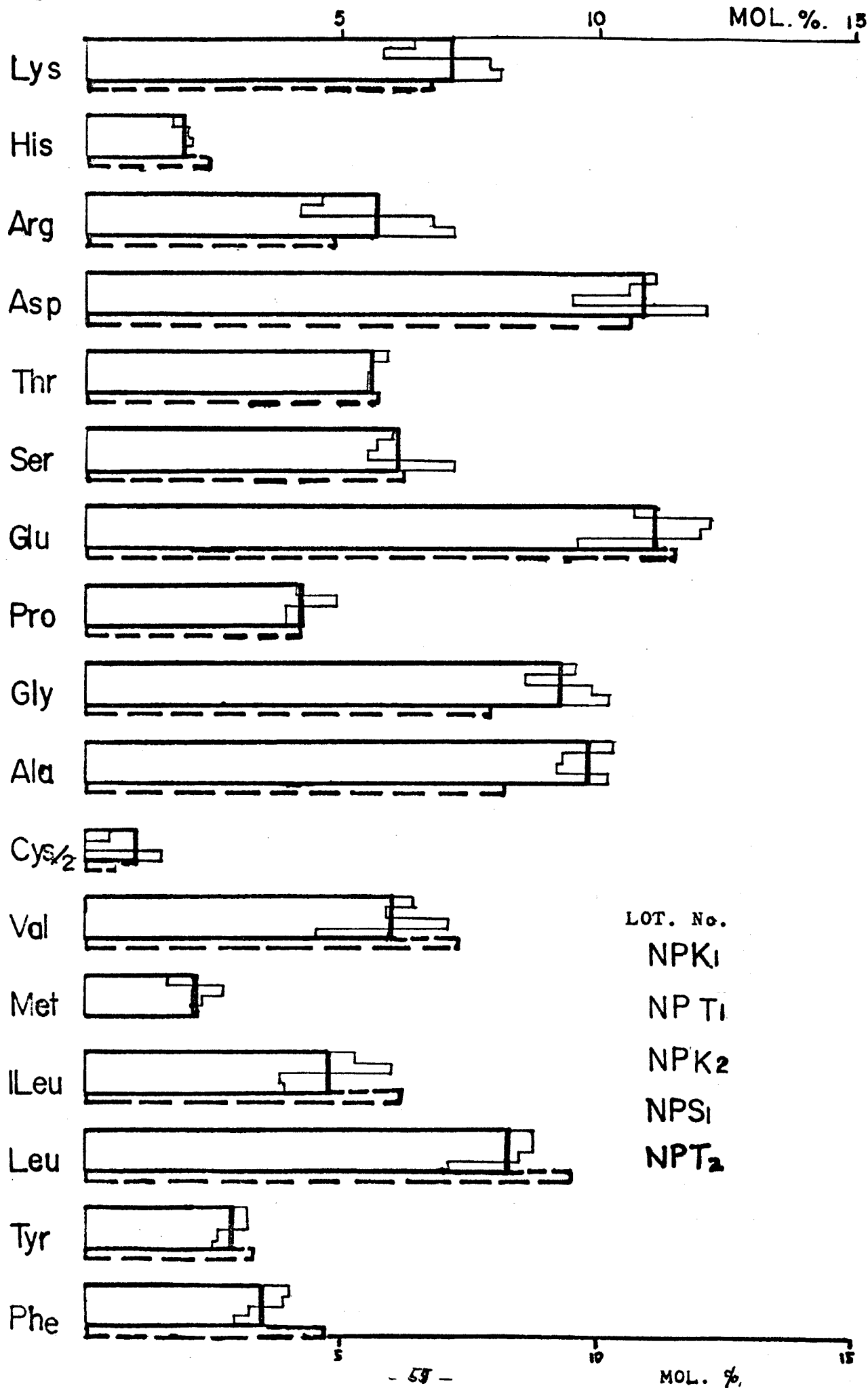Fig. 14 AMINO ACID COMPOSITION OF THE NUCLEAL ~~FRACTION~~ PROTEIN.

MOL. %.

Lys

His

Arg

Asp

Thr

Ser

Glu

Pro

Gly

Ala

Cys/2

Val

Met

ILeu

Leu

Tyr

Phe

LOT. No.

NPK₁

NP T₁

NPK₂

NPS₁

NPT₂

MOL. %.

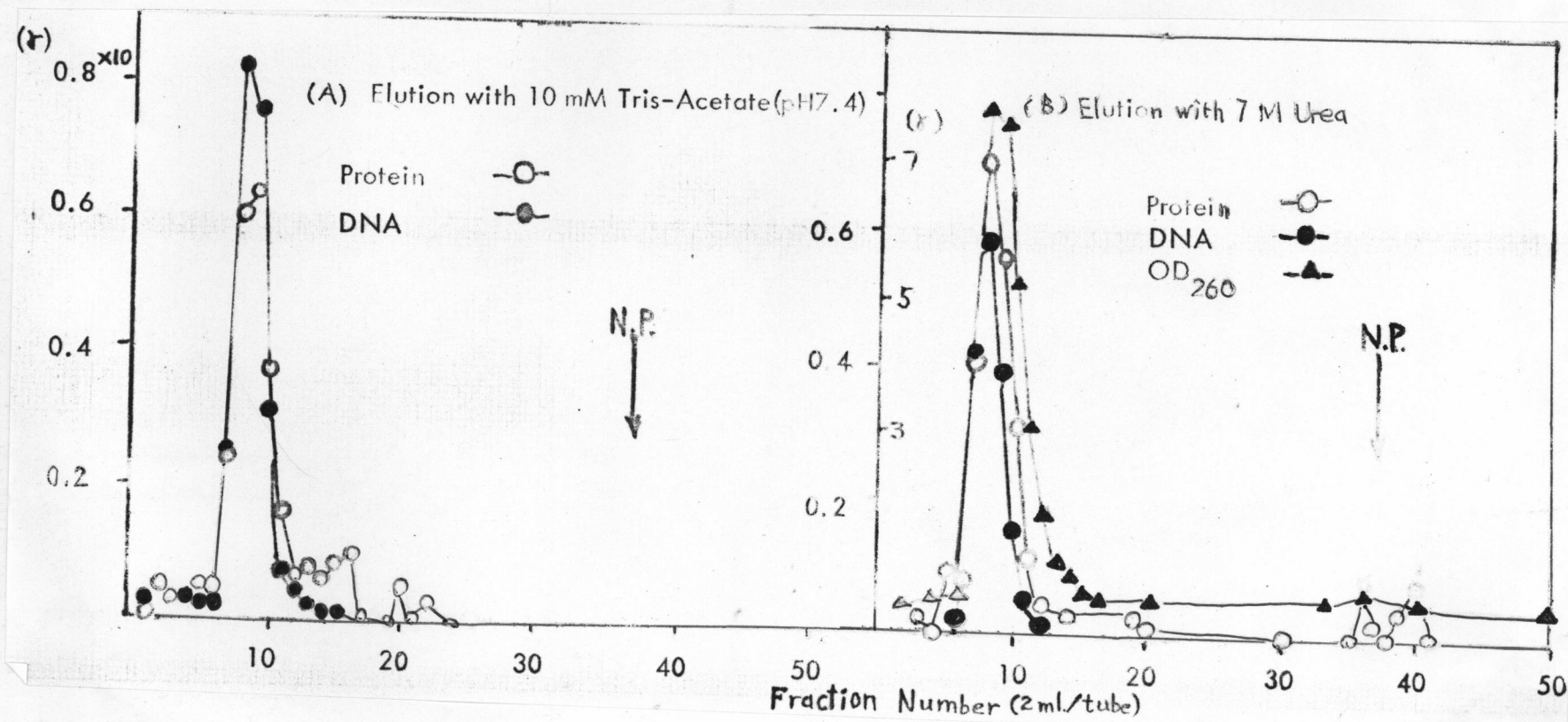Fig. 15: The Column Chromatography of Deoxyribonucleoprotein.

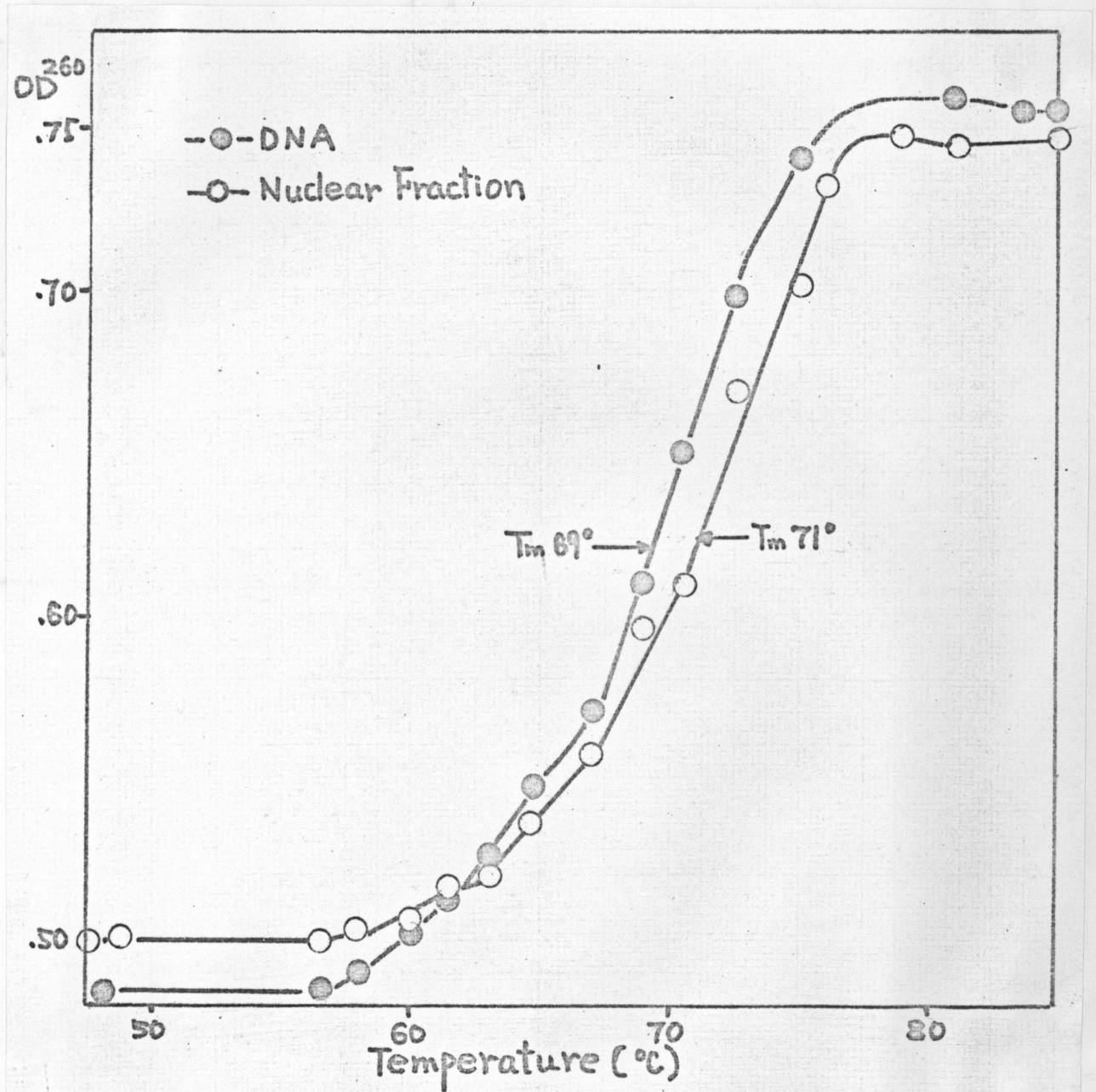Fig. 16:  Melting Profile of DNA from Whole Cell and
the Nuclear Fraction.

Fig. 17.    Melting Profile of DNA and recombined DNP