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Studies on the Binding of B(a)P diol epoxide to DNA and Chromatin

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Running Title: BaP Diol Epoxide Binding to DNA Chromatin

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DISCLAIMER

Introduction

Benzo(a)pyrene [B(a)P] is a ubiquitous environmental pollutant and its toxic, mutagenic, and carcinogenic effects are a consequence of the metabolic conversion to reactive intermediates by the mixed function oxidase system [1-4]. These reactive metabolites of B(a)P are capable of interacting with nucleic acids and cellular proteins [5-9]. Although the interaction of these carcinogens with any of the cellular components may eventually lead to tumor formation, it is currently thought that a covalent DNA-B(a)P adduct may be one of the primary events in the initiation process of carcinogenesis [5,9].

In recent studies the ultimate carcinogenic form of B(a)P appears to be the (+)7B,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydro benzo(a)pyrene [(+)B(a)P diol epoxide-anti] [10-16]. B(a)P diol epoxide has been shown to form covalent adducts with RNA and DNA *in vitro* and *in vivo*, and the major adduct isolated was N^2(7,8,9-dihydroxy-7,8,9,10-tetrahydro benzo(a)pyrene-10-yL) guanine nucleoside. Other covalent adducts have now been isolated and characterized involving deoxyadinine, deoxycytidine, [17] and the N^7 position of deoxy-guanine [18]. Covalent binding of carcinogens to DNA has been shown to destabilize the DNA and interfere with DNA dependent transcription *in vitro* [19,20].

However, since eukaryotic DNA is associated with histones, which are responsible for packaging the DNA into discrete subunits called nucleosomes [21-26], the binding of carcinogens to chromosomal DNA may be subject to additional constraints. Nucleosomes in all eukaryotes examined to date contain 140 basepairs of DNA which are wrapped around a histone core, containing two of each H2A, H2B, H3, H4. At the primary level of organization the nucleosomes

are arranged like beads on a string[21], each nucleosome is connected to the next one by a spacer region, consisting of DNA which is variable in length and seems to have histone H1 and some non-histones associated with it [34]. Recent evidence has also shown that the binding of carcinogens to chromatin is not uniformly distributed [27,28]. However, since current data suggests that nuclease digestion may differentially hydrolyse DNA of "active" and "inactive" chromatin [34,26,35], these results should be carefully evaluated. We have therefore studied the <u>in vitro</u> interaction of B(a)P diol epoxide (anti) with chicken erythrocyte chromatin. Chicken erythrocyte chromatin was chosen since it has been well characterized, it is an example of inactive genome and the inter nucleosomal spacer DNA is rather constant containing 60-70 basepairs [30]. Consequently the binding of a compound to nucleosomal and internucleosomal DNA can be more precisely quantitated.

Our studies have revealed that the binding of B(a)P diol epoxide (anti) with chromosomal DNA appears to involve intercalation and covalent binding. This reaction can be differentially inhibited by a competing nucleophile such as cysteine. Analysis of the distribution of the carcinogen to mononucleosomes (ν_1) and dinucleosomes (ν_2) revealed that the internucleosomal DNA bound 3-4 times more carcinogen than the nucleosomal DNA. Furthermore, dissociation of labeled chromatin showed that this ratio is no longer detectable after re-constitution.

Materials and Methods

Preparation of carcinogen labeled nuclei, chromatin and nucleosome.

Chicken erythrocyte nuclei were isolated from either fresh or frozen chicken blood [29]. The nuclear suspension was diluted with isolation buffer [10 mM Tris (pH 7.0), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40] to contain 1.4 x 10^9 nuclei/ml, in 1.0 mM $CaCl_2$, 0.1 mM phenylmethanesulfonylfluoride (PMSF). Micrococcal nuclease (60 units/ml) was added, and the digestion at 37°C was stopped after 10 min by the addition of EDTA (10 mM final concentration). The digested nuclear suspension was dialyzed for 24 h with three changes of 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF. The lysed nuclear suspension was centrifuged at 10,000g for 10 min at 4°C. The supernatant containing solubilized chromatin was concentrated on a Amicon UM 10 membrane, and further purification was achieved by centrifugation through sucrose gradients (Fig. 3). Chromatin from fractions 41-54 (Fig. 3) was pooled and dialyzed against 10 mM Tris (pH 7.0), 0.1 mM PMSF at 4°C for 12 h. It was then reacted with [^{14}C] (±)trans 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (specific activity, 29.4 mCi/mmol) at 37°C for 30 min under yellow light. The reaction mixture was subsequently dialyzed against 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF at 4°C for 48 h with four buffer changes. The final dialysis step was against 10 mM Tris (pH 7.0), 0.1 mM PMSF. The dialysate containing oligonucleosomes (ν_n) was made 3 mM $CaCl_2$ and redigested with micrococcal nuclease (60 u/ml) for 5 min at 37°C. Nucleosome monomers (ν_1) and dimers (ν_2) were isolated after centrifugation through sucrose gradients (Fig. 4). The purified ν_1 and ν_2 were dialyzed against 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF, and their specific activity was determined.

Binding of B(a)P in the presence of cysteine.

Chicken erythrocyte DNA, chromatin and mononucleosomes were dissolved in 100 mM Tris (pH 7.0), 0.1 mM PMSF and 0.14 M KCL containing a variable amount of

cysteine. (±) B(a)P diol epoxide (anti) was added and the reaction mixture was dialysed as described above. The specific activities of non dialysable material are shown in Fig. II (closed symbols). Since chromatin precipitated in the presence of 0.2 MKCL the above reaction was repeated in the absence of KCL. Under these conditions chromatin remained soluble (Fig. II, open symbols).

Purification of DNA.

Chromatin, ν_1 and ν_2 were repeatedly extracted with phenol:chloroform (1:1 v/v) followed by chloroform and ether extractions. The purified DNA was precipitated with ethanol at -20°C. The dried DNA pellet was dissolved in 0.2 mM EDTA (pH 7.0). A known volume (0.1 ml) was routinely dissolved in 10 ml Aquasol (New England Nuclear) and the radioactivity was determined, using a Packard (Model 3385) scintillation counter.

Purification of histone core complex.

For the isolation of histone core complex, purified chicken erythrocyte nuclei were labeled with (±) B(a)P diol epoxide in the isolation buffer (see above). This reaction mixture was extensively dialysed against 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF. The subsequent chromatin suspension was made 2.0 M NaCl by slow addition of solid NaCl with gentle mixing. The resulting viscous solution was left for 12 hours at 4.0°C and centrifuged in a Beckman 50 Ti rotor at 48,000 rpm for 12 hours. The pellet (i.e. DNA and residual proteins) was solubilized in 0.2 mM EDTA (pH 7.0), and the DNA was purified as described above. The supernatant solution containing histones and some non-histone proteins, was concentrated by vacuum dialysis. The core histone proteins (H2A, H2B, H3, and H4) were purified by centrifugation through a 5-20% linear sucrose gradient containing 2.0 M NaCl, 10 mM Tris (pH 7.2), and 0.1 mM dithiothreotol (DTT) for 44 hours at 50,000 rpm in an SW 50.1 rotor at 4°C[41].

The histone core was isolated and the specific activity was determined by use of the value $E_{230(1cm)}^{0.1\%} = 4.2$.

Reconstitution of carcinogen chromatin complex

Chromatin was isolated, labeled and dialysed as described above except that in this experiment [^{3}H](±)B(a)P diol epoxide (anti) (S.A 269 mCi/mmole) was used. Aliquots of labeled chromatin were made, 0.65 M and 2.0 M in NaCl concentration, by slow addition of solid NaCl. Reconstitution was achieved by stepwise dialysis for at least four hours against: A) 2.0 M NaCl, 0.1 mM DTT, 10 mM Tris (pH 7.0), B) 1.4 M NaCl, 0.1 mM DTT, 10 mM Tris (pH 7.0), C) 1.0 M NaCl, 0.1 mM DTT, 10 mM Tris (pH 7.0), D) 0.3 M NaCl, 10 mM Tris (pH 7.0), E) 0.1 M NaCl, 10 mM Tris (pH 7.0), F) 10 mM Tris (pH 7.0). All solutions contained 0.1 mM PMSF and 0.2 mM EDTA (pH 7.0), and final dialysis for 12 hours was against 0.2 mM EDTA (pH 7.0), and 0.1 mM PMSF. The specific activities of reconstituted chromatin is shown in Table II. Further digestion of this labeled chromatin to mononucleosomes and oligonucleosomes was obtained by redigestion of reconstituted chromatin (Fig. VI), using the parameters described in Fig. V. Aliquots of each individual pool (A, B and C) were further digested to mononucleosomes and the specific activities are shown in Table III.

Fluorescence spectrophotometry

All fluorescence measurements were made with a conventional fluorescence spectrophotometer (Perkin Elmer MPF44) at room temperature. The reaction buffer was 10 mM Tris (pH 7.0), containing a variable amount of DNA or 200 µg of mononucleosomes. The reaction mixture was equilibrated at room temperature and the reaction in each experiment was started by the addition of 10 µl (8.6 µg) B(a)P diol epoxide in tetrahydrofarane:triethylamine (19:1 v/v) which was mixed

by rapid inversion. The λex = 327 nm, the λem was either maintained at 379 nm for continuous measurement (Fig. I), and/or scanned from 300 nm to 475 nm Fig. I (inset).

Results and Discussion

In order to establish initial conditions and parameters for the reaction of B(a)P diol epoxide (anti) with various nucleophiles, we have studied the rate of breakdown of B(a)P diol epoxide in buffer and buffer containing different amounts of DNA, either as free DNA or as nucleohistone complex.

Fluorescence

It was found that the rate of breakdown of B(a)P diol epoxide to tetraoles could be monitored by fluorescence spectrophotometry, since B(a)P diol epoxide has no detectable fluorescence spectrum in aqueous medium, while the tetraoles show a fluorescence spectrum analogous to one obtained by pyrene adducts [36] (Fig. I). The rate of tetraole formation from B(a)P diol epoxide in solution at fixed emission and excitation wavelengths can therefore be monitored. Discontinuous monitoring of the spectrum at time intervals during this reaction revealed that the increase in relative fluorescence intensity was due to an increase in the magnitude of the pyrene-like spectrum (Fig. I inset), and not due to any other form of spectra. Free tetraoles added to the reaction buffer produced an identical spectrum which did not change with time. In the case of solvolysis (Fig. IA) an initial fast reaction was observed while a slower reaction continued for about 35 minutes; after this no further changes were observed. The presence of a nucleophile such as DNA, or monomucleosomes appeared to abolish the slow reaction observed in A. It should be pointed out that the relative intensity decreased with increasing amounts of nucleophile. Fluorescence quenching of a variety of dyes such as acriflavine, proflavine and quinacrine, in the presence of DNA, has been observed by a number of investigators [37-39]. This phenomenon was identified with intercalation into G-C rich regions of DNA and

the mechanism of quenching has been related to the formation of a charge-transfer complex in the excited state involving π electron interactions. Consequently, the fluorescence quenching observed when B(a)P diol epoxide is reacted with DNA would appear to indicate that the interaction involves not only covalent binding to the exocyclic amino group of guanine, but also intercalation[40]. The latter may be responsible for the decrease in fluorescence intensity. Furthermore, the reaction in the presence of a nucleophile was complete within 10 minutes, and thus an incubation time of 30 minutes at 37°C would be more than adequate to ensure completion of the reaction.

Addition of 540 µg DNA to the reaction mixture containing only solvolysis products i.e., tetraoles (Fig. IA) decreased the intensity to the height shown in curve D (Fig. I). Since the maximum level of fluorescence intensity in curve D was obtained by reacting the B(a)P diol epoxide with 25 µg DNA, this would suggest that the reaction involving B(a)P diol epoxide and DNA enhanced (≈20 fold) intercalation. It is not clear at this time whether covalent binding is a prerequisite to intercalation or vice versa.

Similar in the reaction of the diol epoxide with purified mononucleosomes the decrease in relative intensity would suggest that this reaction is not of the same magnitude in the nucleohistone complex as that found with free DNA. This may indicate that the presence of histones decreases the availability of binding to chromosomal DNA. In other words, it may well be that the presence of histones in eukaryotic chromatin confers an additional level of specificity on the reaction of carcinogens with DNA.

The effect of cysteine on the binding of B(a)P diol epoxide

To inhibit the reaction of the diol epoxide with DNA, chromatin and mononucleosomes we have used a competing nucleophile, cysteine. The effects of

cysteine on adduct formation are shown in Fig. 2. Free DNA contained almost twice the number of non dialysable molecules as ν_1 and chromatin. The lower incorporation of radioactivity into chromatin, compared with ν_1 (closed symbols), could be attributed to a solvent affect, since in the presence of 0.2 M KCl chromatin precipitated. By changing the ionic strength of the medium (in the absence of KCl) chromatin remained soluble and the incorporation of adducts was increased (open squares, Fig. 2). The presence of cysteine therefore would largely abolish the covalent type interaction and at [cys] >3.2 mg/ml 85% of the radioactivity associated with DNA could be removed by repeated phenol extraction. Repeated extraction of chromatin or ν_1 with phenol removed only 20% of the bound carcinogen. This data would therefore suggest that under our in vitro condition DNA incorporated a substantial amount of non-covalently bound material. In chromatin or mononucleosomes about 80% of the carcinogen seems to be covalently bound to the DNA.

Distribution of B(a)P adducts in chromosomal components

In order to examine the distribution of the carcinogen with respect to chromosomal DNA and histone proteins, in detail, isolated chicken erythrocyte nuclei were labeled with B(a)P diol epoxide. The labeled nuclei were lysed and the resulting chromatin component, DNA and proteins were purified as described in materials and methods. The phenol purified DNA had a specific activity of 29,629 cpm/mg DNA and the purified histone core proteins (H2A, H2B, H3, and H4) had a specific activity of 4,476.5 cpm/mg histone. Consequently, only 15% of the radioactivity was associated with the core histone while the remainder was associated with chromosomal DNA. The binding of B(a)P diol epoxide to the core histone complexes is thus a minor component, in contrast with other

studies in which the parent hydrocarbon has been used[8,32]. It would therefore be important to know if some other metabolites of B(a)P are involved in binding to chromosomal proteins.

Distribution B(a)P adduct in nucleosomal and internucleosomal DNA

Since it is now apparent that most of the in vitro binding of the diol epoxide to chromatin involves the formation of covalent adducts with DNA we have examined the distribution of adducts in chromosomal DNA, w.r.t. nucleosomal and internucleosomal regions. The isolation of chromatin (ν_n) (Fig. 3), labeling and redigestion has been described in Materials and Methods. The isolated ν_1 and ν_2 (Fig. 4) were pooled, concentrated, and the specific activities of ν_1 and ν_2 from 3 independent experiments are shown in Table I. These experiments showed that ν_2 contained more bound carcinogen per unit DNA than did ν_1.

Furthermore, phenol extraction of ν_n, ν_1, and ν_2 revealed that in each case 75 ± 5% of the carcinogen was associated with the DNA. Since in chicken erythrocyte chromatin the internucleosomal region appears rather constant at 60-70 base pairs of DNA and the core ν_1 contains approximately 140 base pairs[30,31], we have estimated that the internucleosomal region bound 3.8 ± 0.5 times more carcinogen than the core nucleosome. This increase in binding to the internucleosomal region appears to be due to the increased accessibility of the internucleosomal DNA, since phenol extraction does not change the ratio of specific activities. Furthermore, since chicken erythrocyte chromatin may be viewed as a 200-210 base pair repeat containing a 140 base pair ν_1 plus a 60-70 base pair spacer region, the specific activity of chromatin calculated from our experimental findings was within 10% of the experimental value observed for chromatin.

Reconstitution of chromatin containing B(a)P adducts

We have dissociated and reconstituted labeled chromatin to examine whether the presence of B(a)P adducts would interfere with the assembly process of nucleosomes and if the distribution of adducts remains similar after reconstitution. In this manner one might be able to use the distribution of adducts as a probe to evaluate the fidelity of reconstituted chromatin in vitro. Furthermore, some of the answers could then be related to the in vivo situation of chromosomal assembly in the presence of carcinogens during the S-phase of the cell.

Chromatin was isolated as previously described, treated with [^{3}H] B(a)P diol epoxide (anti) and extensively dialysed to a specific activity of 14,520.0 cpm/A260. Aliquots of this labeled chromatin were made 0.65 M NaCl, to dissociate histones H1 and H5, and 2.0 M NaCl, to dissociate all histones and some non histone protein, from chromosomal DNA. Reassociation was achieved as described in Materials and Methods. The specific activities of the reconstituted chromatin is shown in Table II.

In order to establish parameters for redigestion with micrococcal nuclease, labeled chromatin was redigested, as shown in Fig. V. This revealed that chromatin had been subjected to slow digestion in the absence of added enzyme, presumably by residual micrococcal nuclease activity, and that the digestion to multimers was optimum in 3 min, while complete digestion to mononucleosomes took place in 15-20 min (Fig. V). The control and reconstituted chromatin solutions were redigested to monomers and oligomers of nucleosomes (Fig. VI A, B and C). Each pool (1-5) was isolated and the specific activities were determined (Table III). Aliquots of each pool were redigested to mononucleosomes using the digestion parameters shown in Fig. V (E). The specific activities of the purified ν_1 from each pool are shown in Table III.

One of the striking features of this analysis showed that the mononucleosomes of the 2.0 M NaCl dissociated and reassociated chromatin(Table III C) have the same specific activity as chromatin, while the control (Table III A), showed the typical non uniform distribution of the carcinogen w.r.t. nucleosome v/s internucleosomal region. This would indicate that in the 2.0 M NaCl dissociated chromatin, reconstitution leads to random distribution of histones. This suggests that the presence of B(a)P adducts does not hinder the formation of nucleosomes and that a compound such as B(a)P diol epoxide can be used to ascertain the fidelity of reconstituted chromatin. A second feature that we have observed consistently is the high specific activity of mononucleosomes obtained from short digestion of labeled nuclei, or chromatin with micrococcal nuclease. This could be attributed to either a subpopulation of ν_1 that contain spacer-regions, which are much more susceptible to binding of the carcinogen or to some other macromolecule (i.e., RNA or nuclear protein) associated with the internucleosomal region that can be removed by micrococcal nuclease digestion.

Conclusion

The data presented here, strongly suggests that the interaction of B(a)P diol epoxide (anti) with free DNA or chromosomal DNA involved, apart from covalent binding, intercalation of the covalent and non-covalent bound carcinogen, and that in the case of chromatin or mononucleosomes intercalation appeared to be decreased. This decrease may be a function of changes in DNA conformation, steric hindrance or both due to the presence of chromosomal proteins. Furthermore in the presence of a competing nucleophile such as cysteine, the covalent binding of B(a)P diol epoxide could be greatly diminished and our data suggests that little if any non-covalent association occurred in chromatin.

Analysis of the distribution of carcinogen with respect to chromosomal components revealed that most of the adducts were associated with chromosomal DNA and that the internucleosomal DNA contained 3-4 times more carcinogen than nucleosomal DNA. Reconstitution of labeled chromatin showed that removal of the very lysine rich histones by 0.65 M NaCl did not affect the distribution of the carcinogen, while "complete" dissociation using 2.0 M NaCl and reassociation revealed randomization of the bound carcinogen.

The reconstitution experiments showed that the presence of carcinogen did not interfere with the reassociation of histones to DNA, and that B(a)P diol epoxide could be used to evaluate the fidelity of reconstituted chromatin *in vitro*.

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Fig. I.

Kinetics of tetraole formation from B(a)P diol epoxide measured by fluorescence spectrophotometry; λex = 327 nm, λem = 378 nm. B(a)P diol epoxide (8.6 μg) was added to each reaction mixture. A, 10 mM Tris (pH 7.0); B, 0.5 μg DNA/ml; C, 100 μg DNA/ml as mononucleosomes; D, 25 μg DNA/ml; E, 100 μg DNA/ml; F, 500 μg DNA/ml; G, 1000 μg DNA/ml.

Inset shows typical spectrum obtained at any point during the reaction. This spectrum is identical to the spectrum of tetraoles having excitation maxima at 378 nm, 397 nm and 420 nm.

Fig. II.

Incorporation of B(a)P diol epoxide into DNA, chromatin, and nucleosomes as a function of cysteine concentration. Chicken erythrocyte DNA, chromatin and nucleosomes (A_{260} = 1.0) was reacted with 1.0 µg B(a)P diol epoxide (anti) in the presence of cysteine. Closed symbols represent buffer containing 0.14 M KCL, open symbols represent buffer without KCL. DNA (●, ○), mononucleosomes (▲), chromatin (■,□).

Fig. III

Optical density profile of sucrose gradient. 2.5 ml chromatin (A_{260} = 83.0) was layered on a 5-25% linear sucrose gradient containing 0.2 mM EDTA (pH 7.0), 20 mM NaCl and was centrifuged for 14 h at 23,000 r.p.m. in an SW 25.2 rotor at 4°C. The gradients were fractionated with an ISCO density gradient collector (Model 640) equipped with an ISCO UV-5 absorbance monitor. Nucleosome monomers, dimers and oligomers are shown as ν_1, ν_2 and ν_n.

Fig. IV

(a) Chromatin obtained from pool ν_1 (Fig. III) was reacted with B(a)P diol-epoxide and redigested with micrococcal nuclease as described in the text. ν_1 and ν_2 were purified by ultracentrifugation through a 5-25% sucrose gradient containing 0.2 mM EDTA (pH 7.0), 20 mM NaCl by use of an SW 41 Ti rotor at 39,000 r.p.m. for 12 h at 4°C. The fractions containing ν_1 and ν_2 were pooled, concentrated, and subjected to another ultracentrifugation run under the same conditions to yield purified ν_1 (b) and ν_2 (c).

Fig. V.

Optical density profiles obtained from sucrose gradients. The conditions are similar to those described in Fig. IV. Chromatin (A_{260} = 6.6/ml) was incubated at 37°C in 10 mM Tris (pH 7.0), 0.1 mM PMSF, 3 mM $CaCl_2$ and 100 units/ml micrococcal nuclease. The reaction was stopped with addition of EDTA (10 mM final concentration) and 0.5 ml was layered on a 5-25% sucrose gradient.

Fig. VI.

Optical density profiles of sucrose gradients. The conditions are as those described in Fig. III. A, control chromatin; B, reconstituted 0.65 M dissociated chromatin; C, reconstituted 2.0 M dissociated chromatin. Pool 1, 2, 3, 4, and 5 represent ν_1, ν_2, ν_3, ν_4 and $\geqslant \nu_5$.

DISTRIBUTION OF LABELED CARCINOGEN IN CHROMATIN

FRACTION	EXPERIMENT I SPECIFIC ACTIVITY	EXPERIMENT II SPECIFIC ACTIVITY	EXPERIMENT III SPECIFIC ACTIVITY
	(cpm/A_{260})	(cpm/A_{260})	(cpm/A_{260})
CHROMATIN	11,805.0	9641.0	6461.6
ν_1	4443.0	4213.0	2452.0
ν_2	6250.0	6690.0	3580.0

Chromatin was reacted with B(a)P diol-epoxide as described in the text. The ratio of DNA to B(a)P diol-epoxide in experiments I and II was 100:1 and in experiment III, 200:1. Assuming that $\nu_2 = 2\ \nu_1$ + spacer, the spacer region contained 3.8 ± 0.5 times more carcinogen than ν_1.

SPECIFIC ACTIVITY OF RECONSTITUTED CHROMATIN

CHROMATIN	DISSOCIATION CONDITION	cpm/A_{260}
ν_n	0.00 M NaCl (control)	14,814.9
ν_n	0.65 M NaCl	13,993.9
ν_n	2.00 M NaCl	14,940.0

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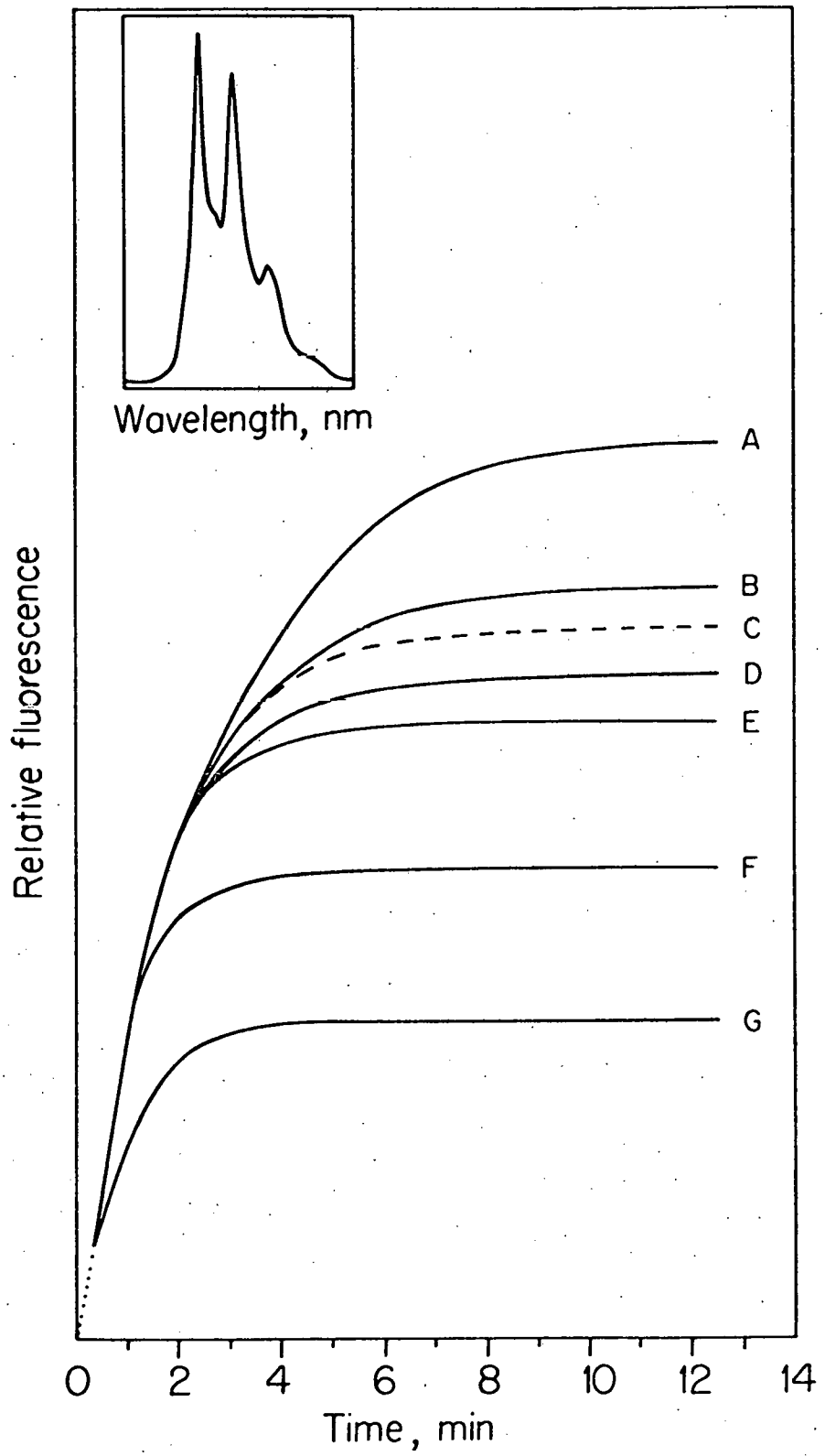
SPECIFIC ACTIVITIES OF MONO- AND OLIGONUCLEOSOMES OBTAINED FROM RECONSTITUTED CHROMATIN.

POOL	cpm/A_{260}	cpm/A_{260} (ν_1*)
1A	21,514.1	6,569.3
2A	15,886.4	7,358.5
3A	14,789.3	7,307.6
4A	14,508.2	—
5A	13,170.2	6,168.8
1B	21,704.5	8,433.8
2B	17,564.1	9,158.0
3B	15,103.4	7,878.0
4B	14,189.2	—
5B	13,424.5	7,388.5
1C	27,000.0	16,490.1
2C	18,205.0	16,363.6
3C	15,315.8	15,595.7
4C	14,727.3	—
5C	15,396.2	15,079.4

*Each individual pool was redigested to mononucleosome and the specific activity of purified ν_1 was determined. Pool, $1 = \nu_1$; $2 = \nu_2$; $3 = \nu_3$; $4 = \nu_4$; $5 \geq \nu_5$.

A, control chromatin; B, reconstituted 0.65 M NaCl dissociated chromatin; C, reconstituted 2.0 M NaCl dissociated chromatin.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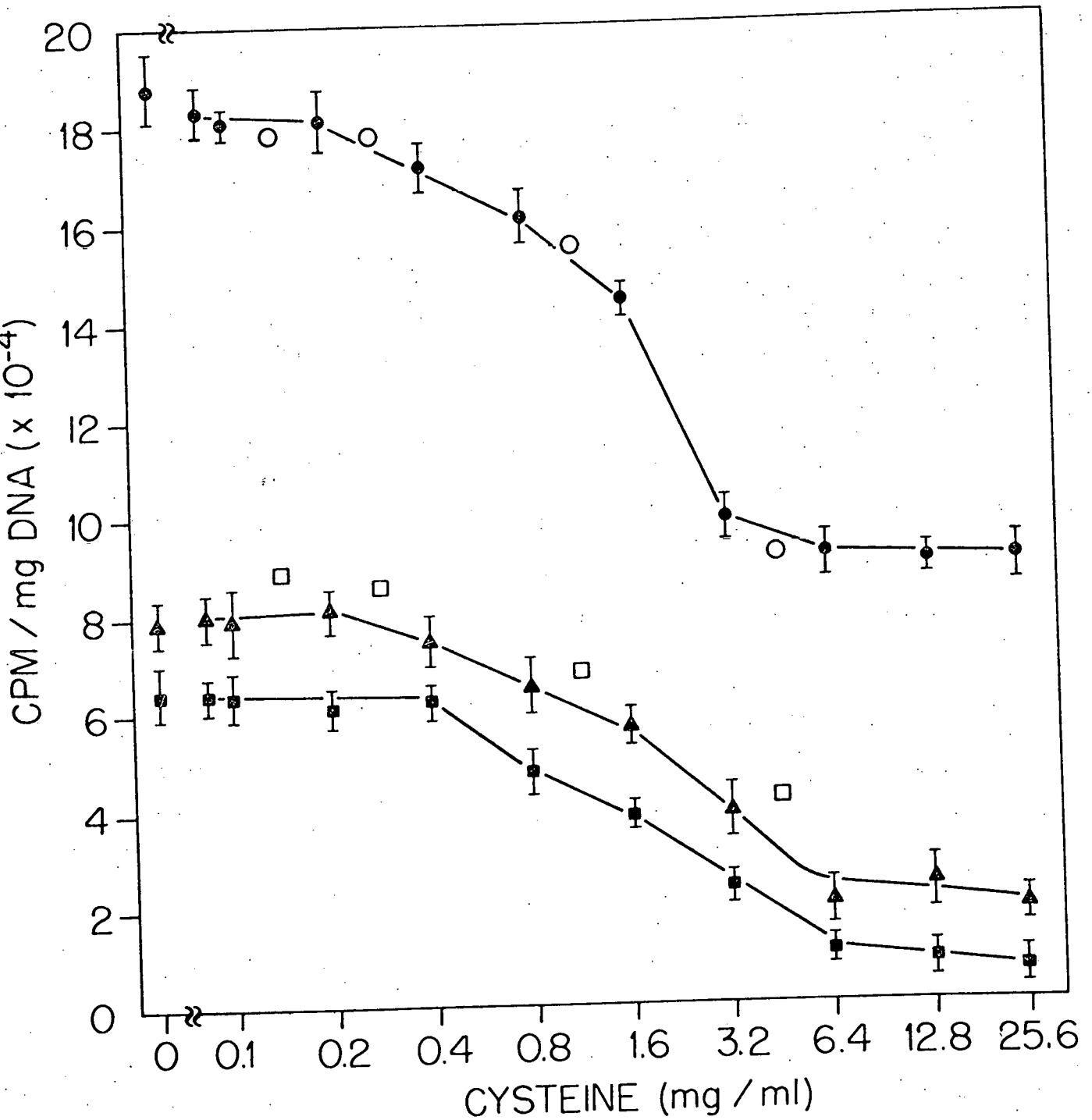
Time, min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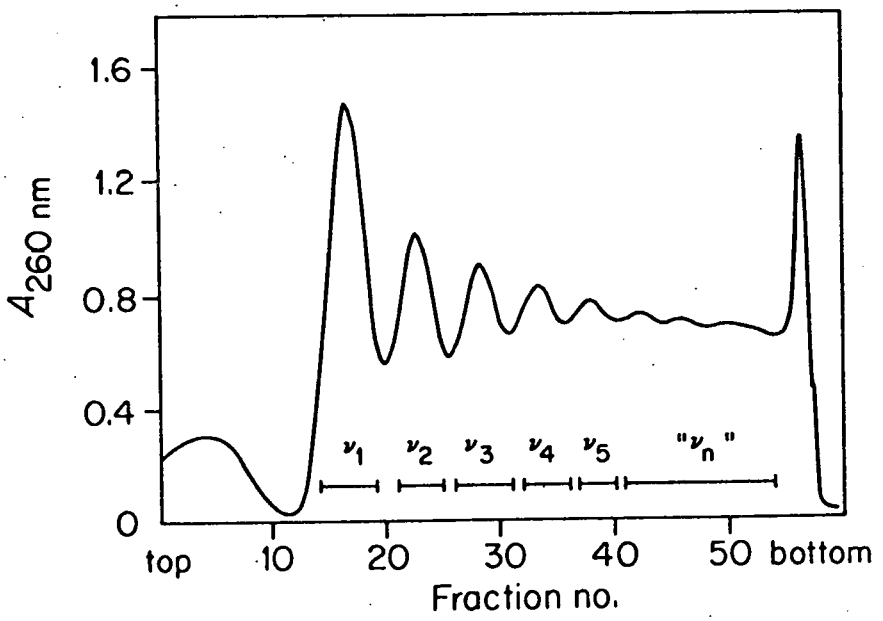
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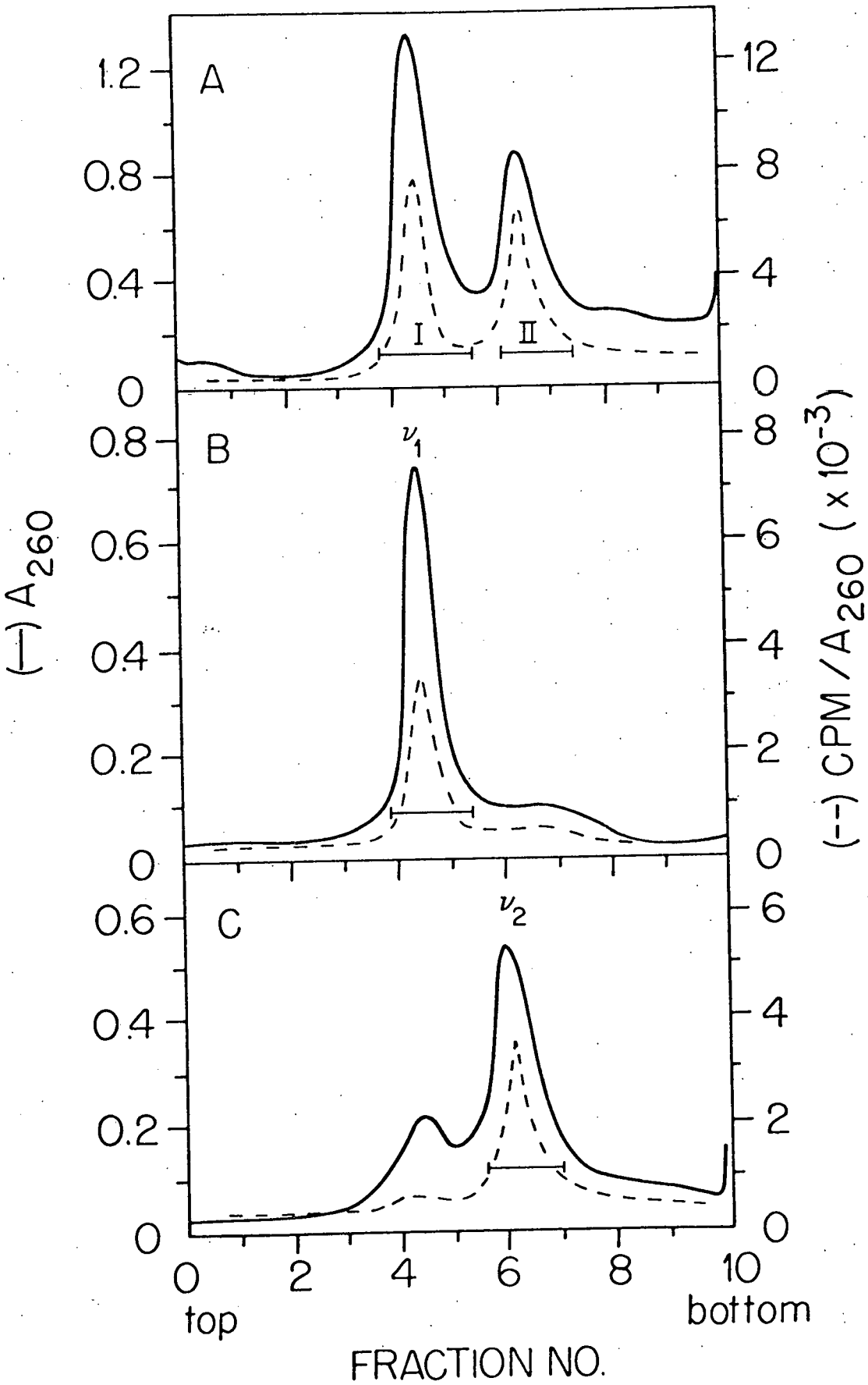
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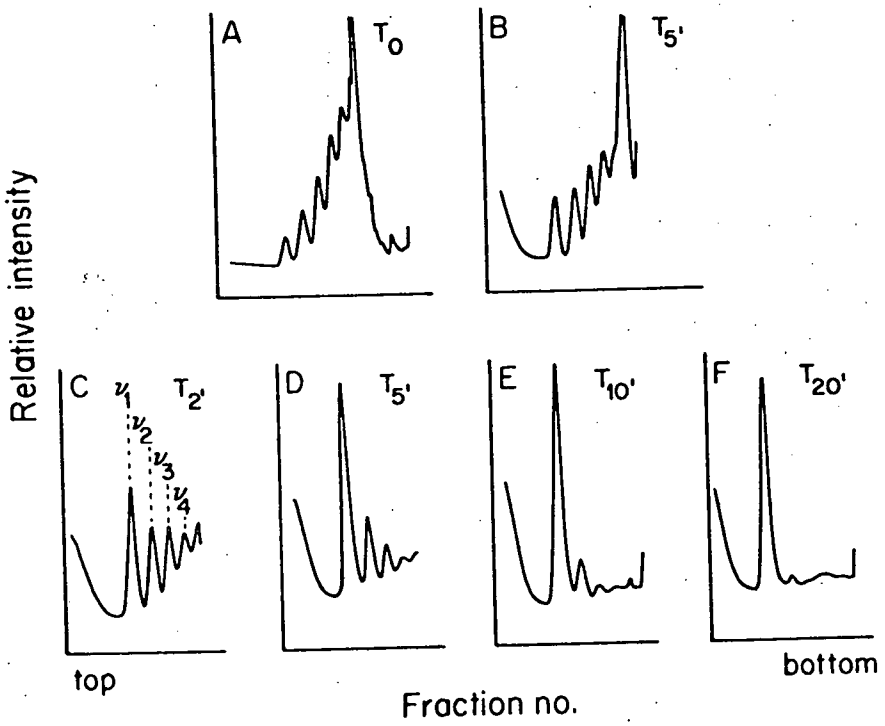
FRACTION NO.
(—) A_{260}
(--) CPM / A_{260} (x 10^{-3})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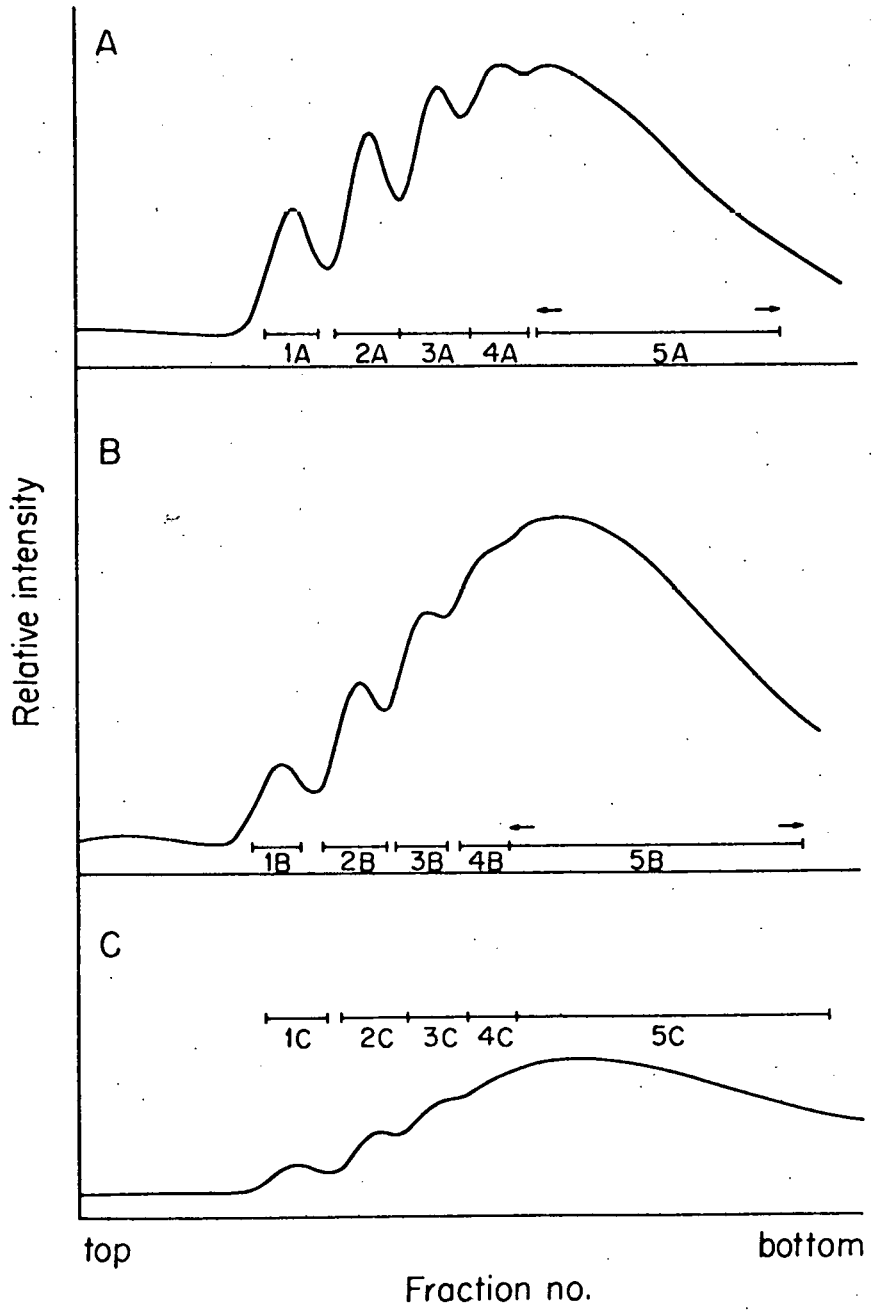
Relative intensity
top
bottom
Fraction no.


A
1A
2A
3A
4A
5A
B
1B
2B
3B
4B
5B
C
1C
2C
3C
4C
5C
Relative intensity
top
bottom
Fraction no.

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