CELL-FREE PROTEIN SYNTHESIS: THE ASSOCIATION OF VIRAL RNA AND E. COLI RIBOSOMES.*

by

R. Haselkorn, V. A. Fried, and J. E. Dahlberg

Committee on Biophysics

University of Chicago

The hypothesis that information specifying amino acid sequences in proteins is carried from the gene to ribosomes by messenger RNA[1] is now supported by an overwhelming body of experimental facts. Following the pioneer work on phage-infected bacteria[2], the most convincing proof of the messenger hypothesis came from experiments utilizing synthetic polynucleotides to direct the synthesis of polypeptides in a cell-free system from E. coli[3]. Some time ago we observed that RNA from several plant viruses stimulated the incorporation of amino acids into protein in the E. coli system[4]; that specific protein can be made in this manner has been shown using RNA from the bacteriophage f2[5].

Several plant viral RNA's have been characterized extensively, and our familiarity with one of them, TYMV-RNA, prompted us to use it to define the complex carrying our protein synthesis in the E. coli system. In this direction, three basic types of experiment have been carried out, all utilizing zone centrifugation through sucrose gradients[6]. In the first, viral RNA and E. coli ribosomes are mixed, fractionated on the gradient, and the RNA located. Secondly, the RNA-ribosome mixture is fractionated, and the individual fractions are assayed for their ability to incorporate amino acids[7]. Finally, the RNA-ribosome mixture is allowed to incorporate amino acids, then fractionated, and the RNA and newly made protein located. All three experiments give essentially the same result, namely, that in the cell-free system approximately 10% of the ribosomes participate in protein synthesis, and the active complex consists of one 70s ribosome and one molecule of viral RNA. In this paper we present the results of experiments of the first kind.

Materials and Methods.

E. coli ribosomes were prepared as described previously[4]. TYMV was labeled with P^{32} by growing chinese cabbage plants in Hoagland's solution No. 1 for several weeks[8], then inoculating with virus, and transferring the plants to the same solution lacking phosphate, other than .1 - 1.0 mC $^{32}PO_4^{=}$ per liter. Six to ten days later the young leaves showing chlorosis typical of TYMV infection were harvested, minced, and the sap filtered through muslin. There followed a centrifugation at low speed to remove large debris, and a ninety minute centrifugation at 40,000 rpm in the No. 40 rotor of the Model L Spinco to pellet the virus. The pellet was dissolved in a small volume of 0.01 M EDTA, and in each cup of the SW 39 rotor 2.0 ml of this solution were layered on top of 3.0 ml of CsCl solution having a density of 1.30. After 3 hours at 35,000 rpm the virus (= 1.49) was completely sedimented, while leaf protein, lipid, and polysaccharide contaminants floated, and could be decanted. The virus pellets were taken up in 0.05 M Tris buffer, pH 7.5, and freed of excess CsCl by another high speed centrifugation. Finally, these pellets were taken up in 0.05 M Tris and shaken with water-saturated phenol as previously described[9]. This procedure routinely afforded RNA containing 10^6 c.p.m. per mg.

Linear gradients of sucrose (5-20%) were formed using a device like that described by Britten & Roberts[6]. After centrifugation the tubes were placed in a jig whose bottom consisted of a rubber stopper in which a syringe needle was embedded. The plastic tube could be forced down onto the needle, the stopper then forming

a tight seal to the bottom of the tube. Drops emerging from the needle were collected in Shevsky-Stafford tubes, the volume and absorbancy of each fraction were measured, and then the entire fraction was transferred to ringed planchets, dried, and counted in a thin end-window gas flow counter.

Figure 1 shows the result of centrifuging P^{32}-labeled TYMV for two different times. In each case the half-width of the zone of virus is approximately one-tenth the total column height; presumably most of the broadening of the zone occurs during the layering of material onto the column and loading the rotor into the centrifuge. In the figures presented below, zones occupying more than one-tenth the column height indicate heterogeneity with respect to sedimentation coefficient.

Amino acid incorporation was measured in the complete system as described previously[4], except that H^3-leucine and H^3-aspartic acid were used in place of the C^{14}-labeled mixture of amino acids. After incubation for 30 minutes at 36°C., the reaction was terminated by the addition of one-tenth volume of 50% TCA. The precipitates were collected by centrifugation and washed twice by solution in 0.1 N NaOH containing cold leucine and aspartic acid and re-precipitation with 5% TCA. The final pellet was dissolved on 1.0 ml of hyamine 10-x, taken up in 14 ml of toluene-PPO-POPOP scintillator fluid, and counted in a Packard liquid scintillation counter.

Results.

Viral RNA attaches to E. coli ribosomes in the ionic environment in which protein synthesis is observed; no factors other than Mg^{++} appear to be necessary for attachment. This is shown in Figure 2, in which a 0.2 ml zone containing ribosomes and P^{32}-labeled TYMV-RNA in 0.01 M Mg^{++}, 0.01 M Tris, pH 7.5 was analysed on a gradient containing the same ionic composition. In this medium the ribosomes exist as a mixture of 70s and 100s particles; the P^{32}-labeled RNA is seen to move faster than 100s, and in fact occupies a region corresponding to 120-150s.

The distribution of ribosomes among sedimentation coefficient classes is affected by a number of factors in addition to Mg^{++} concentration. One of these is the monovalent salt concentration; since we found initially that optimum amino acid incorporation was observed when 0.07 M KCl was present in addition to Mg^{++}, it was of interest to note the effect of KCl on viral RNA attachment[4]. In Figure 3b, the charge for the gradient was identical to that for 3a; the experiment differs only in the addition of 0.07 M KCl to the gradient itself. First, it can be seen that the sedimentation coefficient of the RNA-ribosome complex is decreased to 80-100s; secondly, the RNA:ribosome ratio of individual fractions is approximately twice that in the gradient lacking KCl. The calculation of the RNA:ribosome ratio for each fraction in Figure 3b is shown in Table 1. This was done by first determining the amount of

RNA from the known specific activity of the RNA and the total counts for each fraction, converting to absorbancy units, subtracting the RNA absorbancy from the total, and dividing the remainder by the extinction coefficient for E. coli ribosomes. The weight ratio of RNA to ribosomes is given in the seventh column, and the molar ratio in the last. For conversion to the molar ratio, the molecular weight of TYMV-RNA was taken as 2.1×10^6 and of 70s ribosomes as 2.7×10^6. For fractions 5, 6, and 7 most of the absorbancy is contributed by ribosomes from the 70s region, rather than from the complex itself. Making a correction based on similar runs with ribosomes alone, the molar ratio for fractions 5, 6, and 7 is raised to 0.3, 1.0, and 0.4 respectively. Note that radioactivity attributable to complexes is distributed over half the gradient, a spread well outside the limits for a single species (0.1 of the column height, see Fig. 1). Comparison with Figure 3a suggests that the leading region (130-170s) is comprised of a small class of particles stable in the presence of KCl, while the peak region (80-100s) is comprised of those that have dissociated. Analytical ultracentrifugation of E. coli ribosomes in 0.01 M Tris, 0.01 M Mg^{++} shows a mixture of 100s and 70s particles; in the KCl-containing medium one finds 70s and 50s particles in the ratio of approximately 2:1[11]. Thus the shift in the peak of radioactivity from 150s in Fig. 3a to 80-100s in Fig. 3b can probably be attributed to

the dissociation of 100s particles. The RNA:ribosome ratio of 0.5 calculated for fractions 2, 3, and 4 in Table 1 is consistent with the interpretation that these fractions contain complexes composed of two 70s ribosomes and one molecule of RNA. Fractions 5, 6, and 7 (80s-100s) should then contain complexes composed of one 70s ribosome and one molecule of RNA, and have an RNA:ribosome ratio of 1.0. Qualitatively this is observed, although the nature of the calculation precludes the assignment of an accuracy better than a factor of two to the ratio for these fractions. The conclusion that these complexes have the suggested composition rests primarily on their low sedimentation coefficient and the comparison between gradients with and without KCl.

Systematic variation of the RNA:ribosome ratio in separate gradients makes it possible to determine the stoichiometry of the interaction between ribosomes and RNA. In Figure 4a, b, and c, three experiments are shown in which the molar ratio of RNA to ribisomes is 0.48, 0.18, and 0.12 respectively. By comparing the distribution of P^{32} in such gradients with the distribution observed when viral RNA is run alone under identical conditions, an estimate of the fraction of RNA bound can be obtained. Figure 5 shows a plot of the fraction of RNA bound as a function of the RNA:ribosome ratio. It can be seen that saturation is obtained at a ratio of approximately 0.1. If one measures amino acid incorporation into protein as a function of the RNA:ribosome ratio in the complete system[4], the incorporation goes through a maximum at approximately the same ratio, as shown also in Figure 5.

This result indicates that only those ribosomes which function in protein synthesis bind viral RNA, and that by either test only 10% of the ribosomes in the cell-free system are functional.

The attachment of viral RNA to ribosomes is reversible. This can be demonstrated in several ways, of which one is illustrated in Figure 6. In this case ribosomes and RNA in 0.01 Mg^{++} were layered on a gradient containing 10^{-4} M Mg^{++}. In the latter medium 70s ribosomes are dissociated into 50s and 30s particles. When this occurs the RNA falls off and is found at a position corresponding to 30s, the sedimentation coefficient of free viral RNA. Reversibility can also be shown by the ability of P^{32}-labeled viral RNA to replace cold viral RNA bound to ribosomes. The reversibility is sufficiently complete that the order of addition of labeled and unlabeled RNA to ribosomes does not affect the fraction of label bound; the only pertinent variable is the final RNA:ribosome ratio.

It was of interest to determine which of the subunits of the 70s particle contains the binding site(s) for RNA. Accordingly, 50s and 30s particles were separated by centrifugation in a low Mg^{++} gradient, concentrated, and each mixed with viral RNA in high Mg^{++}. Gradient analyses of these interactions are shown in Figure 7a and b. This experiment has been performed four times with different preparations of ribosomes; in each case attachment to 30s particles was observed while in three of the four experiments attachment to 50s particles was observed. The preparation of 50s

particles which did not bind RNA was also unable to combine with 30s particles to form 70s particles although they had been derived from 70s particles[12]. We have on several other occasions observed irreversible dissociation of 70s into 50s and 30s; as might be expected, such particles are inactive in the amino acid incorporation system as well as being unable to bind P^{32}-labeled viral RNA.

Discussion.

The existence of a cell-free system carrying out specific protein synthesis permits us to formulate two separate questions: what is the nature of the machinery in the cell-free system, and what relation does this machinery bear to that operative in vivo? It should be clear that the results cited above provide information concerning the first question only.

The first point to be made is that the active complex requires only Mg^{++} and such cofactors as are already present in washed E. coli 70s particles for its formation. This result has already been obtained for poly U[13, 14, 15] and E. coli messenger RNA[16]. The contention that viral RNA requires energy to attach to ribosomes is not supported by our results[13].

In 0.01 M Mg^{++} E. coli ribosomes exist as 70s and 100s particles. In this milieu the complex of viral RNA and ribosomes moves faster than 100s, and might be comparable to the "heavy" 100s particles described by Risebrough et al[17].

The addition of KCl to the gradients (Fig. 3) demonstrates that in the case of the viral RNA-ribosome complex heavy 100s particles are artifacts in the sense that they are not obligatory for amino acid incorporation. Gilbert has recently shown that KCl dissociates 100s particles in the crude extract of *E. coli* as well, although in his experiment the *activity* for protein synthesis continued to move faster than 100s[15]. The sedimentation coefficient of the viral RNA-ribosome complex, together with the ratio of radioactivity to ultraviolet absorbancy in fractions from the complex region in KCl-containing gradients indicates that the complex contains at most two, and more likely one, 70s ribosome together with one molecule of viral RNA.

Published reports indicate that the active complex in reticulocytes *in vivo* consists of a string of ribosomes connected by a molecule of RNA[18, 19]. It is not yet clear whether a similar situation is found in bacteria, although Gilbert's experiment[15] suggests that. A polysome-like structure appears to be formed by poly U *in vitro*, but no such structure has yet been demonstrated with messenger or viral RNA. On the contrary, the present work suggests that viral RNA is incapable of forming such structures *in vitro*. Regardless of that handicap, polypeptide synthesis can be efficiently directed by viral RNA *in vitro*: we conclude that one 70s ribosome is sufficient machinery to carry out protein synthesis, a conclusion also reached by Gilbert.

Ishihama et al. reported that E. coli messenger RNA attached to 70s particles only when the particles were first dissociated in low Mg^{++} and then re-associated in the presence of the RNA[16]. We do not find this requirement for viral RNA. Ishihama et al. found that messenger RNA could attach to both 30s and 50s particles; we find the same true of viral RNA. Furthermore, an examination of the stoichiometry of association with the ribosomal subunits indicates that a given number of 30s or 50s particles is capable of binding nearly 80% of the RNA bound by the same number of 70s particles. Since S-RNA attaches specifically to 50s particles[20], and messenger attachment is only slightly enhanced by association with 30s particles, it is tempting to suggest another role for the 30s particle: could it be supplying the amino acid polymerase?

Summary.

Plant viral RNA associates with E. coli ribosomes in the absence of cofactors other than Mg^{++}. The complex thus formed appears to consist of one molecule of viral RNA and one 70s ribosome, and is capable of carrying out polypeptide synthesis. In a typical preparation of ribosomes, 10% are capable of binding viral RNA; the same fraction functions in the amino acid incorporation assay. The association of viral RNA with ribosomes is reversed in 10^{-4} M Mg^{++}. Both 30s and 50s particles appear to contain site(s) for viral RNA attachment.

We should like to thank J. Ofengand for gifts of ribosomes and supernatant in the early stages of this work; L. Johnson for technical assistance; and E. P. Geiduschek for numerous helpful suggestions.

* This work was supported by Grant E-4448 from the U. S. Public Health Service.

REFERENCES

1. Jacob, F. and J. Monod, J. Mol. Biol. 3: 318 (1961).
2. Brenner, S., F. Jacob and M. Meselson, Nature 190: 576 (1961).
3. Nirenberg, M.W. and J. H. Matthei, these PROCEEDINGS 47: 1588 (1961).
4. Ofengand, J. and R. Haselkorn, Biochem. Biophys. Res. Comm. 6: 469 (1962).
5. Nathans, D., G. Notani, J. Schwartz, and N. Zinder, these PROCEEDINGS 48: 1424 (1962).
6. Britten, R.J. and R. B. Roberts, Science 131: 32 (1960).
7. This type of experiment was suggested to us independently by W. Gilbert and J. Ofengand.
8. Hoagland, D. R. and D. I. Arnon, Calif. Ag. Expt. Sta. Circ. No. 347 (1950).
9. Haselkorn, J., J. Mol. Biol. 4: 357 (1962).
10. Yankofsky, S. and S. Spiegelman, these PROCEEDINGS 48: 1069 (1962).
11. The ratio of 70s to 50s ribosomes in 0.05 M Tris, 0.07 M KCl, 0.01 M Mg^{++} is dependent upon temperature and upon concentration, decreasing as the temperature is raised from 2°C. to 25°C., and as the solution is diluted, as one expects for an equilibrium of the type A + B C.
12. Green, M. and B. D. Hall, Biophys. J. 1: 517 (1961).
13. Barondes, S. and M. Nirenberg, Science 138: 813 (1962).
14. Spyrides, G. and F. Lipmann, these PROCEEDINGS 48: 1977 (1962).
15. Gilbert, W., J. Mol. Biol., in press.
16. Ishihama, A., N. Mizuno, M. Takai, E. Otaka, and S. Osawa, J. Mol. Biol. 5: 251 (1962).
17. Risebrough, R., A. Tissieres and J. Watson, these PROCEEDINGS 48: 430 (1962).

18. Warner, J., A. Rich, and C. E. Hall, Science 138: 1339 (1962).
19. Warner, J., P. Knopf, and A. Rich, these PROCEEDINGS 49: 122 (1963).
20. Cannon, M., R. Krug, and W. Gilbert, unpublished results.

TABLE 1

Calculation of RNA:Ribosome Ratio

FRACTION	µg RNA	A_{260} RNA	A_{260} TOTAL	A_{260} RIBOSOMES	µg RIBOSOMES	µg RNA / µg RIBOSOMES	MOLAR RATIO
1	.3	.007	.042	.035	2.0	.15	.20
2	.4	.009	.027	.018	1.0	.40	.51
3	.7	.016	.052	.036	2.0	.35	.45
4	1.4	.032	.098	.066	3.7	.38	.49
5	2.3	.053	.238	.185	10.3	.22	.28
6	3.2	.074	.571	.497	27.6	.12	.15
7	3.2	.074	1.285	1.211	67.2	.05	.06
8	2.7	.062	2.500	2.440	136.0	.02	.03
9	1.7	.039	1.590	1.550	86.0	.02	.03
10.	1.4	.032	.453	.421	23.4	.06	.08
11.	1.05	.024	.152	.128	7.1	.15	.19
12.	1.0	.023	.163	.140	7.8	.13	.17

Data from Fig. 3b. Each fraction was 0.4 ml. The extinction coefficient for TYMV-RNA was taken as 23 and for *E. coli* ribosomes as 18 for 1 mg/ml at 260 mµ. See text for details of calculation.

LEGENDS TO FIGURES

Fig. 1. Resolution of the 5-20% linear sucrose gradient. Centrifugation of P^{32}-labeled TYMV (S=115) at 35,000 rpm, 25°C. for the times indicated.

Fig. 2. Attachment of TYMV-RNA to ribosomes. Centrifugation at24,000 rpm for 2 hours at 25°C. 1.0 mg of ribosomes and 4 µg P^{32}-labeled RNA were applied to a gradient containing 0.01 M Tris, 0.01 M Mg^{++}. Solid line indicates absorbancy, dashed line radioactivity.

Fig. 3. Effect of KCl on the RNA-ribosome complex. Centrifugation at 35,000 rpm for 45 minutes at 25°C. 400 µg ribosomes and 20 µg P^{32}-labeled RNA in 0.1 ml were applied to each gradient. Gradient in (a) contained 0.01 M Tris, 0.01 M Mg^{++}; in (b) contained 0.05 M Tris, 0.07 M KCl, 0.01 M Mg^{++}. Solid line indicates absorbancy, dashed line radioactivity.

Fig. 4. Stoichiometry of the interaction between TYMV-RNA and *E. coli* ribosomes. Centrifugation at 35,000 rpm for 45 minutes at 25°C. Gradients contain 0.05 M Tris, 0.05 M KCl, 0.01 M Mg^{++}. To each was applied 0.1 ml containing 300 µg ribosomes and (a) 110 µg, (b) 42 µg, (c) 27 µg P^{32}-labeled RNA. Solid line indicates absorbancy, dashed line radioactivity.

Fig. 5. Correlation between RNA binding and amino acid incorporation. Open circles were obtained from six

separate gradients as in Fig. 4. The same cold RNA was used for isotope dilution in the gradients as was used in the amino acid incorporation assays, filled circles.

Fig. 6. Dissociation of the RNA-ribosome complex. Centrifugation at 35,000 rpm for 60 minutes at 25°C. 300 µg ribosomes and 7 µg P^{32}-labeled RNA in 0.1 ml of 0.01 M Tris, 0.01 M Mg^{++} was applied to a gradient containing 0.01 M Tris, 0.0001 M Mg^{++}. Solid line indicates absorbancy, dashed line radioactivity.

Fig. 7. Attachment of TYMV-RNA to 30s and 50s particles. Centrifugation at 35,000 rpm, 25°C., for (a) 45 minutes and (b) 60 minutes. 0.01 ml containing (a) 250 µg 50s particles and 24 µg RNA and (b) 180 µg 30s particles and 48 µg RNA was applied to gradients containing 0.05 M Tris, 0.05 M KCl, and 0.01 M Mg^{++}.

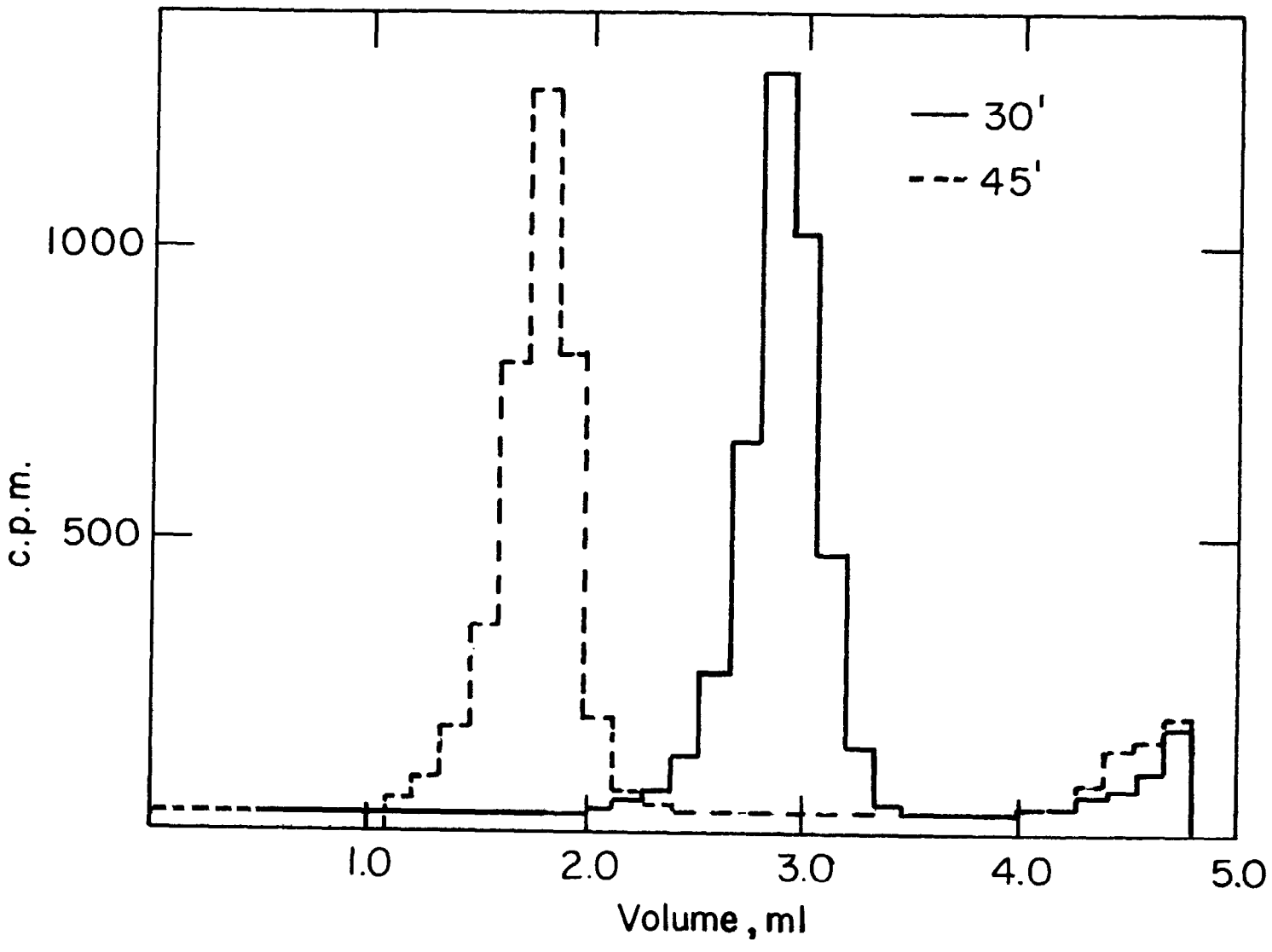

Fig. 1

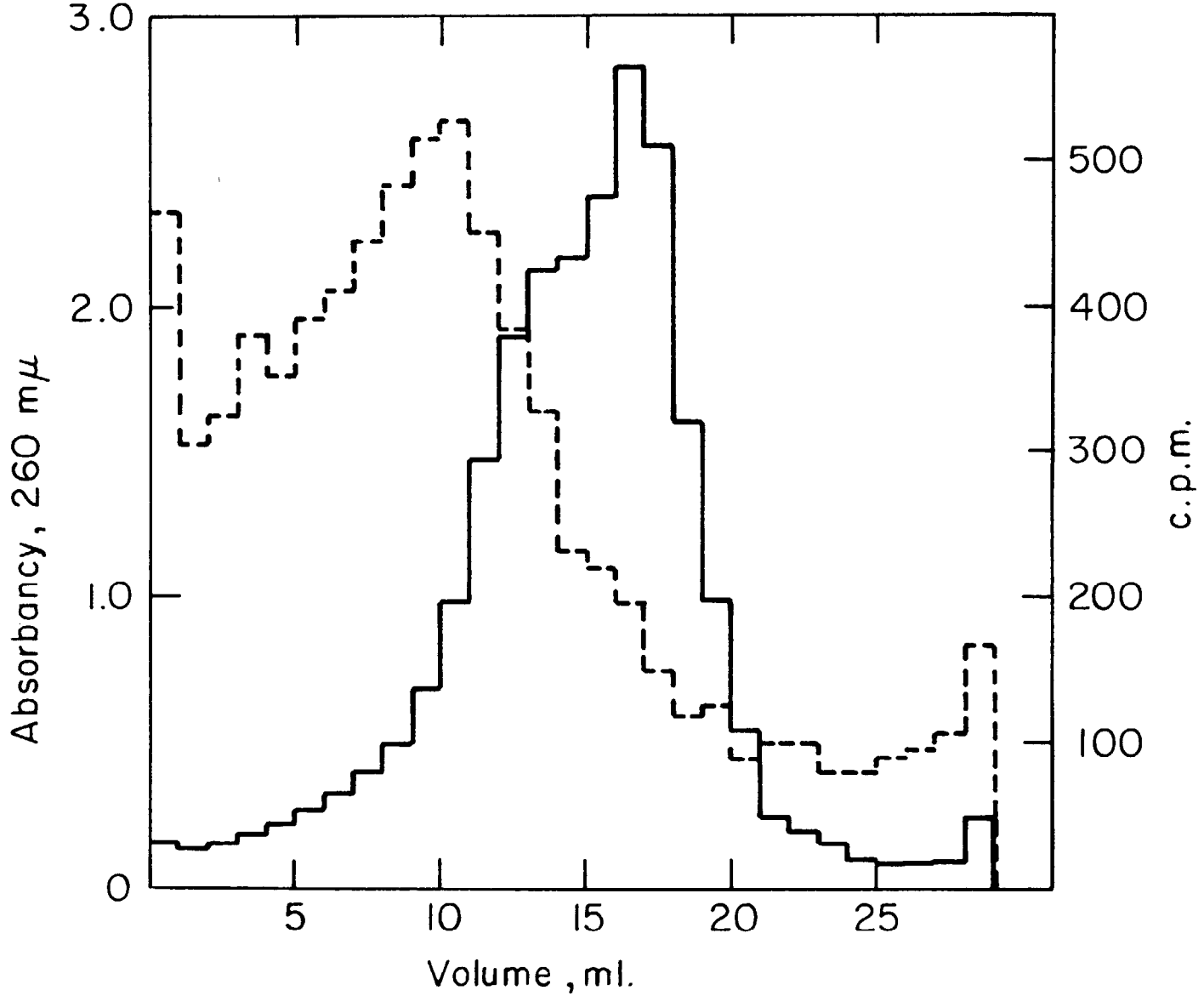
3.0
2.0
1.0
0
Absorbancy, 260 mμ
500
400
300
200
100
c.p.m.
5
10
15
20
25
Volume , ml.

Fig. 2

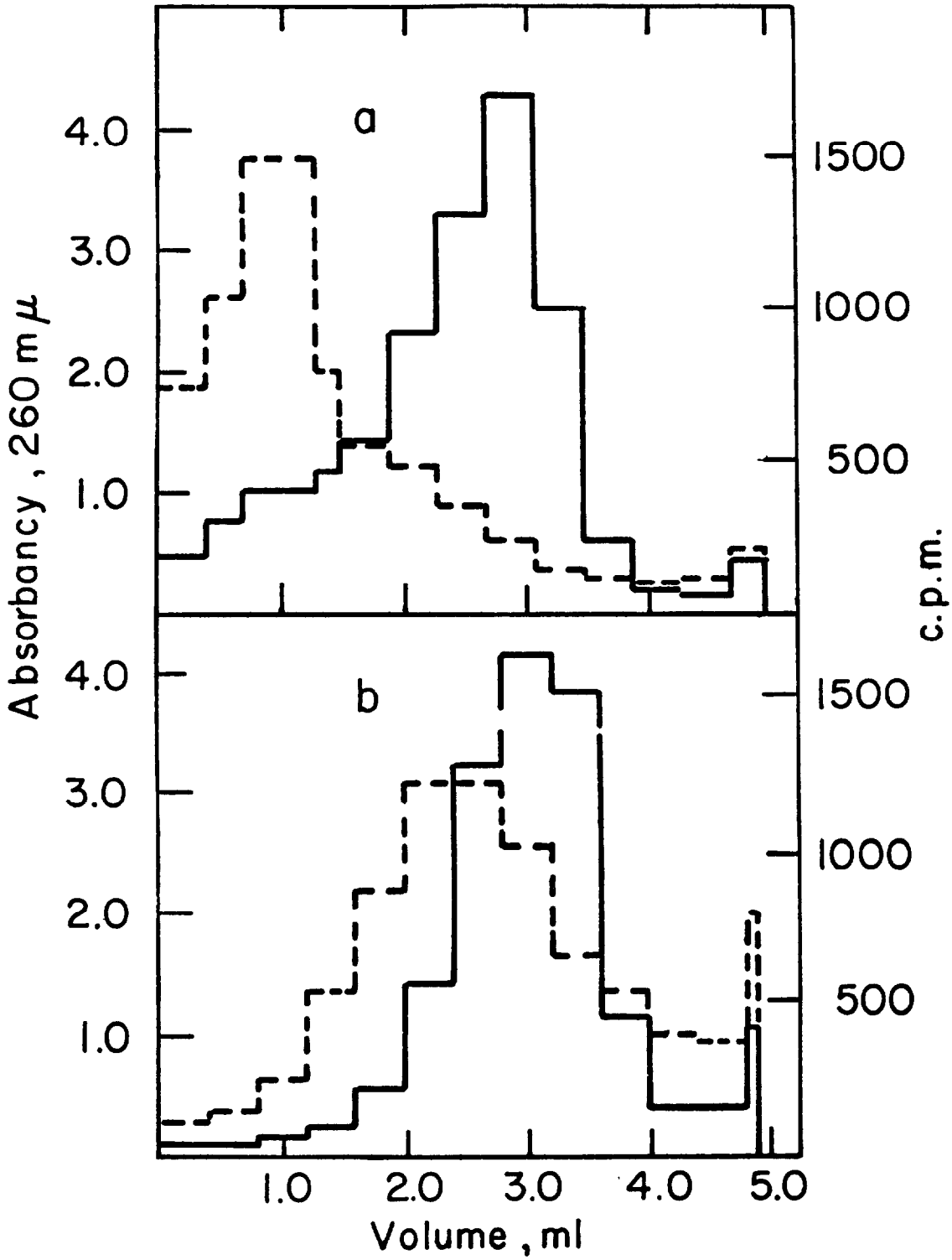
a
b
4.0
3.0
2.0
1.0
1500
1000
500
c.p.m.
Absorbancy , 260 mμ
1.0
2.0
3.0
4.0
5.0
Volume , ml

Fig.3

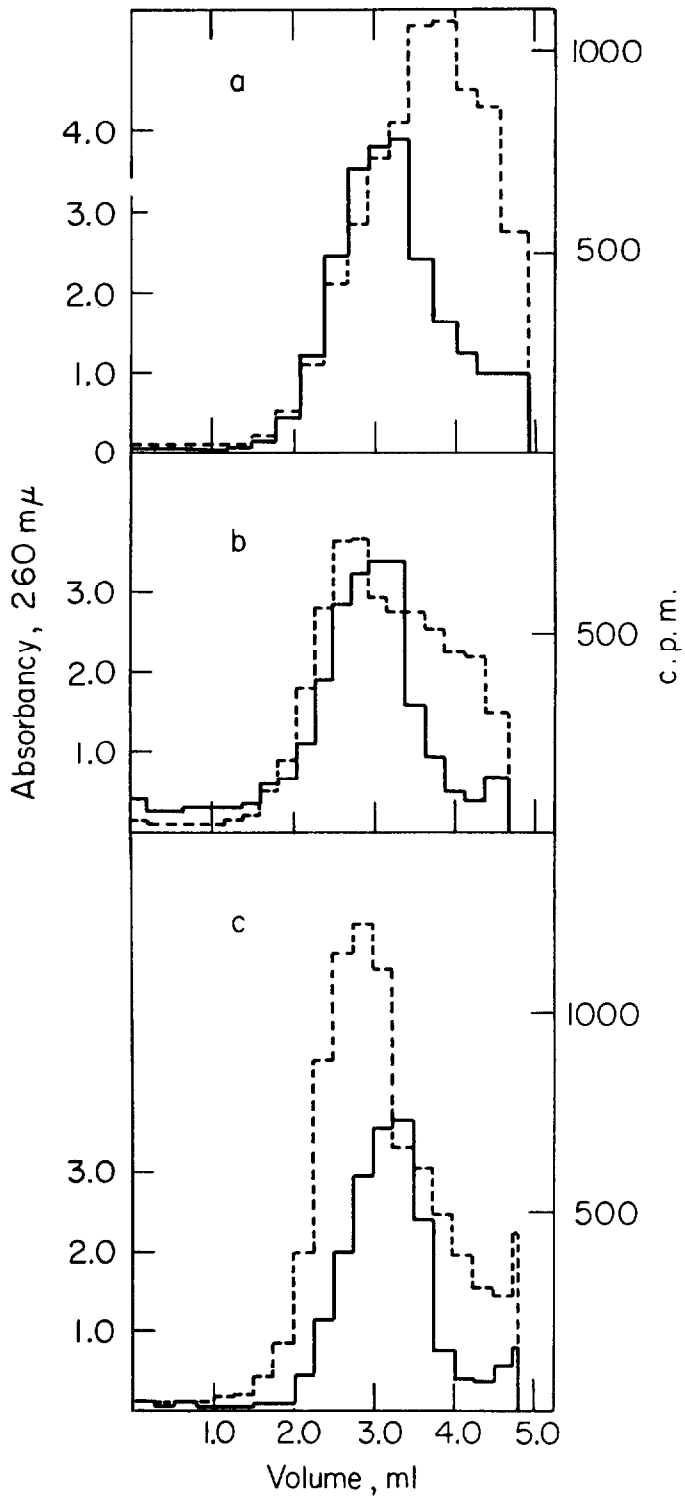
a
b
c
4.0
3.0
2.0
1.0
0
1000
500
Absorbancy, 260 mμ
c.p.m.
1.0
2.0
3.0
4.0
5.0
Volume, ml

Fig. 4

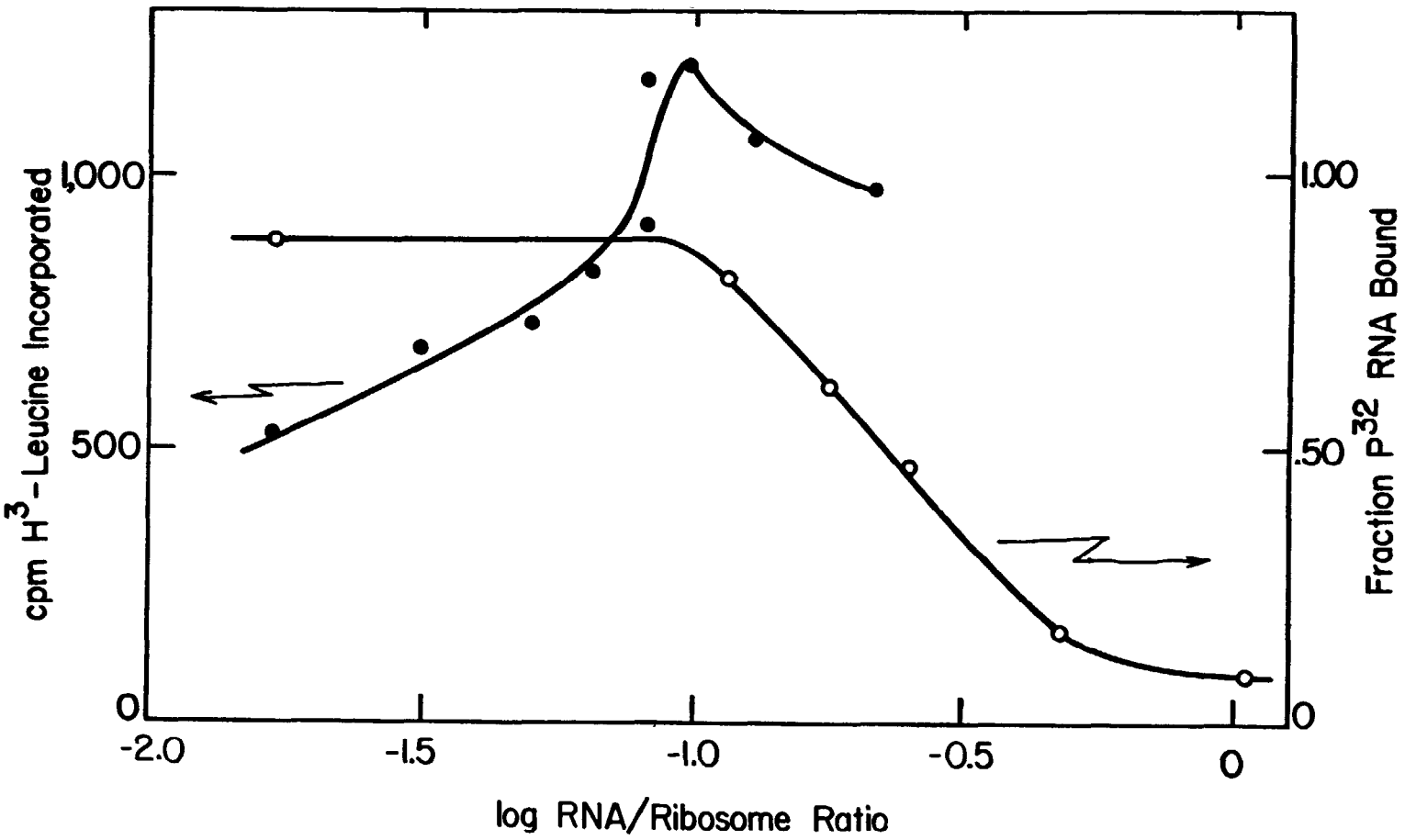
cpm H3-Leucine Incorporated
1,000
500
0
1.00
.50
0
Fraction P32 RNA Bound
-2.0
-1.5
-1.0
-0.5
0
log RNA/Ribosome Ratio

Fig. 5

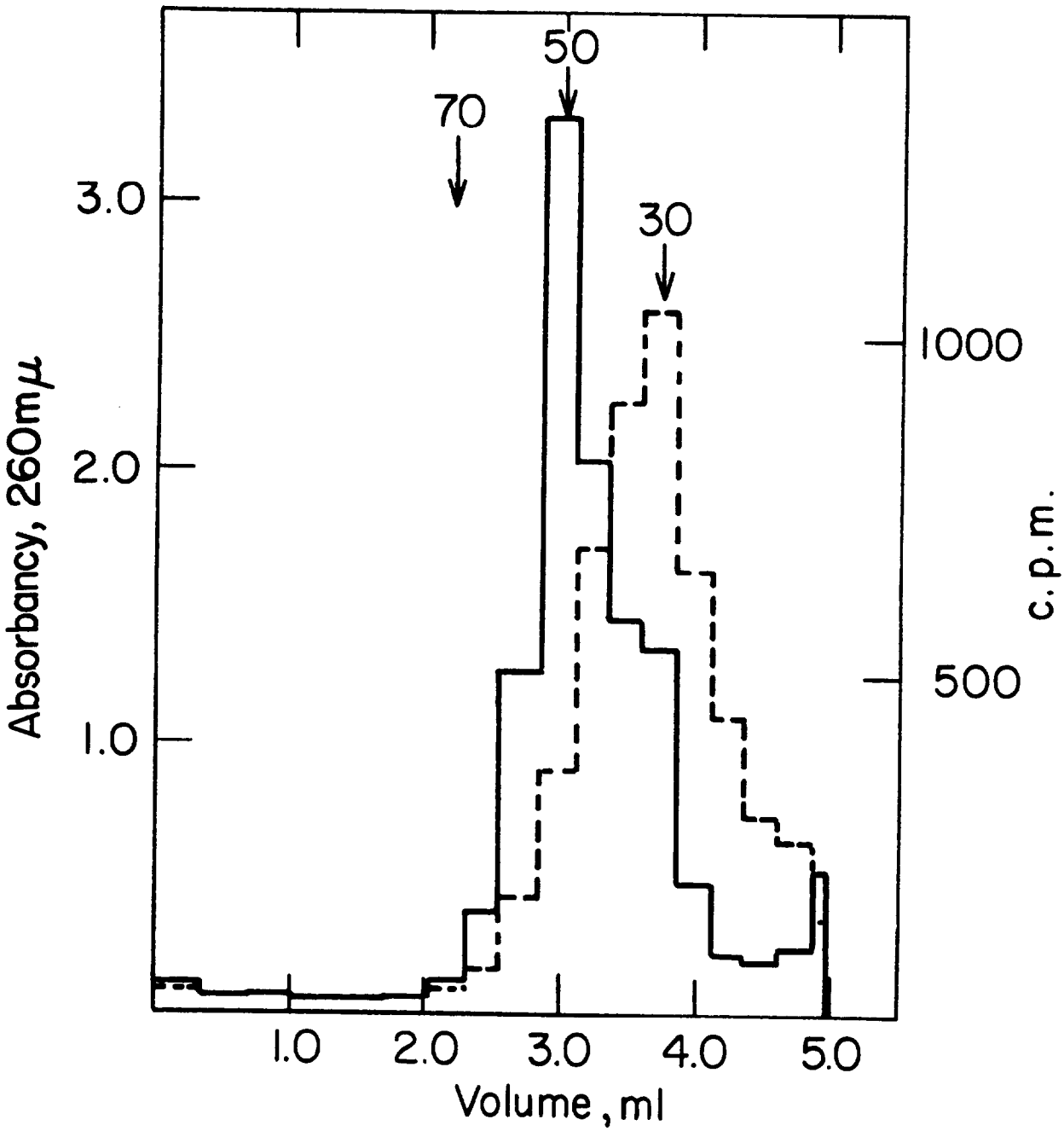
50
70
30
3.0
2.0
1.0
1000
500
Absorbancy, 260mμ
c.p.m.
1.0
2.0
3.0
4.0
5.0
Volume, ml

Fig.6

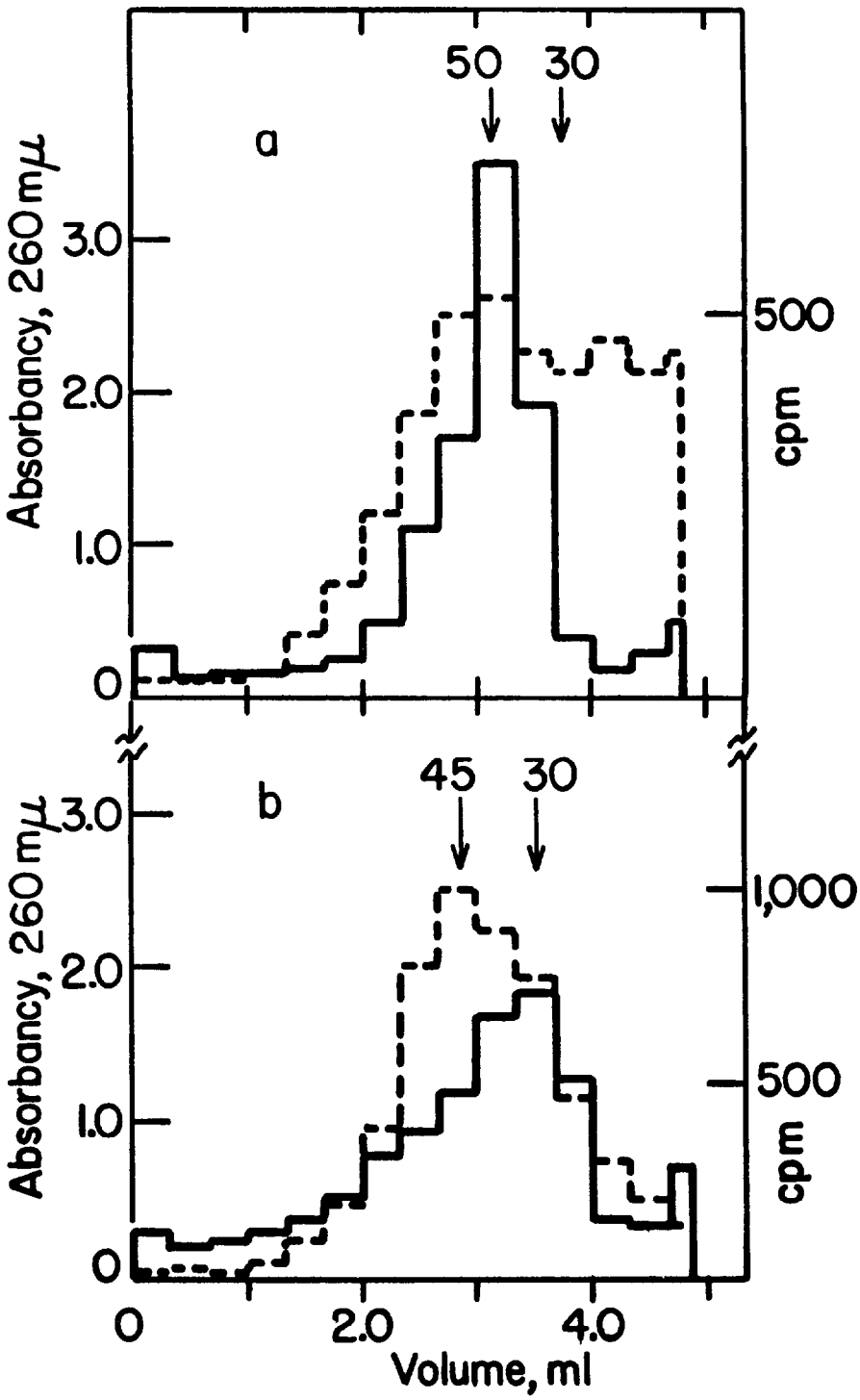
a
50
30
Absorbancy, 260 mμ
3.0
2.0
1.0
0
500
cpm
b
45
30
Absorbancy, 260 mμ
3.0
2.0
1.0
0
1,000
500
cpm
0
2.0
4.0
Volume, ml

Fig. 7