Subunit Equilibria of the 7S Nerve Growth Factor Protein*

Andrew P. Smith,† Lloyd A. Greene,‡ H. Ronald Fisk, Silvio Varon, and E. M. Shooter

ABSTRACT: Iodination of the 7S nerve growth factor protein with ^{125}I introduces the label into all three types of subunits. The specific activities of the α and γ subunits are similar while the specific activity of the β subunit is about one-third that of the other two subunits. Iodination does not affect the electrophoretic or sedimentation properties of the 7S nerve growth factor protein or its subunits. Addition of ^{125}I-labeled α subunits to unlabeled 7S nerve growth factor protein at neutral pH results in the incorporation of radioactivity into the latter. All this radioactivity is subsequently recovered in the α subunits of the 7S nerve growth factor protein. The rate at which free and complexed α subunits exchange is greater at 27° than at 4°, half the expected exchange occurring in 30 min at the higher and in 300 min at the lower temperature.

Incubation of an excess of an unlabeled individual α subunit with 7S nerve growth factor protein results in the appearance of all the other individual α subunits in the free subunit pool, suggesting that all the α subunits exchange with each other and do so at the same rate. If, in this type of experiment, the individual α subunit is labeled with ^{125}I but the 7S nerve growth factor protein is not, then the new individual α subunits in the free α-subunit pool are unlabeled. No corresponding exchange of ^{125}I-labeled γ or β subunits with the 7S protein is observed. These results suggest that 7S nerve growth factor protein is in moderately rapid equilibrium with its α subunits at all pH values. The existence of such equilibria among the multiple forms of the 7S protein may explain why they cannot be separated by electrophoresis or chromatography and also why gradients in the types of both α and γ subunits are found when the 7S protein is so analyzed. Changes in the equilibrium concentrations of the 7S nerve growth factor protein and its γ subunits or intermediate complexes on dilution or with changing pH account for the observed differences in the enzyme kinetics of the 7S protein and its γ subunits.

The 7S species of the nerve growth factor protein contains three different types of subunits, α, β, and γ (Varon *et al.*, 1967a, 1968). These subunits are of about the same size but differ significantly in net charge. The basic β subunit is the only one to elicit a response in the usual bioassay. The γ subunits on the other hand have an esterase enzymatic activity (Greene *et al.*, 1968, 1969). The three subunits recombine when mixed at neutral pH to form a high molecular weight protein with the physicochemical, enzymatic, and biological properties of the 7S nerve growth factor protein (Varon *et al.*, 1968). Unlike the β subunit, the α and γ subunits have been shown to contain more than one protein species. These arise from multiple forms of the 7S species present in the original preparation, forms which have the same general subunit composition but which differ in the type of individual α and γ subunit they contain (Smith *et al.*, 1968). These multiple 7S species differ only with respect to electrophoretic mobility, the latter being determined by the type of α but not of γ subunit present in the complex.

In spite of their different mobilities, the 7S species are not clearly resolved when the original preparation is subjected to electrophoresis or ion-exchange chromatography. Also a gradient of γ subunits appears in the migrating 7S nerve growth factor protein zone, γ^1 predominating on the low and γ^3 on the high mobility side of the zone, a result which would not be anticipated from the known constancy of the mobilities of 7S species containing a given α subunit but differing γ subunits (Smith *et al.*, 1968). These observations as well as studies on the enzymatic properties of the 7S species (Greene *et al.*, 1969) suggested that the multiple forms of the latter may, like hemoglobin (Guidotti *et al.*, 1963), be in mobile equilibrium with their subunits at all pH values. The present paper describes the use of ^{125}I-labeled 7S nerve growth factor protein and its subunits in examining this question and reports results which confirm that the α subunits are in moderately rapid equilibrium with parent 7S $\alpha\gamma\beta$ complex at pH values where no visible dissociation occurs. A brief account of this work has been presented (Smith *et al.*, 1969).

Materials and Methods

The 7S nerve growth factor protein was isolated by the methods of Varon *et al.* (1967b). The α, γ, and β subunits were obtained by chromatography on CM-cellulose at acid pH (Smith *et al.*, 1968) from unlabeled or ^{125}I-labeled 7S nerve growth factor protein. Individual α and γ subunits were isolated as described by these authors from either unlabeled or ^{125}I-labeled α-subunit pools or from unlabeled γ-subunit pools, respectively. The 7S species $\alpha^4\gamma\beta$ was made by mixing α^4, γ, and β subunits in 0.05 M Tris-Cl buffer (pH 7.4) in approximately equal amounts by weight, sedimenting the mixture in a 5–20% sucrose gradient, isolating the 7S pool, and freeing it from sucrose by dialysis and concen-

* From the Departments of Genetics and Biochemistry, and the Lt. Joseph P. Kennedy Laboratories for Molecular Medicine, Stanford University School of Medicine, Stanford, California (APS, HRF, and EMS) and the Department of Chemistry (LAG) and Biology (SV), University of California at San Diego, La Jolla, California. *Received August 18, 1969.* This research was supported by U. S. Public Health Service research grants from the National Institutes of Neurological Diseases and Stroke (NB 04270 and 07606) and National Science Foundation Grants GB 4430 and GB 6878.

† National Science Foundation Predoctoral Fellow.

‡ National Science Foundation Trainee.

tration against the Tris-Cl buffer. The 7S nerve growth factor protein was characterized by its sedimentation and electrophoretic properties, and by its biological activity in tissue culture (Levi-Montalcini *et al.*, 1954). The methods used for sucrose gradient sedimentation and acrylamide gel electrophoresis, as well as for ion-exchange chromatography, have been described previously (Smith *et al.*, 1968). Isoelectric focusing on acrylamide gels was carried out as described by Wrigley (1968). The protein sample made 5% in sucrose was layered on the anodic end of the gel. The carrier ampholytes used to produce a pH gradient from 3 to 10 were obtained from LKB-Produkter AB, Sweden. Buffer pH values are specified at 25°.

Iodination of the 7S nerve growth factor protein was carried out by an adaptation of the method used by Greenwood *et al.* (1963) to label human growth hormone. To the sample, consisting of 5 mg of 7S nerve growth factor protein in 500 μl of 0.05 M phosphate buffer (pH 6.8), was added 0.5 mCi of carrier-free Na^{125}I (New England Nuclear), followed by the oxidizing agent, 40 μg of chloramine T. The mixture was allowed to react for 10 min in an ice bath and for an additional 5 min at room temperature. Excess ^{125}I was then reduced with 190 μg of sodium metabisulfite for 10 min at room temperature. The labeled 7S nerve growth factor protein was then separated from radioactive iodide on a 5 × 0.5 cm Dowex 1X-8 column, which had been flushed previously with the (pH 6.8) phosphate buffer.

Labeling efficiency, defined as the fraction of ^{125}I atoms added to the original reaction mixture which passed through the Dowex column and which were trichloroacetic acid insoluble, was on the order of 50%. This technique permitted the recovery of about 80% of the 7S nerve growth factor protein, without the use of carrier protein, with a specific activity of 0.05–0.10 μCi/μg, corresponding to roughly one iodine atom per 200–400 molecules of the 7S species. By physicochemical criteria, the labeled protein had many of the properties of the original unlabeled 7S preparation (see Results).

Measurement of radioactivity was made in a Nuclear Chicago well-type crystal scintillation counter. Because of the high penetration of the X-radiation from ^{125}I, samples were counted in glass tubes with negligible quenching. Column and sucrose gradient fractions were thus counted directly; radioactivity present in acrylamide gels was assayed by slicing the gels into equal disks approximately 1 mm thick and then counting directly each segment in a glass tube. Counting efficiency was 46%. All samples from regions including peaks of radioactivity were counted to a standard deviation no greater than ±3%. For samples from regions of several unresolved peaks, counts were taken to a standard deviation smaller than ±2%.

Results

Characterization of the ^{125}I-Containing 7S Nerve Growth Factor Protein. Evidence that the iodinated 7S nerve growth factor protein was physicochemically similar to the unlabeled protein was obtained in several ways. When subjected to electrophoresis in the Tris tricinate buffer system, the 7S nerve growth factor protein typically appears as a single major band, along with minor bands of higher mobility representing the individual α subunits produced by the slight

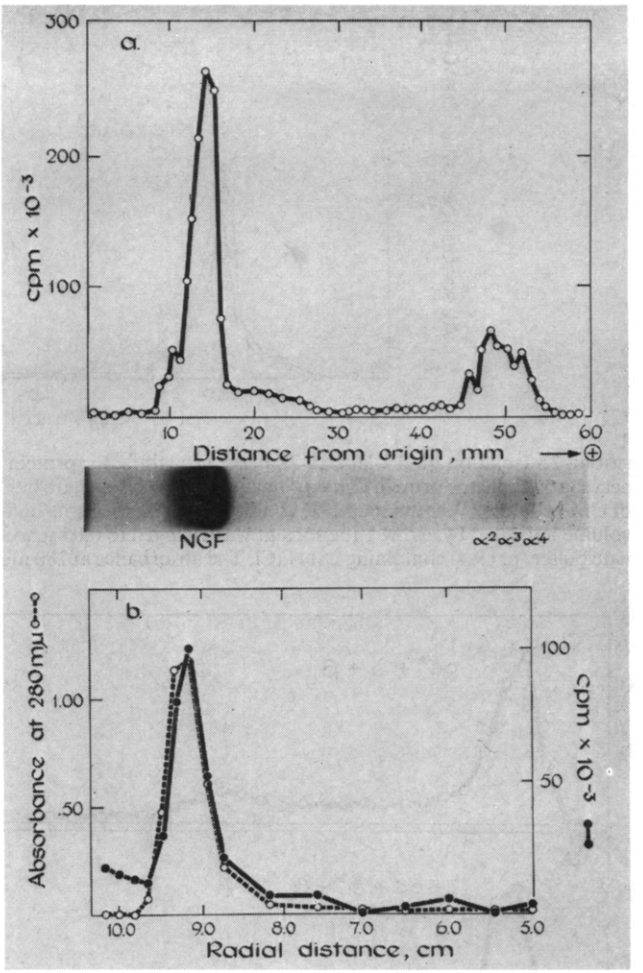

FIGURE 1: Characterization of ^{125}I-labeled 7S nerve growth factor on two resolving systems. (a) Electrophoretic analysis in the Tris tricinate system (pH 8.3) of 110 μg of ^{125}I-labeled 7S nerve growth factor (specific activity 20,000 cpm/μg). Migration time 2.25 hr. Gel stained with Naphthol Blue Black, photographed, and then cut in 1-mm segments, each of which was counted for radioactivity. (b) Sedimentation analysis of 22 μg of the same ^{125}I-labeled 7S nerve growth factor protein preparation mixed with 400 μg of unlabeled 7S protein. Protein layered on top of 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4) and sedimented in a SB-405 rotor (International B-60 ultracentrifuge) at 60,000 rpm for 13 hr at 4°. Fractions (3 drops) were collected from bottom of tube and their absorbance at 280 mμ and radioactivity (cpm) was measured.

dissociation at the resolving pH of 8.3. Figure 1a shows that the labeled protein gave the same pattern and that the radioactivity profile paralleled that of protein, giving a major peak at the position of the 7S protein, and a group of smaller peaks in the α-subunit region. Thus, labeled 7S nerve growth factor protein appears to be electrophoretically identical with the unlabeled species. When the labeled 7S nerve growth factor protein was sedimented in a sucrose gradient, in the presence of an additional 20-fold excess of unlabeled protein, the patterns of radioactivity and protein again coincided (Figure 1b). Thus the labeled material, like the original protein, has a sedimentation coefficient close to 7 S.

The labeled 7S nerve growth factor protein, mixed with a 100-fold excess of unlabeled protein, was also subjected to the ion-exchange chromatographic procedure for the separa-

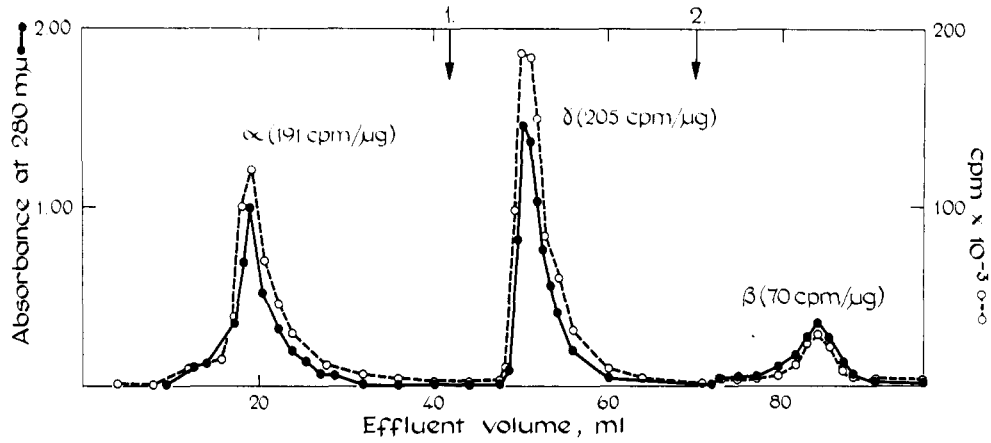

FIGURE 2: Isolation of ^{125}I-labeled 7S nerve growth factor protein subunits. A mixture of 110 μg of ^{125}I-labeled and 14 mg of unlabeled 7S nerve growth factor protein dialyzed against 0.05 M Na acetate buffer (pH 4.0) containing 0.15 M NaCl and applied to a 0.9 × 10 cm column of CM-cellulose (Whatman CM32) equilibrated with the same buffer. The column was eluted with the same buffer at 17 ml/hr. At an effluent volume indicated by arrow 1 the solvent was changed to 0.05 M acetate buffer (pH 4.0) containing 0.4 M NaCl and at arrow 2 to 0.05 M glycinate buffer (pH 9.4) containing 2 M NaCl. The absorbance at 280 mμ and radioactivity (cpm) of each fraction was measured.

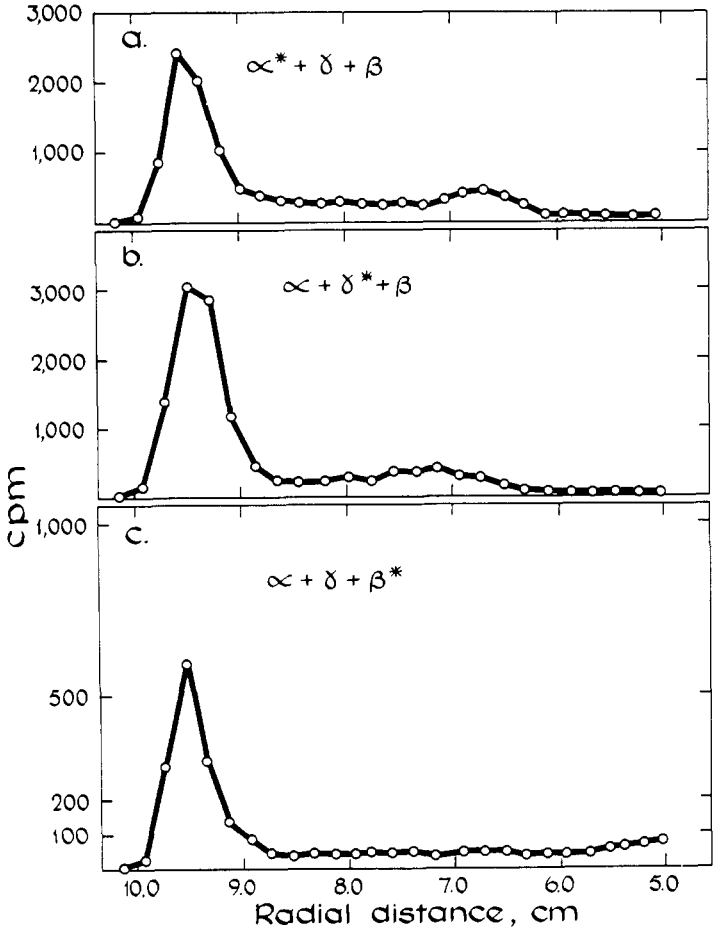

FIGURE 3: Recombination of the ^{125}I-labeled subunits to form radioactive 7S species. Each recombination mixture consisted of approximately 70 μg of α, 90 μg of γ, and 50 μg of β subunits in 0.05 M Tris-Cl buffer (pH 7.4), one subunit being labeled. After incubation for 2 hr at 4° the mixture was layered on top of a 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4) and sedimented in a SB-405 rotor at 60,000 rpm for 13 hr at 4°. Fractions (3 drops) collected from the bottom of the tube and assayed for radioactivity: (a) using ^{125}I α subunits of specific activity 140 cpm/μg, (b) using ^{125}I γ subunits of specific activity 140 cpm/μg, (c) using ^{125}I β subunits of specific activity 35 cpm/μg.

tion of the constituent subunits (Smith *et al.*, 1968). As shown in Figure 2, radioactivity appeared with protein at all three elution steps. This is evidence that the labeled nerve growth factor protein contained ^{125}I in all three of its subunits, although the extent of labeling and thus the specific activity of the β subunit was considerably lower than that of α or γ subunits. This fact is not immediately obvious from an inspection of the absorbance and radioactivity profiles in Figure 2 because the ratio of absorbance at 280 mμ to protein concentration, while approximately the same for the α and γ subunits, is lower for the β subunit (Smith, 1969). The specific activities recorded in Figure 2 were determined by measurement of radioactivity and protein concentration of the concentrated pool of each subunit peak. Since the three subunit types have approximately the same molar content of tyrosine residues (D. Strauss and E. M. Shooter, unpublished data) it is also apparent that a number of these residues are less accessible in the β than in the α or γ subunits.

The labeled protein in each of the three subunit fractions sedimented together with excess unlabeled subunit protein, with a sedimentation coefficient of about 2.5 S (Figure 4a, b,c). Also, when labeled subunit was mixed with two remaining subunits which were not labeled, a major portion of the label (Figure 3) and protein (not shown) sedimented in the 7S region of the gradient. The iodination procedure affects, therefore, neither the size and net charge of the 7S species and its subunits nor, in a gross sense, the ability of the subunits to reconstitute the high molecular weight 7S complex.

The biological activity of the labeled 7S nerve growth factor protein was about 60 BU/μg, which is the same level observed for typical unlabeled preparations. Because of the low degree of labeling, it is not possible to infer whether nerve growth factor protein molecules which actually contain ^{125}I are biologically active. However, it is clear that the labeling procedure itself in no way impairs the biological activity of the 7S nerve growth factor protein.

The Use of Labeled Subunits to Determine the Subunit Equilibria of the 7S Nerve Growth Factor Protein. The hypothesis that the 7S nerve growth factor protein may be in rapid equilibrium with one or more of its constituent subunits was tested in several ways, although the basic design of each of

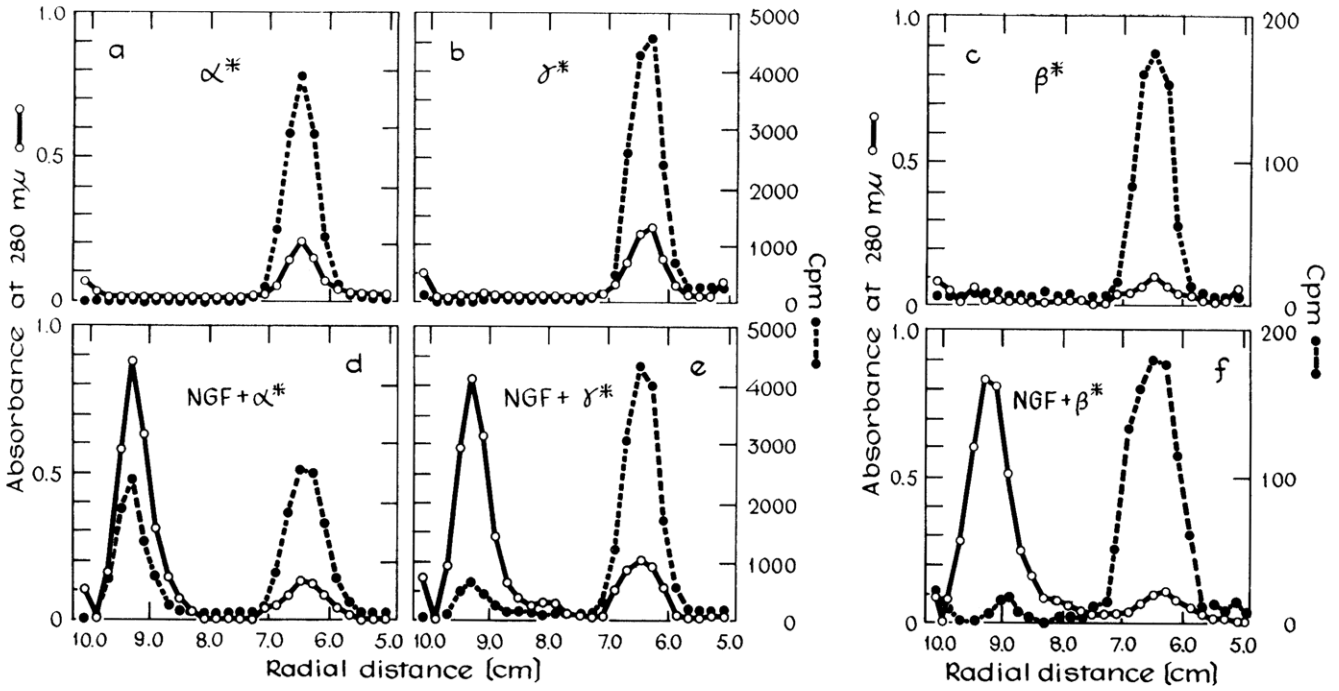

FIGURE 4: Exchange of ^{125}I-labeled subunits with the 7S nerve growth factor protein. Analyses in the top line are of (a) 65 μg of ^{125}I α subunit (specific activity 360 cpm/μg), (b) 65 μg of ^{125}I γ subunit (specific activity 360 cpm/μg), and (c) 60 μg of ^{125}I β subunit (specific activity 30 cpm/μg). Analyses in the bottom line are of (d) 65 μg of ^{125}I α subunit + 270 μg of 7S nerve growth factor protein, (e) 65 μg of ^{125}I γ subunit + 270 μg of 7S protein, and (f) 60 μg of ^{125}I β subunit +270 μg of 7S protein. These mixtures were incubated for 4 hr at 4°. All subunits were in 0.05 M Tris-Cl buffer (pH 7.4) and the final volume of the mixture was 100–200 μl. All samples were layered on top of 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4) and sedimented at 60,000 rpm for 13 hr at 4°. Fractions (3 drops) were collected from the bottom of the tube and their absorbance at 280 mμ and radioactivity (cpm) measured.

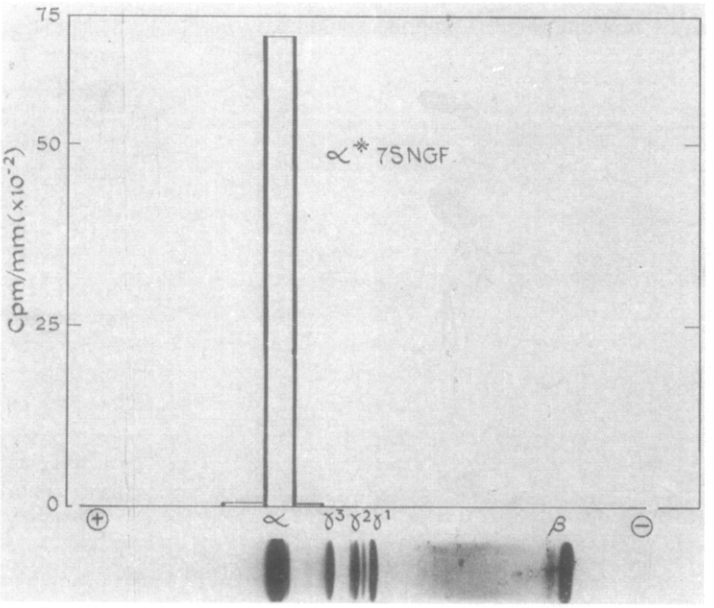

FIGURE 5: Recovery of the ^{125}I label from 7S nerve growth factor protein after incubation with ^{125}I-labeled α subunit. The pool of the 7S component from the experiment described in Figure 4d was concentrated by vacuum dialysis against H_2O to approximately 200 μl. A volume corresponding to 100 μg of protein, made 5% in sucrose, was layered on top of a 1.3 cm, 7.5% acrylamide gel containing carrier ampholytes for a pH 3 to 10 gradient. Electrophoresis was carried out at 50 V for 4 hr and the gel stained with 0.1% Coomassie Brillant Blue R250 in 20% trichloroacetic acid. Segments (1 mm) were counted directly for radioactivity.

the experiments was similar. All the exchange experiments were performed in 0.05 M Tris-Cl (pH 7.4), at protein concentrations between 1 and 3 mg/ml and for periods of a few hours at either 4 or 25°. The most direct method was to determine whether exchange of a given subunit with the corresponding subunit in the 7S nerve growth factor protein occurred when a labeled subunit was mixed with the unlabeled parent complex. The extent of exchange was determined by separating the subunit and parent species by sedimentation and estimating the amount of label in the previously unlabeled 7S nerve growth factor protein. When this was done for each of the subunits in turn (Figure 4d,e,f) it was found that only for the α subunit did a sizable fraction of the subunit label appear in the 7S nerve growth factor protein. In this experiment approximately 40% of the original label moved to the 7S region of the gradient (Figure 4d). In contrast, after the same

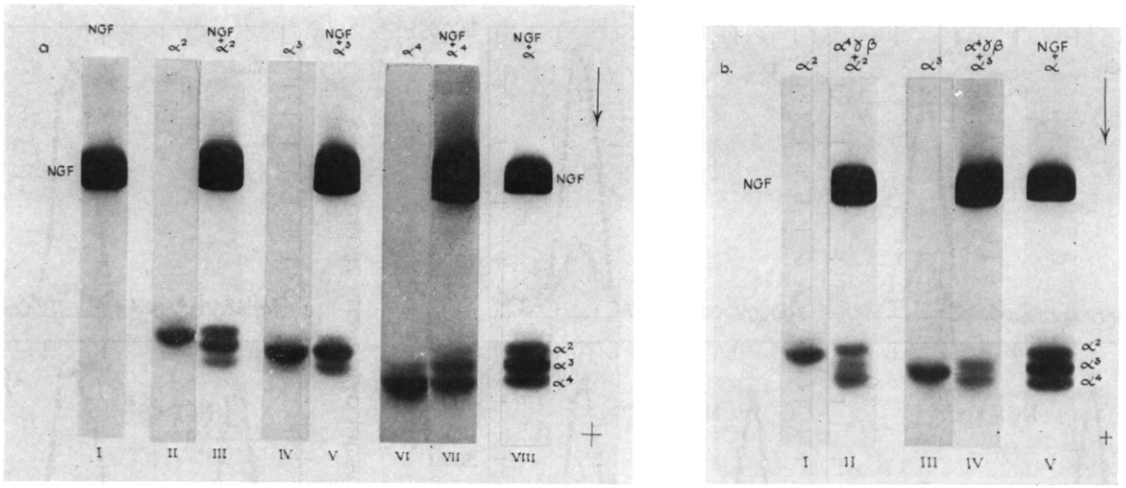

FIGURE 6: Exchange of individual unlabeled α subunits with (a) 7S nerve growth factor protein and (b) the 7S species $\alpha^4\gamma\beta$. Samples containing either an individual subunit alone or in mixture with the 7S nerve growth factor protein were incubated for 2 hr at 25° in 0.05 M Tris-Cl buffer (pH 7.4) and then analyzed electrophoretically in the bistris–tes system (pH 7.55). Migration time was 1.5 hr, stained with Naphthol Blue Black; (a) (I) 200 μg of 7S nerve growth factor protein, (II) 26 μg of α^2 subunit, (III) 50 μg of α^2 subunit + 250 μg of 7S nerve growth factor protein, (IV) 25 μg of α^3 subunit, (V) 47 μg of α^3 subunit + 250 μg of 7S nerve growth factor protein, (VI) 25 μg of α^4 subunit, (VII) 50 μg of α^4 subunit + 250 μg of 7S nerve growth factor protein, (VIII) 50 μg of α subunit + 150 μg of 7S nerve growth factor protein. Volume of all samples made up to 120 μl with 0.05 M Tris-Cl buffer (pH 7.4). (b) (I) 26 μg of α^2 subunit, (II) 50 μg of α^2 subunit + 160 μg of $\alpha^4\gamma\beta$, (III) 25 μg of α^3 subunit, (IV) 25 μg of α^3 subunit + 160 μg of $\alpha^4\gamma\beta$, (V) 50 μg of α subunit + 150 μg of 7S nerve growth factor protein. Volume of all samples made up to 120 μl with 0.05 M Tris-Cl buffer (pH 7.4).

length of incubation less than 10% of the radioactivity was found in the 7S nerve growth factor protein when either labeled γ or β subunits were used (Figure 4e,f).

That the radioiodine label remained with the α subunit after incorporation into the 7S nerve growth factor protein was checked by isolating the 7S component and analyzing it, after concentration, by the isoelectric focusing technique on acrylamide gel. Under the conditions obtaining during the establishment of the pH gradient in the gel, the 7S nerve growth factor protein dissociates and the individual subunits move to equilibrium position in the gel corresponding to their isoionic points. After electrophoresis and staining, 1-mm sections of the gel were cut out and counted for radioactivity. The results showed that virtually all of the label remained with the α subunit, none appearing in the γ or β subunits (Figure 5). The appearance of ^{125}I in the 7S nerve growth factor protein after incubation with excess ^{125}I-labeled α subunit appears therefore to be the result of the exchange of free and bound α subunits rather than a nonspecific transfer of ^{125}I from the labeled α subunits to the unlabeled parent protein. Further evidence in favor of this view comes from the finding that approximately the same degree of labeling of the 7S nerve growth factor protein occurred when it was incubated under the same conditions with α subunits containing [^{3}H]-leucine. The ^{3}H-labeled α subunits were prepared from 7S nerve growth factor protein labeled by *in vivo* incorporation of [^{3}H]leucine (S. Varon, J. Nomura, and C. Raiborn, unpublished data).

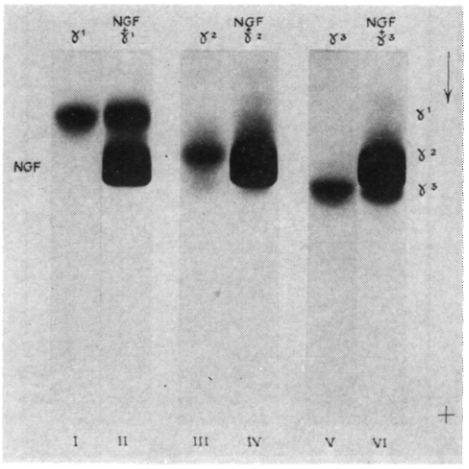

FIGURE 7: Lack of exchange of individual γ subunits with the 7S nerve growth factor protein. Samples containing either an individual subunit alone or in mixture with the 7S nerve growth factor protein were incubated for 2 hr at 25° in 0.05 M Tris-Cl buffer (pH 7.4) and then analyzed electrophoretically in the bistris–tes system (pH 7.55). Migration time was 1.5 hr, stained with Naphthol Blue Black; (I) 40 μg of γ^1 subunit, (II) 40 μg of γ^1 subunit + 250 μg of 7S nerve growth factor protein, (III) 30 μg of γ^2 subunit, (IV) 30 μg of γ^2 subunit + 250 μg of 7S nerve growth factor protein, (V) 40 μg of γ^3 subunit, (VI) 40 μg of γ^3 subunit + 250 of 7S nerve growth factor protein. Volume of all samples made up to 120 μl with 0.05 M Tris-Cl buffer (pH 7.4).

Subunit Exchange with Individual Subunits. While the experiment reported above indicates that free α subunits readily exchange with α subunits in the 7S nerve growth factor protein, it does not distinguish among the behaviors of the individual α subunits. This was accomplished by using electrophoresis rather than sedimentation to separate the subunits from the parent 7S protein. The system of choice for electrophoresis was the bistris–tes[1] buffer system which resolved at

[1] Abbreviations used are: bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

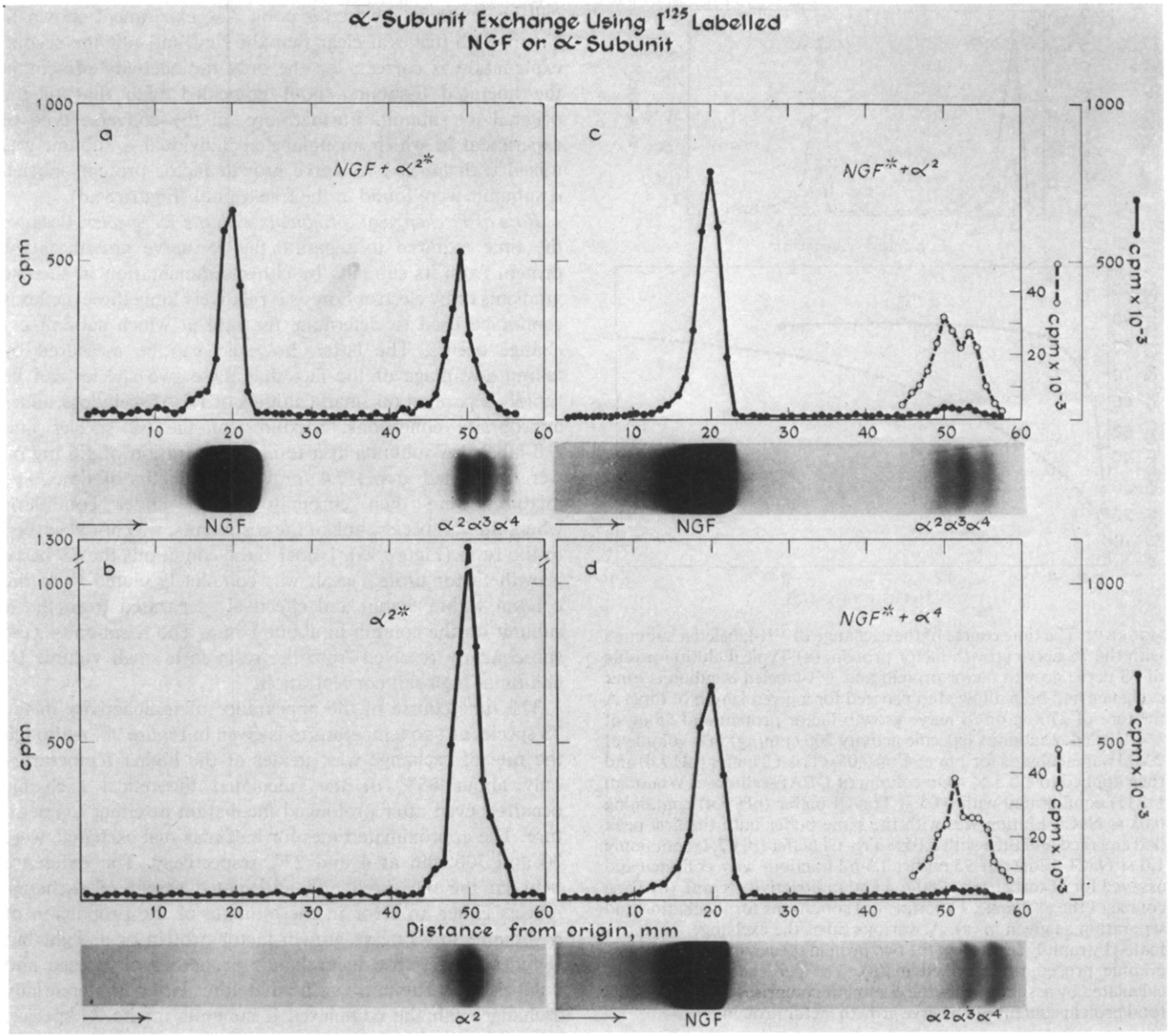

FIGURE 8: Exchange of individual α subunits with the 7S nerve growth factor protein. (a) A mixture of 250 μg of 7S nerve growth factor protein and 50 μg of ^{125}I-labeled α^2 subunit (specific activity 90 cpm/μg) was incubated for 2 hr at 25° in 0.05 M Tris-Cl buffer (pH 7.4). (b) 50 μg of ^{125}I-labeled α^2 subunit used in (a). (c) A mixture of 250 μg of ^{125}I-labeled 7S nerve growth factor protein (specific activity 13,000 cpm/μg) and 25 μg of α^2 subunit was incubated for 2 hr at 25° in 0.05 M Tris-Cl buffer (pH 7.4). (d) A mixture of 200 μg of ^{125}I-labeled 7S nerve growth factor protein (specific activity 13,000 cpm/μg) and 25 μg of α^4 subunit was incubated for 2 hr at 25° in 0.05 M Tris-Cl buffer (pH 7.4). Samples were analyzed electrophoretically in the bistris-tes system (pH 7.55), migration time 1.5 hr. Gels were stained with Naphthol Blue Black and then cut into 1-mm segments for counting.

pH 7.55, that is, below the upper pH stability limit of the 7S protein.

Figure 6a shows the results of the experiments in which individual (unlabeled) α^2, α^3, or α^4 subunits were added to 7S nerve growth factor protein and the mixture subsequently separated by electrophoresis. In each instance the analysis of the mixture showed the appearance of other α subunits besides the one added initially. This is the result that would be expected if all the α subunits were freely exchangeable with an added excess of any one α subunit. Moreover, the amount of an α subunit displaced from the 7S nerve growth factor protein should be in direct proportion to its contribution to the total α-subunit pool in the parent protein. Since the α^3 subunit is typically present in greatest amount and the α^2 and α^4 subunits in approximately equal but lesser amounts (Figure 6a, VIII), then more α^3 subunit should be displaced than α^2 or α^4 subunits. The analyses in Figure 6a suggest that qualitatively this is what is found. Also it follows that if the 7S species contains only one of the individual α subunits then this subunit can be displaced by exchange with any of the other individual α subunits. The analyses in Figure 6b confirm this prediction. The addition of α^2 or α^3 subunits to an artificially prepared 7S species containing essentially only the α^4 subunit resulted in the appearance only of this subunit in each instance. The trace of α^3 subunit noted in the displacement reaction with the α^2 subunit arose

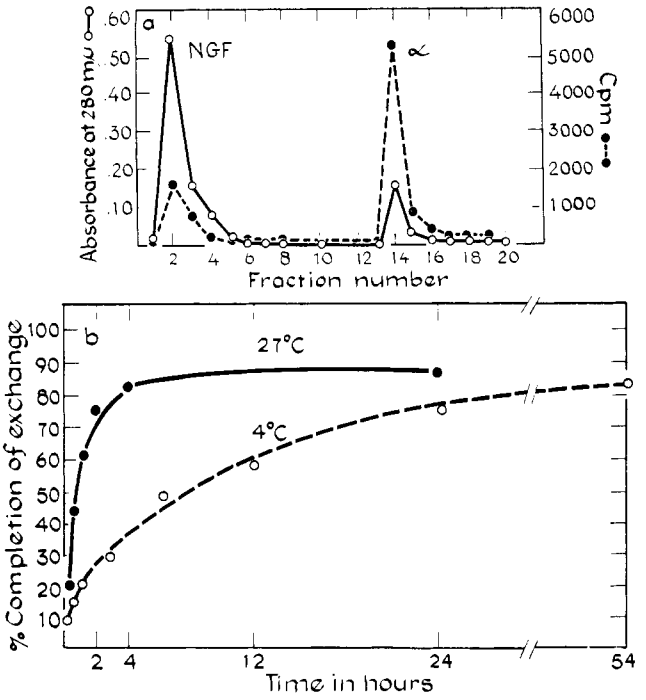

FIGURE 9: The time course of the exchange of ^{125}I-labeled α subunits with the 7S nerve growth factor protein. (a) Typical elution profile of 7S nerve growth factor protein and ^{125}I-labeled α subunits after exchange had been allowed to proceed for a given length of time. A mixture of 270 μg of 7S nerve growth factor protein and 55 μg of ^{125}I-labeled α subunits (specific activity 200 cpm/μg) in a volume of 200 μl was incubated for 1 hr at 4° in 0.05 M Tris-Cl buffer (pH 7.4) and then applied to a 0.5 × 4 cm column of DEAE-cellulose (Whatman DE11) equilibrated with 0.05 M Tris-Cl buffer (pH 7.4) containing 0.08 M NaCl. Elution was with the same buffer until the first peak had emerged and then with 0.05 M Tris-Cl buffer (pH 7.4) containing 1.0 M NaCl. Flow rate 25 ml/hr; 0.5-ml fractions were collected and assayed for absorbance at 280 mμ and radioactivity (cpm). (b) Time course of the exchange. Experimental conditions for incubation and separation as given in (a). At various times the exchange was terminated by rapidly separating the two protein species by the chromatographic procedure described in (a). The per cent exchange was calculated by assuming that the α subunit comprises one-third of the total protein content of 7S nerve growth factor protein.

from trace contamination of the α^4 subunit used in the preparation of the specific, α^4-subunit-containing, 7S species.

In contrast to the free exchange of the individual α subunits it was found (Figure 7) that addition of an individual γ subunit to the 7S nerve growth factor protein did not result in the appearance of the other individual γ subunits. This result is in keeping with the very low degree of exchange of γ subunits as a whole with the 7S nerve growth factor protein, as indicated by the sedimentation experiments using labeled subunit described above (Figure 4e).

A final and rigorous proof of α subunit exchange can be made using an ^{125}I-labeled individual α subunit, *e.g.*, ^{125}I-labeled α^2 subunit, and the unlabeled 7S species. If exchange occurs it should result in incorporation of part of the ^{125}I-labeled α^2 subunit into the 7S species and the appearance of the α^3 and α^4 subunits in the free α pool, but in unlabeled form. On the other hand, if the α^2 subunit is simply converted into the other α subunits, perhaps through the known enzymatic activity of the γ component of the 7S protein (Greene *et al.*, 1968, 1969), then labeled α subunits other than α^2 will be found in the free α pool. The experiment shown in Figure 8a,b makes it clear that the first and not the second explanation is correct, for the only radioactivity present in the liberated α-subunit pool coincided with that of the original α^2 subunit. Furthermore, in the converse type of experiment in which an unlabeled individual α subunit was mixed with labeled 7S nerve growth factor protein, labeled α subunits were found in the free α pool (Figure 8c,d).

Rate of Exchange of α Subunits with the 7S Species. Because the time required to separate the 7S nerve growth factor protein from its subunits by either sedimentation in sucrose gradients or by electrophoresis is relatively long, these methods cannot be used to determine the rate at which subunit exchange occurs. The latter, however, can be measured by taking advantage of the fact that these two species can be rapidly separated on small columns of DEAE-cellulose under appropriate conditions. Mixtures of the 7S species and ^{125}I-labeled α subunits at a total concentration of 1.6 mg/ml were incubated at pH 7.4 for varying lengths of time. The mixtures were then chromatographed under conditions where the 7S species, unlike the α subunits, was not absorbed to the resin (Figure 9a). Under these conditions the 7S nerve growth factor protein peak was completely eluted from the column within 5 min and effectively separated from the α subunit on the column in about 1 min. The α subunits were subsequently resolved from the resin in a small volume by elution at high salt concentration.

The time course of the appearance of radioactivity in the 7S species at two temperatures is given in Figure 9b. Although the rate of exchange was greater at the higher temperature only about 85% of the calculated theoretical exchange occurred even after prolonged incubation at either temperature. The approximate times for half-maximal exchange were 30 and 300 min at 4 and 27°, respectively. The difference between the measured and anticipated extent of exchange reflects either an error in the estimate of the proportion of α subunits in 7S nerve growth factor protein or a slight but significant difference in exchange properties of labeled and unlabeled α subunits such that the latter preferentially exchange with the complexed α subunits in the 7S species. That the second explanation is probably correct was shown in the following way. The products of the recombination of mixtures of ^{125}I-labeled α subunits with unlabeled γ and β subunits were analyzed by acrylamide gel electrophoresis at pH 7.55 and the amount of the labeled α subunits in the mixtures varied until all the α-subunit protein was complexed into the 7S species as judged by the disappearance of stainable α bands. The sensitivity of the protein staining method is such that an amount of α subunit equivalent to 5% of the total protein in the gel would be easily detected. However subsequent radioactivity measurements on sections of the gel showed that under these same conditions between 15 and 20% of the total radioactivity remained in the position of the α subunits. Therefore while at least 95% of the unlabeled α subunits had recombined with the other subunits only 80–85% of the ^{125}I-labeled α subunit had done so. More of the latter could be complexed into the 7S species by addition of increasing amounts of γ and β subunits. The ^{125}I-labeled α subunits have therefore a slightly lower affinity for their complementary γ and β subunits than do their unlabeled counterparts in the same preparation. This also accounts for the fact that the sedimentation analyses of mixtures chosen

for maximun recombination also show a slight excess of free labeled subunits (Figure 3).

Discussion

The data obtained in this investigation suggest that the 7S nerve growth factor protein is in equilibrium at pH 7.4 and an ionic strength of 0.05 with free α subunits but to a much lesser extent with γ and β subunits. The initial dissociation which produces α subunits therefore results also in the formation of a complex of γ and β subunits, either by themselves or with other α subunits, which under these conditions is relatively stable. The existence of a mobile equilibrium between a parent protein and its subunits has been previously demonstrated for other proteins, notably hemoglobin (Guidotti *et al.*, 1963; Guidotti, 1967). In hemoglobin an equilibrium exists between the $\alpha_2\beta_2$ tetramers and the two $\alpha\beta$ dimer subunits. However, the rate at which this equilibrium is attained is fast, at least as compared to any protein separation process. It is for this reason that the equilibrium cannot be directly demonstrated by, for example, the isolation of hybrid hemoglobin molecules of the type $(\alpha^A\beta^A)$ $(\alpha^A\beta^S)$ from mixtures of the two parent species $\alpha_2^A\beta_2^A$ and $\alpha_2^A\beta_2^S$. Because of the rapid equilibrium between tetramers and dimers any separation process can only reveal the parent species. Guidotti (1967) was able to prove that hybrid hemoglobins do form by showing that the colligative properties of mixtures of hemoglobins were greater than those calculated for the sum of the separate components, assuming no equilibria and thus no interaction between them. In contrast to hemoglobin, the rate at which equilibrium is attained between the α subunits and the 7S complex is sufficiently slow to allow complete resolution of the two species by sedimentation or electrophoresis and thus the direct measurement of the rate at which labeled free α subunits exchange with unlabeled α subunits in the 7S complex. Also the existence of multiple and distinct forms of the α subunits makes it possible to confirm the existence of the equilibrium with entirely unlabeled species.

The kinetic behavior of the 7S nerve growth factor protein system is more like that of tryptophan synthetase than hemoglobin. Creighton and Yanofsky (1966) using enzyme kinetic measurements found that the association and dissociation rate constants for tryptophan synthetase were 6×10^5 M^{-1} sec^{-1} and 1.8×10^{-4} sec^{-1}, respectively, the complex therefore having a half-time of approximately 64 min. This is the same order of magnitude as the half-life of the α-subunit exchange and suggests that the rate-limiting step in this exchange is the slow rate at which the 7S complex dissociates. Bethune and Kegeles (1961a,b) have also pointed out that if a complex sediments at a given fixed rate and if the addition of excess subunits results simply in their appearance in the unassociated form then the association rate constant must be significantly greater than the dissociation rate constant of the complex. This is the behavior of the 7S nerve growth factor protein. Over a wide range of total subunit concentration the 7S species is the complex which is observed, and it must therefore represent the fully aggregated complex of the α, γ, and β subunits. Also, addition of an excess of any one subunit results in its appearance in the free form (Figure 4). The high degree of specificity exhibited by the interaction of the three types of subunits (Varon *et al.*, 1967; Shooter and Varon, 1969; Greene *et al.*, 1969) is also a reflection of the high affinity of the subunits for one another in the 7S complex.

The Enzymatic Properties of the 7S Species in Relation to the Subunit Equilibrium. The properties of an equilibrium like the one outlined above explain the difference in the enzymatic behavior of the 7S nerve growth factor protein and the isolated γ subunits (Greene *et al.*, 1968, 1969). Whereas the latter hydrolyze appropriately substituted argininyl ester or amide substrates with linear kinetics, the 7S preparation shows a characteristic lag phase before reaching maximal velocity. The 7S preparation also displays a significantly lower specific enzymatic activity than do the γ subunits. Thus the lag in reaching maximal reaction velocity is due to the time required for the equilibrium to readjust in favor of more dissociated and more active forms of the γ subunits. It is not observed if the diluted 7S preparation is allowed to stand for a period of time before addition of substrate. Although the enzyme assays were carried out at lower protein concentrations and at different pH values from those in the exchange studies reported here it is still clear from them that the dissociation of the 7S complex is a slow process.

The Effect of the Equilibrium on the Migration of the 7S Species. The existence of a mobile equilibrium also explains the unexpected gradient of individual γ subunits found in a 7S nerve growth factor zone migrating on electrophoresis (Smith *et al.*, 1968). Since the rate of migration of the 7S $\alpha\gamma\beta$ complex is determined only by the type of individual α subunits it contains and is not influenced by variation among the γ subunits it would have been predicted that electrophoresis of the multiple forms of the 7S species would result in a gradient of α but not of γ subunits. However, as a result of the equilibrium, γ subunits appear in a form where their different mobilities are expressed. When the 7S species is re-formed the faster γ species, having on the average migrated further than the slower γ species, have a somewhat greater probability of recombining with the faster α species. Through this continuing process both the faster migrating α *and* γ subunits concentrate in the leading edge of the migrating zone and *vice versa*.

Acknowledgments

We wish to thank Dr. Robert L. Baldwin for his comments and criticisms and Mrs. Elaine S. Shooter for her skilled technical assistance.

References

Bethune, J. L., and Kegeles, G. (1961a), *J. Phys. Chem. 65*, 433.

Bethune, J. L., and Kegeles, G. (1961b), *J. Phys. Chem. 65*, 1755.

Creighton, T. E., and Yanofsky, C. (1966), *J. Biol. Chem. 241*, 980.

Greene, L. A., Shooter, E. M., and Varon, S. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 1383.

Greene, L. A., Shooter, E. M., and Varon, S. (1969), *Biochemistry 8*, 3735.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963), *Biochem. J. 89*, 114.

Guidotti, G. (1967), *J. Biol. Chem. 242*, 3694.

Guidotti, G., Konigsberg, W., and Craig, L. C. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 774.
Levi-Montalcini, R., Meyer, R. H., and Hamburger, V. (1954), *Cancer Res. 14*, 49.
Shooter, E. M., and Varon, S. (1969), *in* Protein Metabolism of the Nervous System, Lajtha, A., Ed., New York, N. Y., Plenum Press (in press).
Smith, A. P. (1969), Ph.D. Thesis, Stanford University, Stanford, Calif.
Smith, A. P., Varon, S., and Shooter, E. M. (1968), *Biochemistry 7*, 3259.
Smith, A. P., Varon, S., and Shooter, E. M. (1969), *Fed. Proc. 28*, 897.
Varon, S., Nomura, J., and Shooter, E. M. (1967a), *Proc. Natl. Acad. Sci. U. S. 57*, 1782.
Varon, S., Nomura, J., and Shooter, E. M. (1967b), *Biochemistry 6*, 2202.
Varon, S., Nomura, J., and Shooter, E. M. (1968), *Biochemistry 7*, 1296.
Wrigley, C. (1968), *Science Tools 15*, 17.

Quantitative Variation in Serine Transfer Ribonucleic Acid During Estrogen-Induced Phosphoprotein Synthesis in Rooster Liver*

Pekka H. Mäenpää and Merton R. Bernfield

ABSTRACT: In roosters, hepatic synthesis of the yolk phosphoprotein, phosvitin, is induced by estrogen treatment. Phosvitin has an unusually high content of serine residues (over 50%), and an attempt was made to correlate phosvitin induction with changes in the benzoylated DEAE-cellulose chromatographic profile of *in vitro* acylated seryl transfer ribonucleic acid. Plasma phosvitin was used as an indicator of phosvitin induction, as newly synthesized phosvitin does not accumulate in the liver. Plasma phosvitin levels increased 150-fold after a single injection of estradiol-17β. Transfer ribonucleic acid and aminoacyl transfer ribonucleic acid ligases were prepared from livers of normal and estrogen-treated roosters. Unfractionated transfer ribonucleic acid isolated during the initial stimulatory period of phosvitin synthesis was acylated with serine to a significantly greater extent than was transfer ribonucleic acid from control animals, regardless of the source or concentration of ligase, concentration of transfer ribonucleic acid, duration of acylation incubation, or isotopic label. At least four seryl transfer ribonucleic acid peaks were observed in chromatograms of acylated transfer ribonucleic acid derived from control or estrogen-treated roosters. A marked relative increase in one major and in one minor peak was found during the rapid phase of phosvitin synthesis. This change in seryl transfer ribonucleic acid chromatographic pattern was independent of the isotopic label and the source of ligase. With decreasing levels of plasma phosvitin these changes in seryl transfer ribonucleic acid diminished and approached the control profile. Identical seryl transfer ribonucleic acid changes were again noted after a second dose of estradiol-17β. Similar chromatographic comparisons of aminoacyl transfer ribonucleic acids for glycine, alanine, valine, leucine, phenylalanine, tyrosine, threonine, methionine, arginine, histidine, lysine, and aspartic and glutamic acids before estrogen injection and during the initial rapid phase of phosvitin synthesis did not reveal major specific changes comparable to those observed in seryl transfer ribonucleic acid. Only small changes in minor species of glycyl, histidyl, and glutamyl transfer ribonucleic acid were observed. The correlation between phosvitin synthesis and specific seryl transfer ribonucleic acid levels, the relative lack of change in other aminoacyl transfer ribonucleic acids, and the predominance of serine in phosvitin suggest that the estrogen-induced seryl transfer ribonucleic acid alterations are related to phosvitin induction.

Multiple species of tRNA specific for a single amino acid (isoaccepting tRNAs) have been described in bacteria and higher organisms (Doctor *et al.*, 1961; Weiss and Kelmers, 1967; Caskey *et al.*, 1968; Yang and Novelli, 1968a). In some instances, isoaccepting tRNA species have been shown to recognize different synonym codons (Weisblum *et al.*, 1962; von Ehrenstein and Dais, 1963; Caskey *et al.*, 1968; Kano-Sueoka *et al.*, 1968; Sundharadas *et al.*, 1968). Changes in the chromatographic profiles of isoaccepting tRNAs have been observed in bacteria after phage infection (Kano-Sueoka and Sueoka, 1966) and during sporulation (Kaneko and Doi, 1966). In higher forms, qualitative and quantitative chromatographic differences have been found during wheat germination (Vold and Sypherd, 1968), and in mammalian cells between tissue types including tumor tissues (Axel *et al.*, 1967; Taylor *et al.*, 1967; Yang and Novelli, 1968a,b; Baliga

* From the Lt. Joseph P. Kennedy Laboratories for Molecular Medicine, Department of Pediatrics and Human Development, Stanford University School of Medicine, Stanford, California 94305. *Received September 10, 1969.* Supported in part by grants from the National Institutes of Health (GM 15086 and HD 02147) and the National Foundation—March of Dimes. P. M. was an Evelyn L. Neizer Postdoctoral Fellow and was also supported, in part, by a grant from Yrjö Jahnssonin Säätiö, Helsinki, Finland.