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Developmental Changes in Microtubule Protein of Chick Brain†

J. R. Bamburg,‡ E. M. Shooter, and L. Wilson*

ABSTRACT: A time-decay colchicine-binding assay and two quantitative polyacrylamide gel electrophoresis systems have been employed to determine the quantities of microtubule protein in the soluble and particulate fractions of chick brain during development. Results with all three methods were identical. The microtubule protein concentration in the soluble fraction of sonicated brains increased from 20% at 5–7 days of development to a maximum concentration of 42% at 13 days of development, then decreased slowly to 25% in the adult. Specificity experiments demonstrated that the binding of colchicine in the particulate fraction of brain was to microtubule protein. No particulate microtubule protein was detected prior to 13 days of development. The particulate microtubule protein concentration then increased slowly from 0.9% of total brain protein at 13 days of development to approximately 2–3% in the adult. The maximum concentration of microtubule protein, as a percentage of total brain protein, was 23–24%, observed between 9 and 17 days of development. The adult concentration was 11–12%. The colchicine binding affinity of microtubule protein appeared to remain constant between 5 days of development in the embryo and the adult. However, the half-life for loss of colchicine binding activity of the protein decreased with increasing age. These results suggest that a change in microtubule protein may be taking place during development of the chick brain.

Microtubules are widely distributed in all eucaryotic cells and tissues, but considerably higher concentrations of microtubule (neurotubule) proteins appear to exist in cells of the central nervous system than in those of other organ systems or tissues (Borisy and Taylor, 1967; Dutton and Barondes, 1969). Estimates of neurotubule protein concentrations in soluble fractions of mammalian brain have ranged between 10 and 40%, and brain has served as a rich source material in the purification of colchicine-binding microtubule proteins (*cf.* Weisenberg *et al.*, 1968; Falxa and Gill, 1969; Bryan and Wilson, 1971; Eipper, 1972).

As an initial approach to questions of regulation of microtubule assembly and function in the central nervous system, we have utilized a time-decay colchicine binding assay procedure, and two independent quantitative polyacrylamide gel electrophoresis procedures to determine accurately the con-

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centrations of microtubule protein in the soluble and particulate fractions of developing chick brain. The colchicine binding activity of microtubule protein has been employed previously much like an enzymatic assay to determine the concentrations of microtubule proteins in various tissues (Redburn and Dahl, 1971; Raff *et al.*, 1971; Yamada and Wessells, 1972). However, the colchicine binding activity of microtubule protein decays in an apparent first-order manner (Weisenberg *et al.*, 1968; Wilson, 1970; Bamburg *et al.*, 1973) and a number of factors such as pH, ionic strength, temperature, and importantly, the concentration of *active* (colchicine-binding) microtubule protein markedly affect the decay rate. Differences in decay rates can give rise to colchicine binding values which do not reflect the true concentrations of microtubule protein present in cell extracts (Bamburg *et al.*, 1973). We have developed a time-decay colchicine binding assay which eliminates the problems associated with varying decay rates (Bamburg *et al.*, 1973). The rate of decay is determined, and by extrapolation to zero time of incubation, the *initial colchicine binding* capacity (*i.e.*, the quantity of colchicine that would have been bound if no decay occurred) is determined. The initial binding capacity is dependent only upon the temperature and concentration of colchicine. In the following study, the use of the time-decay colchicine binding procedure has not only yielded accurate estimates of the quantities of microtubule protein in brain during development, but has provided evidence which suggests that neurotubule protein changes during development of the chick central nervous system.

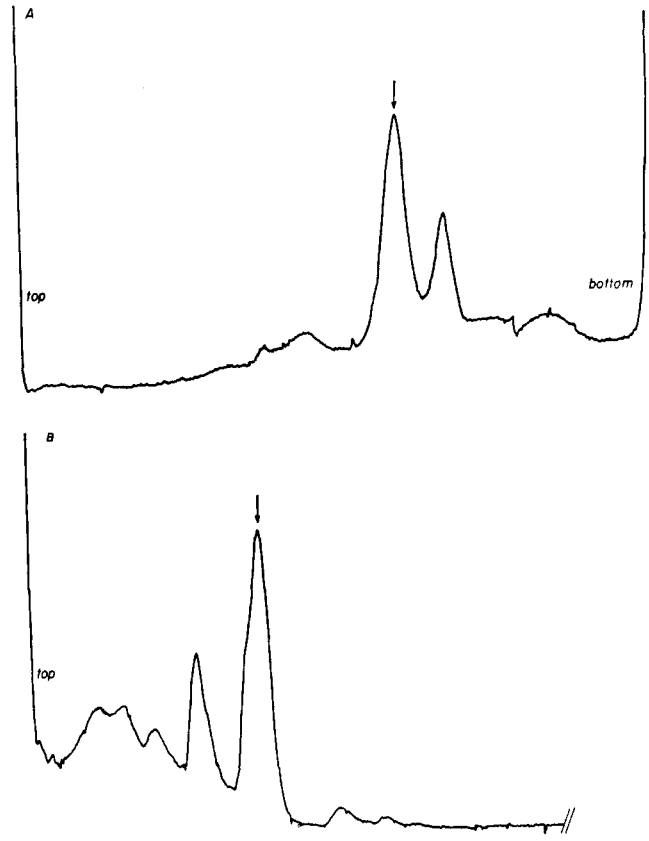

FIGURE 1: Sodium dodecyl sulfate (A) and Tris-glycinate–urea (B) polyacrylamide gel electrophoresis patterns of reduced and carboxymethylated proteins from a supernatant fraction of sonicated 13-day old chick embryo brain. The microtubule protein peak (arrow) comigrates with purified reduced and carboxymethylated chick neurotubule protein (not shown).

Materials

Chemicals. [*methoxy*-^{3}H]Colchicine (ring C) with a specific activity of 5.83 Ci/mmol was obtained from the New England Nuclear Corp. (Boston, Mass.) and diluted to a specific activity of 2.5 Ci/mmol by the addition of purified unlabeled colchicine. [*acetyl*-^{3}H]Colchicine (171 mCi/mmol) was prepared as described by Wilson and Friedkin (1966). Podophyllotoxin was obtained from the Aldrich Chemical Co. (mp 112–114°, λ_{max} 290 mμ in 95% ethanol, ϵ 3750). Vincristine sulfate (97.2% pure) was a gift from the Eli Lilly Co. Bio-Gel P10 was obtained from Bio-Rad Laboratories.

Lumicolchicine. Unlabeled and tritium-labeled β- and γ-lumicolchicine were prepared as described by Mizel and Wilson (1972). Tris(hydroxymethyl)aminomethane (Sigma 7-9) was obtained from the Sigma Chemical Co. Iodoacetamide was a product of Calbiochem Inc. Glycine, *N,N,N'N'*,-tetramethylethylenediamine, acrylamide, and *N,N'*-methylenebisacrylamide were all products of Eastman Organic Chemicals. Sodium dodecyl sulfate was obtained from Fisher Scientific Co. Allied Chemical Co. supplied the Fast Green FCF. All other chemicals were of analytical grade.

Eggs. Fertilized chick eggs were obtained from Kimber Farms, Fremont, Calif., and incubated at 38° in a humidified Jamesway incubator, equipped with an egg turning device which rotated the eggs every 2 hr. The age of the embryos was measured from the start of incubation. The normal hatching time under these conditions of incubation was 21 days.

Methods

Preparation of Chick Brain Fractions. Chick brains (200–250 mg wet weight) from desired age embryos or chicks were removed and sonicated in 4 ml of ice-cold phosphate–glutamate buffer (0.1 M sodium glutamate–0.02 M sodium phosphate, pH 6.75) for 5 sec with a Branson Sonicator (power setting of 1). Centrifugation at 30,000*g* for 15 min at 4° yielded the supernatant and particulate fractions.

Time-Decay Colchicine Binding Assay Procedure. SUPERNATANT FRACTION. Supernatant fractions were diluted to desired protein concentrations with phosphate–glutamate buffer, and incubated at 37° in a water bath. At 2-hr intervals, 0.5-ml aliquots were removed and incubated with 2.7×10^{-6} M radioactive colchicine for 2 hr as described in detail elsewhere (Bamburg *et al.*, 1973). The binding constant is 1–2 $\times$ 10^6 l./mol at 37° for purified 13-day old chick embryo brain microtubule protein (L. Wilson, unpublished data). Bound colchicine was determined after passage of incubation mixtures through 1 $\times$ 18 cm columns of Bio-Gel P10. In samples containing more than 100 μg of microtubule protein/ml, [*acetyl*-^{3}H]colchicine was used; in samples containing less than 100 μg/ml. the higher specific activity [*methoxy*-^{3}H]-colchicine (ring C) was used.

PARTICULATE FRACTION. Whole sonicates as prepared above were incubated at 37°. Aliquots of the whole sonicates, 0.5 ml, were incubated with colchicine for 2 hr at 37° at the time intervals described above. After incubation with colchicine, samples were centrifuged at 30,000*g* for 15 min at 4°. When desired, supernatant fractions were assayed for bound colchicine. The particulate material was washed 3$\times$ with 3-ml volumes of phosphate–glutamate buffer by resuspension and centrifugation, and bound radioactivity was determined after

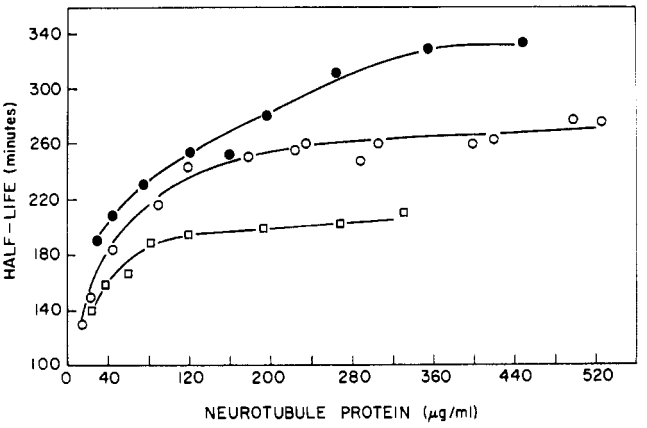

FIGURE 2: Influence of neurotubule protein concentration on half-life in supernatant fractions of sonicated 7-day old (●), 13-day old (○), and adult (□) brain. The concentrations of neurotubule protein in the supernatant brain fractions from each age were determined by Tris-glycinate–urea–polyacrylamide gel electrophoresis (see Results). Time decay colchicine binding assays were carried out on samples of different neurotubule protein concentration obtained by dilution with phosphate–glutamate buffer (see Methods). Initial colchicine binding capacity values for the neurotubule protein of each age supernatant fraction were unchanged at all neurotubule protein concentrations (data not shown); final concentration of colchicine = 2.7×10^{-6} M.

suspension of the particulate material in Bray's solution (Bray, 1960) in a scintillation vial.

Polyacrylamide Gel Electrophoresis Assays. Microtubule protein concentrations in soluble brain fractions were determined by Tris-glycinate–8 M urea–polyacrylamide gel electrophoresis as described in detail previously (Bamburg *et al.*, 1973) and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Shapiro *et al.*, 1967). The use of two different gel electrophoresis procedures, which yielded identical results, reduced the possibility that other contaminating proteins

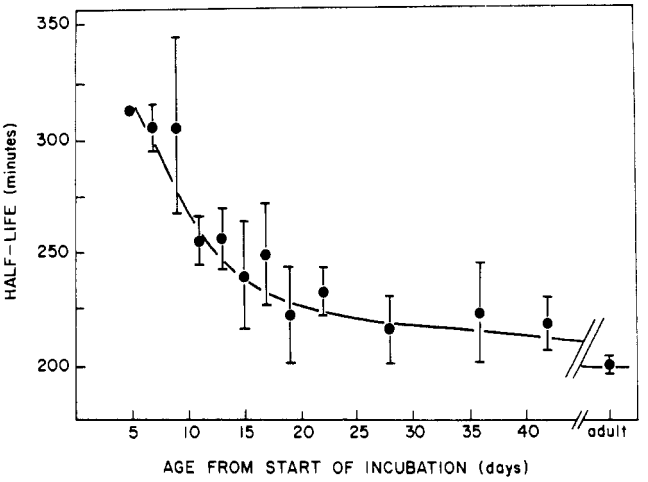

FIGURE 3: Changes in half-life in supernatant fractions of sonicated brain of different ages. Time-decay assays of neurotubule protein in supernatant fractions of sonicated brains were carried out as described under Methods. In all cases, the neurotubule protein concentrations, as determined by polyacrylamide gel electrophoresis (see Figure 5) were greater than 200 μg/ml. Error bars represent the variation at the 95% confidence level, as determined by the "student *t*" test. Between 6 and 12 individual time-decay assays were performed for each age (except at 5 days).

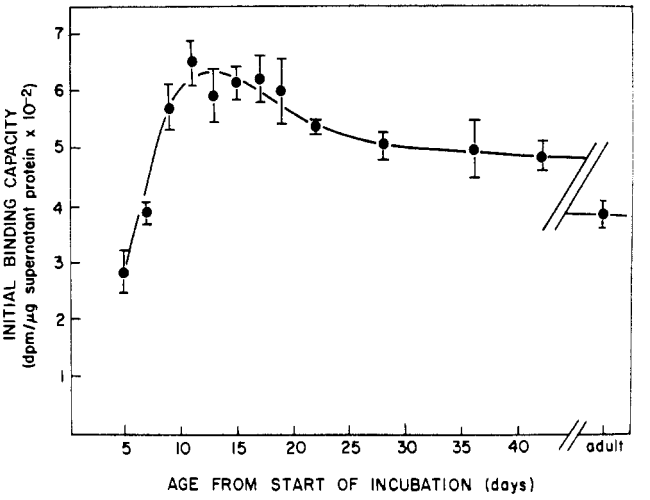

FIGURE 4: Changes in initial colchicine binding capacity in supernatant fractions of sonicated brain of different ages. Initial colchicine binding capacity values were determined from the time-decay assays described in Figure 3.

were migrating in the same zone as microtubule protein. For sodium dodecyl sulfate–polyacrylamide gel electrophoresis, reduced and carboxymethylated samples (25 μl) were applied to the gels in 8 M urea containing 1% sodium dodecyl sulfate. Gels were run for 1 hr at 1 mA/tube, and then for 5 hr at 5 mA/tube. All gels were stained with Fast Green FCF as described elsewhere (Bamburg *et al.*, 1973). Gels were scanned at 640 nm with a Gilford 2000 spectrophotometer equipped with a linear transport system. Typical scans of gel patterns with the sodium dodecyl sulfate and the Tris-glycinate–urea gel electrophoresis systems appear in Figures 1A and B, respectively. The position of the microtubule protein band (arrow) was ascertained by comparison with purified reduced and carboxymethylated chick embryo brain microtubule protein (Bryan and Wilson, 1971). The area under the microtubule protein peak was directly proportional to the amount of microtubule protein applied to the gel (Bamburg *et al.*, 1973).

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951) with standard solutions of either bovine serum albumin or purified chick embryo brain microtubule protein in phosphate–glutamate buffer.

Results

Determination of Neurotubule Protein Concentration in Supernatant Fractions of Sonicated Brain during Development of the Chick. TIME-DECAY COLCHICINE BINDING ASSAY PROCEDURE. The rate of decay of colchicine binding activity is markedly dependent upon the concentration of *active* (colchicine-binding) microtubule protein (Bamburg *et al.*, 1973). The dependence of decay rate on neurotubule protein concentration in supernatant fractions of sonicated 7-day old, 13-day old, and adult brain appears in Figure 2. In all three cases, as the concentration of colchicine-binding protein increased, the decay rate decreased, until a plateau level was reached at a neurotubule protein concentration of approximately 250 μg/ml. Though the general shape of each curve was similar, the plateau half-times were different. The plateau half-times decreased with increasing age, from more than 300

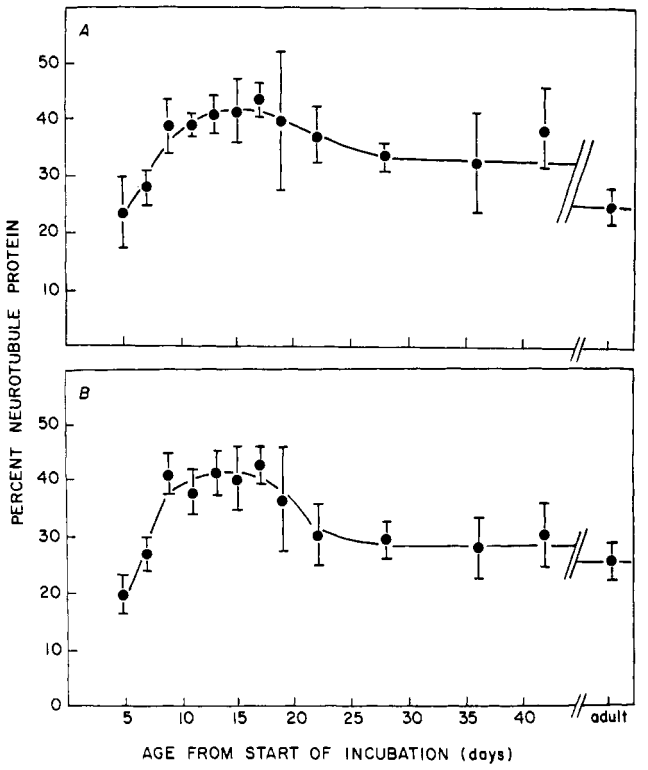

FIGURE 5: Percentage of neurotubule protein in supernatant fractions of sonicated brain of different age chick embryos and adults. Aliquots of the identical supernatant fractions of sonicated brain which were utilized for the time-decay colchicine binding assay (Figures 3 and 4) were applied to sodium dodecyl sulfate gels (A) and Tris-glycinate–urea gels (B). Neurotubule protein content in the supernatant fractions for each age was determined as described under Methods. Error bars represent the variation at the 95% confidence level ("student *t*" test).

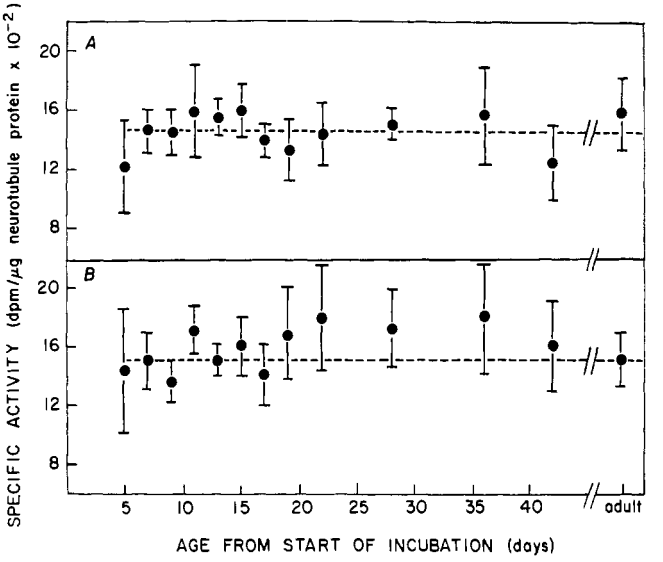

FIGURE 6: Ratio of initial colchicine binding activity to the concentration of neurotubule protein, as determined on Tris-glycinate–urea gels (A) and on sodium dodecyl sulfate gels (B) in supernatant fractions of developing chick brain. The value of initial binding capacity, given in disintegrations per minute of bound colchicine per microgram of total supernatant protein (Figure 4), was divided by the amount of neurotubule protein (micrograms) present in each supernatant fraction (Figure 5).

min at 5–7 days of development, to approximately 200 min in the adult (Figure 3). The developmental pattern of initial colchicine binding capacity in supernatant fractions of chick brain appears in Figure 4. During early embryonic development, initial binding capacity increased sharply between 5 and 11 days of development. Initial binding capacity remained constant until approximately 17 days of development, then it decreased gradually to half the 13-day level in the adult.

QUANTITATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS. The concentration of neurotubule protein in the soluble fraction of chick brain sonicates during development was determined on Tris-glycinate–urea gels, and on sodium dodecyl sulfate gels, utilizing Fast Green FCF stain (see Methods). Results with the two polyacrylamide gel assays were identical (Figure 5). The concentration of neurotubule protein increased sharply from approximately 20% at 5–7 days of embryonic development to more than 40% of the total soluble protein at 9–10 days of development. The neurotubule protein concentration remained constant at 40–42% until approximately 15–17 days of development, then it gradually decreased to a concentration of 25% in the adult.

The pattern of initial colchicine binding capacity (Figure 4) was identical with both quantitative gel electrophoresis patterns (Figure 5). The ratio of initial colchicine binding capacity to concentration of neurotubule protein concentration as determined on Tris-glycine–urea (Figure 6A) and sodium dodecyl sulfate gels (Figure 6B) was the same, and remained constant between 5 days of embryonic development and the adult. Thus, the use of a time-decay colchicine binding assay provided an accurate reflection of the concentrations of neurotubule protein in the supernatant brain fractions. Moreover, the results suggest that the affinity of neurotubule protein for colchicine does not change during development of the chick brain.

Determination of Neurotubule Protein Concentration in Particulate Fractions of Sonicated Brain during Development of the Chick. The percentage of total protein which remained in the particulate fraction of chick brain after sonication increased from 42% at 7 days of development to 63% in the adult (Table I). A similar distribution of protein between the soluble and particulate fractions during development has been reported for mouse brain (Grossfeld, 1968). Neurotubule protein is strongly associated with the particulate fraction of mouse brain, and cannot be solubilized by conventional methods without loss of colchicine binding activity (Feit and Barondes, 1970).

The quantity of neurotubule protein remaining in the particulate fraction of chick brain after sonication was determined by the time-decay colchicine binding procedure. For this study, it was necessary to ensure that the colchicine binding activity detected in the particulate brain fraction was due to the binding of colchicine to microtubule protein, and not to another possible colchicine-binding ligand. Identical with results obtained with soluble microtubule protein (Wilson, 1970; Wilson, unpublished data), colchicine binding activity in the particulate brain fraction from a 32-day old chick was prevented by the addition of podophyllotoxin, or incubation at 0° (Table II). Particulate colchicine binding activity decayed in an apparent first-order manner, and the rate of decay at a particular state of development was similar to the decay rate observed for soluble neurotubule protein of the same age (Figure 7.) The addition of vincristine sulfate markedly decreased the rate of decay, as seen by the twofold increase in bound colchicine after 2 hr of incubation at 37° (Table II).

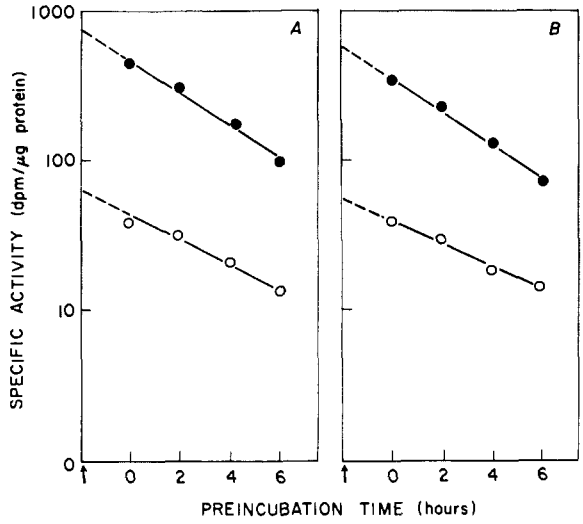

FIGURE 7: Comparison of colchicine binding activity decay rates in supernatant and particulate brain fractions of the same age. Time decay colchicine binding assays were carried out in the supernatant and particulate fractions of a 17-day old (A) and an adult (B) sonicated brain (see Methods). The half-life values for the soluble and particulate fractions of the 17-day old brain were 163 and 226 min, respectively. For the adult brain, values were 160 and 240 min, respectively: (●) supernatant fraction; (○) particulate fraction.

TABLE I: Distribution of Total Protein between the Supernatant and Particulate Fractions of Sonicated Brain during Development of the Chick.[a]

Age from Start of Incubation (Days)	No. of Determinations	% Protein in the Particulate (30,000*g*) Fraction
7	2	42
9	6	46 ± 3.5
11	4	42 ± 2
13	5	44 ± 3.5
15	2	44
17	6	50 ± 3
19	4	54 ± 8
22 (hatched)	2	52
29	1	58
36	2	61
42	2	60
1 year	6	63 ± 1.5

[a] Brains were removed from embryos or chicks, sonicated, and separated into particulate and soluble fractions as described under Methods.

Excess lumicolchicine, an isomer of colchicine which does not bind microtubule protein (Wilson and Friedkin, 1967; Wilson, 1970), did not affect the binding of colchicine.[1]

Since the particulate and soluble neurotubule proteins lost the ability to bind colchicine with similar rates, it was unnecessary to carry out complete time-decay colchicine binding experiments for the determination of *initial-colchicine binding capacity* in the particulate brain fractions. Rather, single point (2 hr, 37°) colchicine binding assays were carried out for each age, and the binding value were corrected for decay utilizing the decay rate of soluble neurotubule protein from the same age. The quantity of neurotubule protein was then calculated from the initial colchicine binding capacity values according to the following relationship: concentration of neurotububle protein in particulate fraction (micrograms/milliliter) = (IBC(particulate)/IBC(soluble))/concentration of neurotubule protein in soluble fraction (micrograms/milliliter), where IBC is the initial colchicine binding capacity given in disintegrations per minute of bound colchicine per microgram of total protein in each fraction. The above relationship assumes that the binding affinity for colchicine of particulate neurotubule protein is the same as that of soluble neurotubule protein (see Discussion). The concentration of neurotubule protein in the particulate fractions of brain during development in the chick appears in Table III, along with the concentration of neurotubule protein in the supernatant brain fractions. No neurotubule protein was associated with the particulate fraction of brain prior to 13 days of development. The concentration of neurotubule protein then increased to a maximum concentration of 3.2% of total brain protein (23% of the neurotubule protein concentration found in the supernatant fraction) at 28 days of development. The maximum concentration of neurotubule protein, as a percentage of total brain protein, was 23–24%, observed between 9 and 17 days of development. The neurotubule protein concentration in adult chick brain was approximately 11–12% (Table III).

Discussion

Approximately one-fourth of the total protein in 11–17-day old embryonic chick brain is neurotubule protein. Although the highest concentrations exist in embryonic brain, neurotubule protein also accounts for a substantial proportion of total brain protein in the adult chick (11–12%). Considerable change occurs in the concentrations of neurotubule protein during development. A very sharp increase in neurotubule protein concentration takes place between 5 days of development (the youngest age examined) and 11 days of development *in ovo*. A plateau level appears to be maintained between 11 and 17 days of development, then the neurotubule protein concentration decreases gradually to the adult level.

A second change in neurotubule protein appears to take place during development, which is reflected by the differences in colchicine-binding activity decay rates at different ages (Figure 3). The half-life for loss of colchicine binding activity is longest during the period of greatest increase in brain neurotubule protein concentration (5–9 days *in ovo*). The half-life then decreases rapidly as development continues. Since these experiments were carried out in crude supernatant extracts of brain, it is possible that the changes in the decay rates may simply reflect changes in the concentration of some factor in solution which alters the rate of decay. Although we cannot yet completely rule out the possibility that external factors are responsible for the increase in decay rate during development,

[1] Considerable nonspecific colchicine binding activity was found at colchicine concentrations greater than 1×10^{-4} M, in both the supernatant and particulate fractions of brain. Interestingly, at low concentrations (2×10^{-6} M), tritium labeled lumicolchicine bound to the particulate fraction of brain. As expected, lumicolchicine was not binding to particulate neurotubule protein since excess colchicine or podophyllotoxin did not affect the lumicolchicine binding activity. Moreover, the binding of lumicolchicine was not temperature dependent.

TABLE II: Specificity of Colchicine Binding Activity in Particulate Fractions of 32-Day Old Chick Brain Sonicates.[a]

[*acetyl*-^{3}H]Colchicine (2.3×10^{-6} M) at	Sp Act. (dpm/μg of Protein)	% of Control Binding Act.
37° (control)	39	100
0°	3	8
37° plus podophyllotoxin (1.65×10^{-5} M)	3	8
37° plus vincristine sulfate (3.1×10^{-4} M)	79	202
37° plus lumicolchicine (5×10^{-6} M)	39	100

[a] A whole brain sonicate from a 32-day old chick was prepared and aliquots (0.5 ml) were incubated with labeled colchicine for 2 hr at 37°. Colchicine binding activity in the particulate material in the presence or absence of other chemical effectors was determined as described under Methods. Specific activity = disintegrations per minute of bound colchicine per microgram of total particulate protein.

TABLE III: Concentration of Neurotubule Protein in Developing Chick Brain.[a]

Age (Days from Start of Incubation)	Neurotubule Protein, % of Total Brain Protein: Supernatant Fraction	Particulate Fraction	Total (Soluble plus Particulate)
5	10.5 ± 2.6	0	10.5
7	15.7 ± 2.9	0	15.7
9	22.5 ± 3.5	0.5	23.0
11	22.1 ± 3.1		
13	23.2 ± 3.6	0.9	24.1
15	22.8 ± 4.7		
17	21.5 ± 3.0	1.8	23.3
22 (hatched)	15.1 ± 3 8		
28	14.1 ± 2.8	3.2	17.3
200	9.4 ± 1.5	2.2	11.6

[a] The concentrations of neurotubule protein in particulate fractions of brain were determined as described in the text. Values for the concentrations of neurotubule protein in the supernatant fractions of brain were those obtained by Tris-glycinate–urea gel electrophoresis (Figure 5B).

two lines of evidence make it very unlikely. First, the addition of an aged brain extract which no longer possessed any colchicine binding activity did not affect the decay rate in fresh brain extracts of various ages, even when the aged brain extract was added in a very concentrated form (Bamburg *et al.*, 1973). Second, neurotubule protein purified from chick brain of a given age loses colchicine binding activity with the same rate of decay at high protein concentrations as does neurotubule protein in crude extracts from embryos of the same age (Bamburg, J. R., and Wilson, L., unpublished data). It is unlikely that a contaminant would have been purified along with the neurotubule protein at the same relative concentration which existed in the crude brain supernatant fraction. Therefore, the change in half-life must be related to a change in the neurotubule protein during development. The change could be due to the presence of different populations of neurotubule proteins with slight differences in primary structure. Alternatively, the differences may reside in the presence of varying levels of tightly bound low molecular weight ligands (*e.g.*, GTP, calcium, *cf.* Weisenberg *et al.*, 1968; Weisenberg and Timasheff, 1970; Weisenberg, 1972), or covalently bound ligands (*e.g.*, phosphate, *cf.* Eipper, 1972).

It is very tempting to speculate that the differences in decay rates of colchicine binding activity during development of the brain are a reflection of the presence of microtubule proteins with different functions. One possibility is that during very early development (until 7–9 days) the major proportion of brain microtubule protein is utilized for cell division in the formation of the mitotic spindle. During later stages of brain development, the major proportion of brain microtubule protein is utilized for one or several aspects of axon function. It is worth noting that the period of maximal neuronal growth and synaptogenesis in the chick embryo coincides with the period of most rapid increase in neurotubule protein concentration (7–13 days of development) (Oppenheim and Foelix, 1972) and moreover, rapid decrease in half-life.

Although some changes do appear to take place in the neurotubule protein during development, it is clear that no change takes place in the quantity of colchicine bound per weight of neurotubule protein. This suggests that the binding constant for colchicine is unaltered during development of the chick brain.

There is considerable interest in the nature of the particulate microtubule protein of brain (Feit and Barondes, 1970; Lagnado *et al.*, 1971). The possibility that neurotubule protein is tightly or even covalently linked to sites on the axon membrane or in the area of the synapse is most intriguing. However, another possibility is that the particulate neurotubule protein arises through a trapping mechanism during sonication or homogenization. The 13-day stage in the embryo, when particulate neurotubule protein concentrations begin to rise, appears to be the stage at which myelinization begins (Oppenheim and Foelix, 1972). Prior to 13 days of development, there is negligible particulate neurotubule protein even though synaptogenesis is almost complete (Oppenheim and Foelix, 1972). Following this period, the increase in neurotubule protein associated with the particulate fraction of sonicated brain approximately parallels the increase in myelinization. The similarity of the colchicine binding properties and decay rates of the soluble and particulate neurotubule proteins suggests that the neurotubule protein associated with the particulate fraction merely results from entrapment of the soluble neurotubule protein in vesicles formed from the myelinated axons. The formation of vesicles containing ribosomes during homogenization of brain has been reported recently (Gambetti *et al.*, 1972).

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Flavine Specificity of Enzyme–Substrate Intermediates in the Bacterial Bioluminescent Reaction. Structural Requirements of the Flavine Side Chain†

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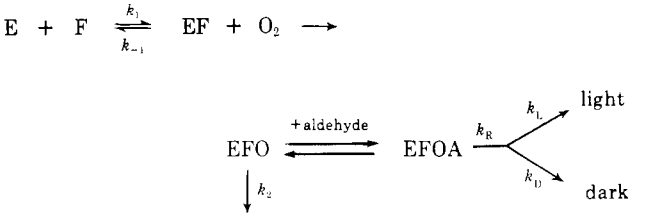
ABSTRACT: The specificity of bacterial luciferase for the side chain of FMN was investigated with the use of flavine analogs. The charge, length, and secondary hydroxyl groups of the flavine side chain affected the stability of the intermediates (enzyme–flavine (EF), enzyme–flavine–oxygen (EFO), and enzyme–flavine–oxygen–aldehyde (EFOA and EFOA′) in the bioluminescent reaction. A negative charge on the side chain was necessary not only for tight binding but good activity and could be supplied either by carboxyl or phosphate groups. This charge had to be located at least 8.4 Å from the N-10 position of the flavine ring. Secondary hydroxyl groups had no effect on flavine binding or activity (initial light intensity I_0) but were implicated in aldehyde binding. The importance of the negative charge in the bioluminescent reaction was further demonstrated by a large stimulation of the activity of neutral flavines by inorganic anions. However, anions had no effect on the binding of neutral flavines. The dissociation constant for inorganic phosphate, measured by stimulation of the activity of the neutral flavines, was the same as that obtained by competitive inhibition with $FMNH_2$, thus demonstrating a specific site for phosphate. The results indicate that the phosphate of FMN binds in a subsite on the enzyme such that it stabilizes the enzyme–flavine intermediates in the bioluminescent reaction.

Bacterial luciferase catalyzes the emission of light "*in vitro*" in the presence of reduced flavine mononucleotide ($FMNH_2$),[1] $^1O_2$, and a long-chain aliphatic aldehyde (Hastings, 1968). A number of intermediates in the bioluminescent reaction have been demonstrated by Hastings and Gibson (1963). A scheme incorporating such intermediates is



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[1] Abbreviations used are: FMN and $FMNH_2$, oxidized and reduced forms of flavine mononucleotide; isoFMN, 6,7-dimethyl-10-(1′-D-ribityl 5′-phosphate)isoalloxazine; 3-MeFMN, 3,7,8-trimethyl-10-(1′-D-ribityl 5′-phosphate)isoalloxazine; EF, enzyme–flavine; EFO, enzyme–flavine–oxygen; EFOA and EFOA′, enzyme–flavine–oxygen–aldehyde. Trivial names were used for compounds of the following series: ω-carboxyethyl-, ω-carboxypentylflavine, 7,8-dimethyl-10-(ω-carboxyalkyl)isoalloxazines; ω-hydroxyethyl-, ω-hydroxypropyl-, ω-hydroxybutyl-, ω-hydroxypentyl-, ω-hydroxyhexylflavine, 7,8-dimethyl-10-(ω-hydroxyalkyl)isoalloxazines; ω-phosphopropyl-, ω-phosphobutyl-, ω-phosphopentyl-, ω-phosphohexylflavine, 7,8-dimethyl-10-(ω-phosphoalkyl)isoalloxazines. For simplicity, flavines are generally referred to in the text as the oxidized forms, but it must be emphasized that it is the reduced forms that are active with luciferase.