Accumulation of an organ-specific protein during development of the embryonic chick brain

By HARVEY P. FRIEDMAN\(^1\) and BYRON S. WENGER\(^2\)

*From the Department of Biology, University of Missouri at St Louis and the Department of Biochemistry, University of Kansas, Lawrence*

In an earlier investigation by the present authors (Friedman & Wenger, 1965) the quantitative development of brain-specific antigens in chick embryos was studied using antisera against whole brain homogenates. The antisera employed were made brain specific by absorption with homogenates of various chicken tissues. Based on this study, the period between 5½ and 12 days of incubation, stages 28 and 38 of the Hamburger & Hamilton (1951) stage series, was considered to be critical for neural differentiation.

Previous studies of the development of brain-specific antigens had generally employed qualitative precipitin tests or specific inhibition of differentiation by antisera and did not measure the accumulation patterns of these antigens (Schechtman, 1948; Ebert, 1950; Flickinger, 1958; McCallion & Langman, 1964).

An opportunity to study the differentiation of the brain with respect to a single, well-characterized tissue-specific protein was presented by the isolation from beef brain of a unique protein termed 'S-100' because of its solubility in 100 % saturated ammonium sulfate (Moore & McGregor, 1965; Moore, 1965). This highly acidic protein becomes immunogenic when complexed with methylated bovine serum albumin (Levine & Moore, 1965) thereby providing the means for such a study.

It had been shown that S-100 protein exhibited relatively little serological variation among a wide range of vertebrate species (Levine & Moore, 1965; Kessler, Levine & Fasman, 1968). This permitted use of antisera against bovine brain S-100 in an assay for the presence of a similar protein in embryonic chicken brain.

The present study, employing antisera against a single brain-specific protein and a highly sensitive, quantitative microcomplement-fixation test, confirms and extends earlier results obtained with absorbed antisera against crude homogenates.

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\(^1\) Author's address: Department of Biology, University of Missouri at St Louis, St Louis, Missouri 63121, U.S.A.

\(^2\) Author's address: Department of Anatomy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
MATERIALS AND METHODS

New Zealand White rabbits weighing 2–3 kg were immunized with a total of 2 mg each of purified beef S-100 protein, kindly supplied by Dr Blake Moore. The protein was complexed with methylated bovine serum albumin (MBSA) according to the procedure of Plescia, Braun & Palczuk (1964) and emulsified with an equal volume of incomplete Freund’s adjuvant before injection into the hind foot pads of the rabbits. Injections were given 3 times weekly for 3 weeks, the first two injections consisting of 125 μg S-100 complexed with MBSA and all subsequent injections containing 250 μg S-100 in the complex.

The rabbits were bledd from the marginal ear vein prior to the 4th and 7th injections and by cardiac puncture 8 to 10 days after the last injection.

Microcomplement-fixation tests were modifications of the quantitative methods of Wasserman & Levine (1961) and Moore & Perez (1966). All stock reagents were diluted for the tests in isotonic N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES, Calbiochem) buffer pH 7.4, containing 0.01 M-TES, 1.5 x 10^-4 M-CaCl2, 0.1 % human serum albumin and 0.14 M-NaCl.

For a highly reproducible source of complement, lyophilized guinea-pig serum (Courtland Laboratories) was reconstituted to its original volume with cold triple distilled water and stored as a stock solution at −80 °C in aliquots of ca. 30 μl. The complement used in each test had been frozen and thawed only once. Rabbit anti-sheep erythrocyte serum (Colorado Serum Co.) was diluted 1 : 3 with cold triple distilled water and stored as stock in small aliquots at −80 °C.

Sheep erythrocytes (Colorado Serum Company), in either Kolmer’s saline or preserved in Alsever’s solution, were washed in buffer and spectrophotometrically standardized to contain 2 x 10^9 cells/ml by the method of Kabat & Mayer (1961). The cells were then sensitized and diluted so that each tube in the complement-fixation test would receive 5 x 10^6 sensitized cells.

The tests were performed in siliconized tubes (6 x 50 mm) prepared by rinsing in ‘Siliclad’ (Clay-Adams, Inc.) and oven-drying. Each tube received 4 μl of antigen followed by 40 μl of a complement-antiserum solution containing enough complement to give ca. 50 % hemolysis. Antisera were inactivated at 56 °C for 30 min before use. Control tubes containing buffer, antigen and complement as well as controls containing buffer, antiserum, and complement were always included. All operations were performed in an ice bath using cold reagents; the contents of each tube were mixed after each addition. The tubes were then incubated for 18 h at 5 °C.

Following incubation, 5 x 10^6 sensitized erythrocytes in a volume of 40 μl was added to each tube. The hemolytic reaction was allowed to proceed for 60 min at 37.5 °C in a shaking water bath. The tubes were then placed in an ice bath and 400 μl of cold buffer added to each to stop the reaction and dilute the
Brain-specific protein

contents for spectrophotometric determination of hemolysis. Released hemoglobin was measured at 413 mμ after centrifugation at 800 × g in a refrigerated centrifuge to sediment unlysed cells.

Double diffusion in agar was performed according to the method of Ouchterlony (1958). Antisera diluted 1:2 with 0.85 % saline were tested against varying concentrations of antigen.

Embryonic test antigens were prepared from New Hampshire Red eggs incubated at 38–39 °C in a forced draft incubator. Embryos were removed at various incubation times, floated in saline to remove adhering yolk and staged according to the Hamburger & Hamilton (1951) stage series. Brains were removed under a stereoscopic microscope using iridectomy scissors and watchmaker's forceps. Skin and mesenchyme were completely removed from older embryonic brains, but this was not always possible for early embryos. Samples were either homogenized immediately in triple distilled water for stock homogenates or frozen on dry ice for later homogenization. Protein determinations were performed on each homogenate using the method of Lowry, Rosebrough, Farr & Randall (1951). Tissue could not be homogenized directly in the TES buffer since the latter was found to interfere with the protein determinations. In a few cases, the sample brains were divided into two equal portions, one homogenized in water for protein determinations and one in TES buffer for complement-fixation.

RESULTS

The antisera obtained showed a single precipitation band when tested in Ouchterlony double diffusion plates against S-100 protein at concentrations of 100 and 200 μg/ml (Fig. 1). No precipitation was obtained with BSA, saline, or stage 34 (8-day) embryonic brain homogenate in the antigen wells. A slight reaction with 7-day post-hatching chick brain homogenate was questionable, being possibly due to fusion of the bands formed by S-100 placed in wells on either side of it.

When tested by the micro procedure used, the antisera fixed complement with as little as 1.25 × 10⁻¹¹ g of the homologous beef S-100 protein (Fig. 2). Cross-reactivity between the anti-beef protein sera and homogenates of brain of adult New Hampshire Red chickens confirmed the presence in chickens of a protein similar to bovine S-100 as noted by Levine & Moore (1965). The reactions of the anti-beef protein sera with chicken brain homogenates were more variable and less sensitive than those with homologous antigen. A curve exhibiting inhibition in the zone of antigen excess was obtained (Fig. 3). Fixation occurred with as little as 1 × 10⁻⁶ g of total brain protein, reached a maximum at about 6 × 10⁻⁶ g and declined at higher concentrations.

The anti-beef S-100 sera were used in microcomplement-fixation tests against homogenates of embryonic chick brains of various developmental stages. Since the absolute amounts of S-100 protein in the homogenates were not known,
Fig. 1. Photograph of agar-diffusion plate showing precipitin lines formed between purified ‘S-100’ and anti-‘S-100’ serum. Antiserum in central well. Key to antigens in peripheral wells:  bsa = bovine serum albumin;  s = saline;  1 = 100 µg/ml S-100;  2 = 200 µg/ml S-100;  34 = stage 34 (8-day) embryonic chick brain;  7 = 7-day post-hatching chick brain.

Fig. 2. Complement-fixation by purified bovine ‘S-100’ protein and rabbit antiserum against bovine ‘S-100’.

Fig. 3. Complement-fixation by adult chicken brain homogenate and rabbit antiserum against bovine ‘S-100’. Note inhibition in zone of antigen excess.
results were related to total protein (Fig. 4) and plotted as total protein against per cent of maximum fixation.

Homogenates of brains at stage 24 (4 days of incubation) reacted variably and only at a total protein content greater than 0·4 μg. 12 different brains were tested, of which 7 fixed complement. Stage 26 brain homogenates (4½ days of incubation) reacted more consistently and gave reactions with as little as 0·12 μg total protein. By stage 29 (6 days of incubation) 0·028 μg of protein was sufficient for fixation. From stages 29 to 39, reactivity gradually rose with reactions occurring with 0·016 μg of protein at stages 39 and 42 (13 and 16 days of incubation respectively). With homogenates of adult brain complement-fixation occurred with 0·008–0·02 μg of protein.

DISCUSSION

These results show rapid accumulation of a protein antigenically similar to bovine S-100 in brains of chick embryos between stages 24 and 29 (4–6 days of incubation) and a gradual rise to adult concentrations by stage 39 (13 days). The microcomplement-fixation test employing antiserum to bovine S-100 was capable of measuring ca. 1 x 10^{-11} g of purified bovine S-100. In cross-reaction with adult chicken brain homogenates, 1 x 10^{-8} g of total protein gave fixation.

Moore, Perez & Gehring (1968) have found about 200 μg of S-100/g of wet bovine brain. Assuming a content of 100 g protein/kg fresh bovine brain (Long, 1961), we calculate a concentration of S-100 of 2 x 10^{-3} g/g of protein. Our
assay detected $1.25 \times 10^{-11}$ g of purified S-100 which is equivalent to ca. $6 \times 10^{-8}$ g of total beef brain protein. Using chicken brain homogenates, fixation occurred with $1 \times 10^{-6}$ g of total brain protein. Thus 170 times as much chicken brain protein is needed to fix complement as is needed with beef brain protein. The difference may be accounted for either by differences in relative concentrations of S-100 in beef and chicken brain or by differences in structure of the protein or both. In any case our assay measures a chicken protein similar to beef S-100 in amounts of the order of magnitude of $10^{-8}$ g or less.

The results of this investigation are generally similar to those of an earlier study of the development of brain-specific antigens using antisera prepared against crude brain homogenates (Friedman & Wenger, 1965). This suggests either that antibodies to S-100 constituted a major portion of those present in our previous sera or that a number of different proteins display similar patterns of accumulation during this period of rapid differentiation. We previously reported the appearance of brain specific proteins between 5½ and 6½ days of incubation (stages 28, 30). Our present data showing the appearance of the S-100 antigen somewhat earlier, e.g. 4–6 days (stages 24, 29) may be due to the greater sensitivity of the microcomplement-fixation test presently used. The general accumulation patterns observed in both studies, however, are quite similar (Fig. 5). Whether one considers the two curves in Fig. 5 to represent the

![Graph showing accumulation of brain antigens during development.](image-url)
same antigen or different antigens with similar accumulation patterns, the present data provide additional support for the hypothesis of Burt & Wenger (1961) that an increase in glucose-6-phosphate dehydrogenase (G6PDH) activity occurring between 4½ and 6 days and persisting through 8 days of incubation is associated with formation of the RNA involved in the synthesis of specific proteins of the differentiating nervous system. Both of our antigen accumulation curves follow a pattern consistent with this hypothesis.

The rapid accumulation of the protein similar to S-100 occurs just after a transient peak in mitotic index (Kallen, 1955) and coincides with a spurt in (G6PDH) activity. Such a temporal sequence would be expected if G6PDH were active in providing ribose, through the pentose cycle, for synthesis of RNA (Horecker & Hiatt, 1958).

Additional support for the hypothesis has been provided by the recent demonstration, using the technique of DNA hybridization, of the appearance and accumulation of a new species of 6–16S RNA in the brain of the embryonic chick between 4 and 7 days of incubation (Johnson, Newmark & Wenger, 1969). This same fraction of heterogeneous RNA has further been shown to provide the synthesis in a cell-free system of an antigen immunochemically identical to S-100 protein (Johnson, 1969).

**SUMMARY**

1. In an earlier study the development of brain-specific antigens was demonstrated using anti-whole brain sera absorbed with homogenates of other tissues. Moore's isolation of a protein unique to nervous system allowed us to study differentiation of chick brain with respect to a single, well characterized, tissue-specific protein.

2. Rabbits were immunized with a total of 2 mg each of purified beef 'S-100' protein-methylated BSA complex in incomplete Freund's adjuvant. Antisera showed single precipitation bands in gel diffusion against homologous antigen. As little as $1.25 \times 10^{-11}$ g of 'S-100' fixed complement with these antisera. Antisera against beef 'S-100' protein showed cross-reactivity with adult New Hampshire Red chicken brain homogenates, indicating the presence of a similar protein in this breed of chicken. Reactions of anti-beef 'S-100' sera with chicken brain extracts were more variable and less sensitive than with homologous antigen.

3. Anti-beef 'S-100' sera were used in microcomplement-fixation tests against homogenates of chicken brains of various developmental stages. Hamburger–Hamilton stage 24 brain homogenates react only variably and only at total protein content greater than 0.4 $\mu$g. Stage 26 brains react more consistently and at protein content as low as 0.12 $\mu$g. At stage 29, 0.028 $\mu$g protein yields positive complement-fixation reactions. From stages 29 to 39, reactivity gradually rises with reactions occurring with 0.016 $\mu$g protein at stages 39 and 42. Adult brain reacts at protein content ranging from 0.008 to 0.020 $\mu$g.
4. These results show rapid accumulation of a protein antigenically similar to 'S-100' between stages 24 and 29 and a gradual rise to adult concentration by stage 39. They suggest the presence of antibodies against 'S-100' in previously used antisera prepared against crude brain homogenates.

RÉSUMÉ

Accumulation d'une protéine spécifique d'organe pendant le développement du cerveau embryonnaire de poulet

1. Dans une étude précédente, le développement d'antigènes spécifiques de cerveau a été démontré en utilisant des immunséums anti-ceseveau total, absorbés par des homogénats d'autres tissus. L'isolement, par Moore, d'une protéine spéciale au système nerveux nous a permis d'étudier la différenciation du cerveau de poulet, en ne considérant qu'une seule protéine tissulaire spécifique bien caractérisée.

2. Des lapins ont été immunisés avec une quantité individuelle totale de 2 mg de protéine 'S 100' de béuf purifiée, BSA, méthylée, associée à de l'adjuvant incomplet de Freund. Les immunséums ont montré une seule bande de précipitation par diffusion en gélose, contre l'antigène homologue. Une quantité aussi faible que $1,25 \times 10^{-11}$ g de 'S 100' suffit à fixer le complément avec ces antiséums. Les antiséums anti-'S 100' de béuf ont montré une réactivité croisée avec les homogénats de cerveau de poulet New Hampshire Red, ce qui indique la présence d'une protéine semblable dans cette souche de poulet. Les réactions de l'anti 'S 100' de béuf avec les extraits de cerveau de poulet se sont montrées plus variables et moins sensibles qu'avec l'antigène homologue.

3. Les sérums anti-'S 100' de béuf furent utilisés pour des micro-réactions de fixation du complément par les homogénats de cerveaux de poulet à différents stades de développement. Au stade 24 de la table de développement de Hamburger et Hamilton, les homogénats de cerveau ne réagissent que de façon variable et seulement pour une quantité totale de protéines supérieure à 0,4 µg. Les cerveaux au stade 26 réagissent de façon plus importante et pour une quantité de protéines de 0,12 µg. Au stade 29, 0,028 µg de protéines donnent une réaction de fixation du complément positive. Du stade 29 au stade 39 la réactivité augmente graduellement avec des réactions positives pour 0,016 µg de protéines aux stades 39 et 42. Le cerveau adulte réagit pour des quantités de protéines de l'ordre de 0,008 à 0,020 µg.

4. Ces résultats mettent en évidence une accumulation rapide d'une protéine antigéniquement semblable à la 'S 100' entre les stades 24 et 29 et son augmentation graduelle jusqu'à la concentration de l'adulte, atteinte au stade 39. Ils suggèrent la présence d'anticorps anti-'S 100' dans les immunséums préparés, au cours d'expériences précédentes, contre des homogénats de cerveau entier.
Brain-specific protein

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