Adult brain antigens demonstrated in chick embryos by fractionated antisera

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INTRODUCTION

The appearance, during development, of antigens which react with antisera against specific adult antigens has been considered to reflect the synthesis of similar substances in the embryo (Clayton, 1960). Within the recognized limitations of serological specificities, where the adult antigens are characteristic of a particular organ or tissue, the appearance in the embryo of cross-reacting substances may be taken as an indication of differentiation in that tissue.

The presence in early embryos of antigens serologically identical to those of adult avian brain has been well established (Burke et al., 1944; Schechtman, 1948; Ebert, 1950; Flickinger, 1958). These investigations, while demonstrating the presence of adult antigens at various developmental stages, were generally not quantitative. For the most part they employed qualitative precipitin tests or specific inhibition of differentiation by antisera.

In the present study antigenic activity relative to total protein was determined at different stages of development in order to reveal temporal patterns. These patterns have been compared with concomitant biochemical and morphological patterns. In addition, information was obtained as to some of the chemical properties of the antigens.

MATERIALS AND METHODS

The antisera were produced by hyperimmunization of rabbits with whole brain homogenates from New Hampshire Red chickens of a local stock. Details of preparation and fractionation will be described elsewhere (Friedman, 1964, in preparation). Complement fixation tests were performed according to Kabat & Mayer (1961). 0·2 ml. vol. of antigen, antiserum and guinea-pig serum containing two exact units of complement were mixed and incubated at 37°C for 75 min. All antisera were inactivated at 56°C for 30 min. before use. One

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exact unit of complement is defined as the minimum amount of guinea-pig serum
diluted 1:20 which gives 100 per cent. hemolysis of a 1 per cent. suspension of
sensitized sheep erythrocytes. Following this 0.4 ml. of a 1 per cent. solution of
sensitized sheep erythrocytes was added and the hemolytic reaction allowed to
proceed for 30 min. at 37°C. The extent of hemolysis was judged visually after
centrifugation to sediment unlysed cells. The amount of complement fixed was
rated on a scale of 0 to +4. Zero signified no fixation of complement (complete
hemolysis, no sedimentation of cells) while +4 signified complete fixation of
complement (no hemolysis, absence of hemoglobin in the supernatant). Approp-
riate controls for anticomplementary activity were run. Sera were absorbed to
completion with liver and in some cases with yolk. This was accomplished by
adding lyophilized egg yolk or acetone powder of chicken liver to the serum and
shaking at room temperature for 30 min. The serum was then separated from the
absorbing material and the process repeated until the serum no longer fixed
complement in the presence of the absorbing antigens. These absorbed sera did
not fix complement when tested with a fresh liver homogenate prepared in 10
volumes of pH 7.2 veronal buffered saline.

Chromatography of antisera was performed on diethylaminoethyl (DEAE)
cellulose using stepwise elution with phosphate buffer of decreasing pH and
increasing ionic strength (method of Peterson & Sober, 1956, as used by Benedict
et al., 1962). Antibody activity was found only in the eluates obtained with
0.0175 M phosphate, pH 6.3 and 0.4 M phosphate, pH 4.7. Based upon ultra-
centrifugal and chemical data, the antibodies in these two active fractions were
identified as being of 7S and 19S types respectively.

In order to investigate the possible lipid nature of the adult antigens, brains
were removed from decapitated animals and homogenized in a Waring blender
with 20 vol. of cold chloroform–methanol (2:1). The homogenate was washed
by immersion of the flask in a ten-fold volume of water for 2 days at 4°C., follow-
ing which it was removed from the wash water and the overlying water–methanol
phase removed by suction and discarded (Folch & Lees, 1951).

The chloroform phase of the washed chloroform–methanol extract was evapor-
ated under reduced pressure at 40°C. and the dry residue suspended in saline for
use in complement fixation tests with anti-brain sera. The chloroform–methanol
insoluble residue was taken up in 3 ml. saline per gram of original brain
tissue. Each of these preparations was tested against both 7S and 19S antibody
fractions.

For preparation of embryonic test antigens New Hampshire Red eggs were
incubated in a forced draft incubator at 38–39°C. for 2 to 12 days. Embryos were
removed, floated in saline to remove any 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.
The brains of older embryos were completely freed of skin and mesenchyme but
this was not always possible for the early embryos. In all cases a major portion
of the skin was removed. Embryos younger than stage 18 were not dissected, the entire embryo being homogenized.

Samples were frozen immediately on dry ice and later homogenized in pH 7.2 phosphate buffered saline for testing against adult brain antisera.

Each homogenate was analysed for protein using the method of Lowry et al. (1951) and tested against 7S and 19S antibody fractions as well as against un-fractionated antiserum.

RESULTS

The results obtained with whole antisera are summarized in Table 1. Adult brain antigens were detected in embryonic brains at all stages studied. Tests for specificity of the antisera showed that they cross-reacted with both chicken liver and yolk. Absorption of the antisera with liver did not diminish the anti-brain titer while absorption with yolk lowered it in all cases (Text-fig. 1). The minimum protein concentration of brain homogenates needed for complete fixation of complement at various developmental stages is shown in column 5,

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of brains in sample†</th>
<th>Antiserum number‡</th>
<th>Absorbed with§</th>
<th>Minimum brain protein completely fixing complement, mg./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;18*</td>
<td>14</td>
<td>1, 3, 4</td>
<td>L</td>
<td>0.20</td>
</tr>
<tr>
<td>&lt;18</td>
<td>14</td>
<td>1, 3, 4</td>
<td>L, Y</td>
<td>0.80–1.1</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>1, 3, 5</td>
<td>L</td>
<td>0.090</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>1, 5</td>
<td>L, Y</td>
<td>0.350</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>1</td>
<td>L</td>
<td>0.096</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>1</td>
<td>L, Y</td>
<td>0.377</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>3, 5</td>
<td>L</td>
<td>0.106</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>3, 5</td>
<td>L, Y</td>
<td>0.328</td>
</tr>
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<td>25</td>
<td>3</td>
<td>3, 5</td>
<td>L</td>
<td>0.125</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>3, 5</td>
<td>L, Y</td>
<td>0.536</td>
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<td>28</td>
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<td>28</td>
<td>4</td>
<td>1, 3, 5</td>
<td>L, Y</td>
<td>0.588</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>1, 3, 5</td>
<td>L</td>
<td>0.075</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>1, 3, 5</td>
<td>L, Y</td>
<td>0.153</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>1, 4</td>
<td>L, Y</td>
<td>0.075–0.150</td>
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<tr>
<td>36</td>
<td>6</td>
<td>1, 3</td>
<td>L</td>
<td>0.030</td>
</tr>
<tr>
<td>36</td>
<td>5</td>
<td>1, 3</td>
<td>L, Y</td>
<td>0.075</td>
</tr>
<tr>
<td>38</td>
<td>4</td>
<td>1, 4</td>
<td>L, Y</td>
<td>0.039</td>
</tr>
<tr>
<td>Adult</td>
<td>1</td>
<td>1, 3, 5</td>
<td>L, Y</td>
<td>0.039</td>
</tr>
</tbody>
</table>

* Hamburger-Hamilton Stages 14–16.
† At least two samples were tested for each stage.
‡ Rabbit number; all antisera tested at 1:32 dilution.
§ L = Liver, Y = Yolk.
TEXT-FIG. 1. Accumulation of brain antigens during development. Units of complement fixed per milligram brain protein plotted against incubation age in days; A = adult. Solid line: antisera absorbed with liver and yolk. Dotted line: antisera absorbed with liver only.

Table 1. For stages younger than 30 this value remain relatively constant. The slight drop in antigenicity at stages 25 and 28 is of questionable significance.

In the 24 hr. between stages 28 and 30 there is a sharp increase in antigenicity. Thus the minimum concentration of brain protein needed for complete complement fixation at stage 28 is 0.588 mg./ml. while 0.153 mg./ml. is sufficient at stage 30. Thereafter a steady increase in antigenicity occurs until the adult level has been reached by stage 38.

When the embryonic brain homogenates of the same stages were tested against chromatographic serum fractions containing antibodies identified as 7S and 19S, respectively, the early brain antigens reacted only with 19S antibodies, while by stage 30 antigens have appeared which react with 7S antibodies (Table 2 and Text-fig. 2). Titers obtained with chromatographic fractions were considerably lower than those obtained with whole antisera. This is probably due to the two- to four-fold dilution attendant upon chromatography as well as to incomplete recovery of protein from the columns. Although all the chromatographic eluates were adjusted to equal protein concentrations prior to testing with the brain homogenates, various factors such as inequality in the proportion of the two antibody species recovered, preferential denaturation of one or the
TABLE 2

Complement fixing activity of chromatographic fractions of antisera against brain homogenates from embryos of various stages

(Notes accompanying Table 1 apply to corresponding columns of Table 2)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of brains in sample</th>
<th>Antiserum number</th>
<th>Absorbed with</th>
<th>Fraction</th>
<th>Minimum brain protein completely fixing complement, mg./ml.</th>
<th>Maximum brain protein failing to fix complement, mg./ml.</th>
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<td>&lt;18</td>
<td>16</td>
<td>1, 4</td>
<td>L, Y</td>
<td>7S</td>
<td>3.5-4.0</td>
<td>&gt; 15.0</td>
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<tr>
<td>&lt;18</td>
<td>16</td>
<td>1, 4</td>
<td>L, Y</td>
<td>19S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>1</td>
<td>L, Y</td>
<td>7S</td>
<td></td>
<td>&gt; 12.0</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>1</td>
<td>L, Y</td>
<td>19S</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>1</td>
<td>L, Y</td>
<td>7S</td>
<td></td>
<td>&gt; 12.0</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>1</td>
<td>L, Y</td>
<td>19S</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>1</td>
<td>L</td>
<td>7S</td>
<td></td>
<td>&gt; 15.0</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>1</td>
<td>L</td>
<td>19S</td>
<td>1.60</td>
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<tr>
<td>25</td>
<td>3</td>
<td>3</td>
<td>L</td>
<td>7S</td>
<td></td>
<td>&gt; 15.0</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>3</td>
<td>L</td>
<td>19S</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>3</td>
<td>L, Y</td>
<td>7S</td>
<td></td>
<td>&gt; 15.0</td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>3</td>
<td>L, Y</td>
<td>19S</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td>30</td>
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<td>7S</td>
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<td></td>
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<td>30</td>
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<td>L, Y</td>
<td>19S</td>
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<td>1, 3, 5</td>
<td>L, Y</td>
<td>7S</td>
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<td>1.50</td>
<td></td>
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<td>5</td>
<td>1, 3</td>
<td>L, Y</td>
<td>7S</td>
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<td>36</td>
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<td>1, 3</td>
<td>L, Y</td>
<td>19S</td>
<td>1.50</td>
<td></td>
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<tr>
<td>38</td>
<td>3</td>
<td>1, 4</td>
<td>L, Y</td>
<td>7S</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>3</td>
<td>1, 4</td>
<td>L, Y</td>
<td>19S</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>3</td>
<td>1, 3, 5</td>
<td>L, Y</td>
<td>7S</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>3</td>
<td>1, 3, 5</td>
<td>L, Y</td>
<td>19S</td>
<td>1.70</td>
<td></td>
</tr>
</tbody>
</table>

Other species, and contamination of the fractions by other serum proteins, could bias the results. This would render unwarranted a direct comparison of the two curves in Text-fig. 2. On the other hand, a comparison of the relationship of the 19S curve at stage 28 and the 7S curve at stage 38 (Text-fig. 2) with the curve for whole homogenate at these two stages (Text-fig. 1) would seem to indicate that they are approximately comparable.

Chloroform–methanol extracts of adult brain tested against each of the two chromatographic fractions fixed complement only with the fraction containing the 19S molecules. A saline suspension of the residue remaining after chloroform–methanol extraction reacted only with the 7S antibody fraction.

DISCUSSION

The existence of at least two distinct brain antigens arising at different stages of embryonic development in the chick has been demonstrated in this study. One

is already present at stage 16, the earliest embryos tested, while the other appears between stages 28 and 30.

Burke et al. (1944) found complement fixing antibodies against adult brain antigens only after 260 hr. of incubation. Schechtman (1948), employing precipitin tests with lyophilized embryonic tissue, was able to detect antigens as early as the primitive streak stage which reacted with antibody against brain of near-hatching chick embryos. These antibodies also reacted with a number of heterologous organs. Since our antisera were absorbed with liver and in some cases with yolk and since Schechtman immunized with saline soluble extracts of lyophilized brain it is likely that different antigens were involved in the two studies.

The antigens found in the earliest stages studied in the present investigation reacted only with 19S γ-globulin which has been shown to fix complement with chloroform–methanol extracts of adult brain but not with the residue left after extraction. The residue, on the other hand, fixes complement only with 7S antibody.

The solubility of the first antigen(s) in chloroform–methanol indicates that at least a portion of the antigenic structure is a lipid, probably a lipoprotein or
glycolipoprotein while the chloroform–methanol insolubility of the second antigen suggests a protein nature. If these results obtained with adult antigens can be assumed to apply to embryonic antigens, we can conclude that lipid antigens reacting with 19S antibodies are present early in development and that at about stage 30 a new species of protein antigen appears which is soluble in saline but not in chloroform–methanol (Text-fig. 2).

The accumulation pattern of the latter deserves particular attention. It appears at a time following a final peak in mitotic activity (Källén, 1955), a later transient rise in glucose-6-phosphate dehydrogenase activity (Burt & Wenger, 1961) and a still later transient rise in whole embryo RNA (Novikoff & Potter, 1948). Thus the new antigen appears at essentially the time which Burt & Wenger (1961, p. 89) have considered to be a period of rapid differentiation that “... should involve the synthesis at a relatively rapid rate of new materials, principally protein, in amounts and types characteristic of the particular portion of the central nervous system”. The present work provides evidence for such differentiation in the form of the initial appearance in detectable quantity and the rapid accumulation of a brain specific protein antigen.

Detailed interpretation of these results is difficult in the absence of further information. Certain observations, however, seem deserving of comment.

When speaking of brain antigens the lipoproteins of myelin immediately come to mind. Our lipid antigens may be associated with myelin but, if so, they would appear to represent a minor component which is shared by other tissues. This is evident from the observation that early embryos, well before the onset of myelination, show appreciable activity against the 19S serum fraction and no striking increase in activity occurs with the advent of myelination. At least some of these early lipid antigens are shared with yolk. It is possible that these antigens are all derived from yolk but are inaccessible for reaction when yolk is used to absorb the antiserum.

Even less can be said concerning the chloroform–methanol insoluble antigen, presumably protein, which reacts with the 7S antibody fraction. It is interpreted to be an indicator of neural differentiation and, as such, holds great interest, particularly if it can be identified with a known component of the brain.

Perhaps the best known protein characteristic of the central nervous system would be cholinesterase. This enzyme does, indeed, increase rapidly during the period between stages 28 and 38 in both spinal cord (Wenger, 1951) and brain (Rogers, 1960). In both cases, however, the accumulation of cholinesterase activity begins before stage 28 and continues after stage 38. Assumption of the existence of more than one antigenic species of enzyme would therefore be required in order to identify our protein antigen with cholinesterase. One can only emphasize the importance of further chemical characterization of the antigen, determination of its intracellular localization and elucidation of its rôle in the development of nervous function.
SUMMARY

1. Antisera produced in rabbits against homogenates of adult chicken brains and absorbed with liver and yolk were used to detect, via complement fixation, the appearance of brain antigens during development of the embryonic chick brain.

2. Reacting antigens are found at the earliest stages studied (stages 14–16, ca. 2 days) but remain at a low level (relative to protein) until after stage 28 ($5\frac{1}{2}$ days), when a rapid increase begins, reaching the adult level by stage 38 (12 days).

3. Antisera were fractionated on DEAE cellulose and the anti-brain activity found in two fractions, identified as 7S and 19S respectively.

4. The 19S antibody reacted with a chloroform–methanol extract of adult brain homogenate but not with the residue, while the 7S antibody reacted only with the chloroform–methanol insoluble residue.

5. When tested against brains of embryos at different developmental stages, the 19S antibody reacted at a low and essentially unchanging level from the earliest stages studied to the adult. The 7S antibody gave no reaction with embryonic brain until after stage 28 at which time a reacting antigen was found which reached the adult level by stage 38.

6. Based on the appearance in detectable quantity, and the rapid accumulation, of a brain-specific protein antigen, the period between stages 28 and 38 is considered to be critical for neural differentiation.

RESUME

Mise en évidence d'antigènes cérébraux adultes chez embryons de poulet par les antisérums fractionnés

1. Des antisérums produits chez des lapins après administration d'homo-génats de cerveau de poulets adultes et absorbés par du foie et du jaune d'œuf, ont été utilisés pour détecter, par fixation du complément, l'apparition d'antigènes cérébraux au cours du développement du cerveau de l'embryon de poulet.

2. On trouve des antigènes réagissants aux premiers stades étudiés (st. 14–16, c. à d. 2 jours) mais ils restent à un niveau bas, par rapport aux protéines, jusqu’après le stade 28 (5 jours $\frac{1}{2}$) où commence un accroissement rapide, atteignant le niveau de l'adulte au stade 38 (12 jours).

3. Les antisérums ont été fractionnés sur DEAE-cellulose et l'activité anti-cerveau a été décelée dans deux fractions, identifiées respectivement comme 7S et 19S.

4. L'anticorps 19S a réagi avec un extrait au chloroforme–méthanol d’-homo-génat de cerveau adulte mais pas avec le résidu, tandis que l'anticorps 7S a réagi seulement avec le résidu insoluble dans le chloroforme–méthanol.

5. Quand on l'a éprouvé en présence de cerveaux d'embryons à divers stades de développement, l'anticorps 19S a réagi à un niveau faible et essentiellement
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inchangé depuis les premiers stades étudiés jusqu’à l’adulte. L’anticorps 7S n’a pas donné de réaction avec le cerveau embryonnaire jusqu’après le stade 28, moment auquel on a trouvé un antigène réagissant qui atteint le niveau ultérieur au stade 38.

6. En se basant sur l’apparition en quantité décelable et sur l’accumulation rapide d’un antigène protéique spécifique, on peut considérer que la période comprise entre les stades 28 et 38 est critique pour la différenciation neuronale.

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REFERENCES


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