EFFECT OF NUTRITION ON THE PRODUCTION OF ACUTE
DISSEMINATED ENCEPHALOMYELITIS IN MICE*

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The experimental production of acute disseminated encephalomyelitis in
mice rests upon the uncertain base of a complex empiricism, and consequently
may be categorized as a kind of phenomenology. Historically, the development
of this particular phenomenology has proceeded from a recognition of the con-
sequences of injection into hosts of homologous and heterologous brain material
(1, 2), the properties of adjuvants in enhancing the pathological effects of such
injections (3, 4), the more precise chemical definition of the injected brain
material incitant (5–8), the efficacy of various modes of injection (9), the role
of hypersensitivity (10, 11), with enhancement by Hemophilus pertussis vaccine
(12), and the recognition of the genetic control of host susceptibility with a
consequent Mendelian analysis of resistant and susceptible mouse genotypes
(11, 13). During the course of the genetic analysis evidence appeared which
suggested that still another facet of this expanding phenomenology was open to
experimental attack; namely, the effect of host nutrition on the capacity to
elicit the disorder. It is the purpose of the present paper to report some experi-
ments which indicate that the susceptibility of some known mouse genotypes
to the experimental production of acute disseminated encephalomyelitis can
be manipulated by nutritional means.

Preliminary Observations of a Nutritional Effect on Susceptibility

A genetic analysis of the difference in susceptibility to experimental acute disseminated
encephalomyelitis (ADE) existing between BSVS mice (100 per cent susceptible) and BRVR
mice (100 per cent resistant) has been reported previously (13). This report suggested that
the resistance of the BRVR mice was due to two equipotent genetic factors either of which,
in single dosage, by simple dominance, was sufficient to confer resistance. It was noted at the
time that a sample of 41 mice obtained in the backcross of the F₁ to the susceptible parental
line (BSVS) exhibited an excessive frequency of susceptibles, 37 per cent instead of the pre-

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1955, Extract No. 13-7. The writers express their gratitude to Miss Sophie L. Skadorwa for
her valuable technical assistance.
dicted 25 per cent. A repetition of this test with an additional 40 backcross mice gave essentially the same results, i.e. 14 of the 40 were susceptible, a frequency of 35 per cent. The correspondence between the two independent tests suggested that the discrepancy from theory was possibly a real one and an attempt was made to analyze the situation further.

Analysis of the distribution of the susceptibles within the test samples failed to reveal any correlation with individual litters but did suggest that susceptibles were randomly distributed among all the litters. The hypothesis was next advanced that the observed excess of susceptibles was due to the incomplete penetrance of the genetic resistance factor, specifically in those instances in which the factor was in single dosage. In the instance of an F1 backcross to the recessive parental stock in a two equipotent factor system, theory predicts that among the resultant genotypes the dominant factor (resistance, in this case) will appear in double dosage with a frequency of 25 per cent, in single dosage, 50 per cent, and fail to appear in the remaining 25 per cent. This latter genotype would be the anticipated completely susceptible type. Actually 35.8 per cent susceptibles had appeared in our tests. The excess of 10.8 per cent susceptibles was assumed to have been derived from among the 50 per cent of the backcross mice which had the factor for resistance in single dosage. The resistant phenotypes of this group was thus reduced from 50 to 50 per cent minus 10.8 per cent, or 39.2 per cent. The penetrance of the single dose resistance factor would thus be 39.2/50 X 100, or 78.4 per cent.

Differences in the genotypic background of the backcross mice as compared with the F1 generation do not permit a direct prediction of the frequency of susceptibles to be expected in the F2 mice on the assumption of 78.4 per cent penetrance of the single dose resistance factor. However, on this assumption a simple calculation shows that the frequency of susceptibles to be expected would be 11.65 per cent. As previously reported (13) a test of 231 such F1 animals revealed an incidence of 6.49 per cent. The probability of experiencing a discrepancy of this size by chance alone is greater than 0.05, but less than 0.1. Thus no clear decision can be made from the data either for or against the hypothesis that penetrance of the resistance factor was less than complete.

In a more direct examination of the hypothetical role of penetrance in these phenomena an attempt was made to affect the degree of penetrance through manipulation of the nutritional environment of the mice. The ideal mouse genotype for this examination would be one with the resistance factor in single dosage. Such stocks were not directly available and their preparation a matter of great practical difficulty because of the historical occurrence of the resistance factor in quadruple dosage in the analyzed, resistant parental homozygote. Instead, use was made of the 50 per cent incidence of this single dose genotype among the progeny of the F1 backcross to the BSVS stock. For this purpose male F1 hybrids of the BRVR X BSVS cross were backcrossed to BSVS dams and the weanling progeny reared in individual screen-bottomed cages for a period of 4 weeks on the following diets, with litters and sexes evenly distributed between two groups. Group 1, consisting of 23 mice, received a diet of natural foodstuffs (diet 100) composed of 66 per cent whole ground wheat, 33 per cent whole dried milk, and 1 per cent NaCl. Group 2, consisting of 21 mice, received a so called synthetic diet (diet 191) composed of vitamin-free casein, glucose, salts, fat, and vitamins. (The precise composition of the diets which will be repeated later and the history of the introduction of diets 100 and 191 in mouse nutrition studies have been described in previous publications (14).) The young adult mice thus prepared were then tested for susceptibility to ADE as described before (11). The results are presented in Table I.

The results provided two comparisons: First, the frequency of susceptibles on the natural foodstuff diet (diet 100), 30.4 per cent, was not significantly different from the frequency (35.8 per cent) encountered in the previous tests in which the animals had been on a dietary regimen of commercial fox chow pellets plus supplementary bread and milk (P > 0.7). Secondly, the synthetic diet (191) lowered the frequency of susceptibility. This was only at the
level of suggestion in the comparison of the effects of diet 100 with that of diet 191 (0.1 >
P > 0.05), but upon combining the experience on the two diets composed of "natural" ma-
terials and comparing the frequency of susceptibles (34/102, or 33.3 per cent) with that en-
countered with diet 191, it was evident that the difference was real (P < 0.02) and that the
nutrition of the mouse could indeed influence susceptibility to acute disseminated encephalo-
myelitis.

The observed nutritional effect on the frequency of susceptibility, however,
now raised problems of interpretation and analysis. If the change of the back-
cross mice to a synthetic diet had been accompanied by a decrease in penetrance
of the single dose resistance factor it would have been expected that suscepti-
bility would have been increased from the 33 per cent hitherto experienced on
natural diets. The observed 4.8 per cent was, of course, just the reverse. On
the other hand, if the shift to the synthetic diet had been accompanied by an
increase in the penetrance of the single dose resistance factor, susceptibility
would have been expected to fall only to the theoretical 25 per cent point;

| TABLE I |

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. tested</th>
<th>No. susceptible</th>
<th>Susceptible per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural diet 100</td>
<td>23</td>
<td>7</td>
<td>30.4</td>
</tr>
<tr>
<td>Synthetic diet 191</td>
<td>21</td>
<td>1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

but the observed decrease in susceptibility to 4.8 per cent was greater and
suggested the additional involvement of the homozygous susceptibles furnished
by genetic recombination in the backcross population. The hypothesis was thus
developed that the homozygous susceptible genotype was phenotypically
dependent upon the nutritional elements of a diet of natural foodstuffs and
that the change to the simplified synthetic diet resulted in a diminution of
susceptibility to an insusceptible phenotype. This hypothesis was capable of
direct test using the homozygous susceptible BSVS stock.

Methods and Materials

Mice.—The mouse genotypes used in these nutritional investigations were (a) the com-
pletely susceptible BSVS strain (44 generations of brother-sister inbreeding), (b) the completely
resistant BRVR strain (33 generations of brother-sister inbreeding), and (c) a backcross popu-
lation prepared by crossing BRVR × BSVS and mating the hybrids back to the parental
BSVS stock. For experiments, mice were taken as weanlings, divided by litter and sex into
dietary groups and housed individually in screen-bottomed cages in an air-conditioned room
at 80°F., 50 per cent relative humidity, and with fluorescent light for 12 hours alternating with
complete darkness for 12 hours. After being reared on the experimental diets for 4 weeks the
mice were tested for susceptibility to ADE as described below. During this test period the
mice remained on the experimental diets.
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Diets.—The diets used were of two main types, "natural" and "synthetic." Their composition is listed below. Distilled water was supplied ad libitum.

"Natural" diets:

<table>
<thead>
<tr>
<th>Diet 100 (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground whole wheat</td>
</tr>
<tr>
<td>Dried whole milk</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Diet 1157 (A stock diet for general management)

- Fox Chow (Ralston Purina Co., St. Louis) ad lib.
- Whole wheat bread moistened with whole milk, supplied fresh daily ad lib.

"Synthetic" diets:

<table>
<thead>
<tr>
<th>Diet 191 (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (taboo, vitamin-free)</td>
</tr>
<tr>
<td>Glucose (cerelose)</td>
</tr>
<tr>
<td>Salts W-2</td>
</tr>
<tr>
<td>l-Cystine</td>
</tr>
<tr>
<td>Water-soluble vitamins</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
</tr>
<tr>
<td>Riboflavin</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
</tr>
<tr>
<td>Ca pantothenate</td>
</tr>
<tr>
<td>Niacin</td>
</tr>
<tr>
<td>Choline chloride</td>
</tr>
<tr>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>Inositol</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Using diet 191 as a basal diet permitted the addition of various supplements, displacing cerelose as necessary. These supplements will be described in the text for the appropriate experiment.

Production of ADE in Mice.—The empirical basis of the production of ADE in mice has been subject to a continuing process of revision in recent years (15, 11, 12). During the investigations reported here one such revision was adopted so that, in course, two techniques were employed. For brevity these two methods will be referred to as the SC6 method and the IC2 method and will be described in the chronological order of their development and use.

SC6 Method.—Mouse brain proteolipide A + B (8, 5) was injected subcutaneously in a series of 6 weekly injections. The injection material was prepared in a modified Freund type adjuvant as follows:

1.4 gm. proteolipide A + B (dry weight basis)
50 ml 0.85 per cent NaCl

1 For composition of salts W-2 see reference 14.
20 mg. merthiolate
250 mg. killed, dried tubercle bacilli, type H37Rv
50 ml. soconol (liquid petrolatum, heavy (U. S. P.), distributed by Socony-Vacuum
Oil Co., New York)

These materials were homogenized for 2 minutes in a Waring blender and the white suspen-
sion, having the consistency of light cream, was stored in the ice chest for use. With time the
tubercle bacilli tended to sediment out, but were easily resuspended by shaking. The emulsion
itself has shown no tendency to separate when kept for periods as long as 6 months, with no
detectable deterioration in encephalitogenic activity. The weekly subcutaneous dose per
mouse was 0.3 ml.

All animals were examined daily for manifest, chiefly neurological, signs of ADE already
described in detail (15). If they showed no sign 12 to 15 days after the sixth injection, their
brains were removed and examined for histopathological lesions. Positive signs were also
confirmed by pathological findings. Animals exhibiting no signs, but with characteristic lesions
upon histopathological examination of the central nervous system were counted as positive
reactors. Attention is drawn to the fact that in a varying number of animals, up to 19 per cent,
objective signs of illness may be overlooked, or be absent, yet characteristic lesions can be
observed in the CNS (16). Finally all animals were scored as either positive reactors, or non-
reactors; i.e., as susceptible or resistant.

IC2 Method.—This method, a modification of the SC6 method above, was devised to shorten
the time necessary to produce ADE and to conserve the preparations of the encephalitogenic
proteolipide A + B (12). Briefly, this involved the shift from the subcutaneous to the intra-
cutaneous route, thus enhancing the hypersensitivity reactions of the animals, and for further
similar enhancement, added a prior intraperitoneal injection of H. pertussis vaccine. In this
way, the number of injections were reduced from 6 to 2. Specifically, 4 days before injection
of proteolipide all animals received, intraperitoneally, an injection of 0.1 ml. of H. pertussis
vaccine (Lederle; phase 1, 60,000 million/ml.). The following day the hair was removed from
the backs of the animals with an electric shaver, and on the 4th day the mice were injected,
intracutaneously in the shaved sites, with a total dose of 0.3 ml. of the proteolipide emulsion
described above. The 0.3 ml. was divided into 3 or more blebs through a 1-inch 21 gauge
needle. 10 days later a second, similarly divided dose of proteolipide emulsion was injected.
The animals were subsequently examined daily for signs of ADE. Such signs usually appeared
7 to 10 days following the 2nd injection of proteolipide. On the 21st post injection day the
experiment was terminated. Histopathologic examination of brains of the animals then followed
as in the SC6 method, with similar scoring of positive reactors and non-reactors.

Histopathological Studies.—Approximately 30 sections obtained from various parts of each
examined brain were stained with hematoxylin-eosin and were studied for histopathological
changes characteristic for ADE (15). Briefly, the outstanding changes in the brain and spinal
cord, the chief tissues which were affected, were characteristic and comprised a productive
inflammation of the blood vessels involving all the vascular coats, especially an infiltration
with monocytic, gllal, and compound granular cells and plasmocytes with only few polymuclear
leucocytes. The infiltration spread out perivascularly into the surrounding parenchyma but
the involved areas were surrounded by apparently normal tissue. Gllal infiltrations and pro-
glelation of glial cells were seen and demyelination was not extensive or

2 In an early observation (15) certain signs of respiratory distress, such as “wheezy respi-
ration,” were reported but in later, other and different stocks of mice, this was not observed.
It is inferred that the respiratory sign is not referable to a specific effect of ADE; rather, this
phenomenon was evoked under the stress of manipulation (see text, infra, discussion of Pas-
turella infection).
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commonly noted. Generally, the cerebellum, the white matter, meningeal, and the subependymal areas revealed these changes of an acute disseminated encephalomyelitis (Figs. 1 and 2); for other details, see reference 15.

Statistical Method.—Tests for the significance of experimental results as departures from purely chance events were conducted by estimation of \( x^2 \) from fourfold contingency tables using the correction of Yates for small numbers.

The Effect of Diet on the Phenotypic Susceptibility of BSVS Mice

At the time of testing the hypothetical nutritional dependence of the phenotypic susceptibility of BSVS mice to ADE, consideration was given to possible supplements which might be added to the “synthetic” diet and which might be implicated in the dietary effect already observed in the instance of the backcross mice; i.e., the difference in effect between “natural” and “synthetic” diets. For example, the vitamin content of synthetic diet 191, reflecting the state of knowledge at the time of its original formulation (14), does not contain supplements of biotin, folic acid, or vitamin B\(_12\). The present availability of these vitamins in pure form made it possible to test their supplementary effects directly.

56 weanling BSVS mice were weighed and divided by litter and sex into 4 groups as indicated in Table II. Each mouse was caged individually and the following diets were supplied: Group 1, diet 100, a “natural” diet of whole wheat and whole dried milk; Group 2, diet 191, a “synthetic” diet; Group 3, diet 1000, the basal diet 191 plus supplements, per kilogram, of 1 mg. of biotin, 10 mg. folic acid, and 50 \( \mu \)g. of crystalline vitamin B\(_12\); Group 4, diet 1157, a stock management diet of commercial fox chow pellets plus bread and milk. The animals were reared on these diets for 4 weeks and weighed weekly. At that time the mice were tested for susceptibility to ADE by the SC\(_6\) method. Upon histopathological examination of the brains.
of the animals, the severity of the observed lesions was graded on a crude scale of "negative" (non-reactor), "mild," "moderate," and "marked." Results are presented in Table II.

These results provide verification for the hypothesis that the phenotypic susceptibility to ADE of the BSVS genotype is indeed nutritionally dependent. The 100 per cent susceptibility again observed on the stock management diet of fox chow, bread, and milk (diet 1157) was reduced to 21 per cent by the synthetic diet, diet 191, \( \chi^2 = 19.87, P < 0.001 \). The supplementation of diet 191 with biotin, folic acid, and vitamin B\(_{12}\) resulted in a restoration of the frequency of susceptibility to 70 per cent \( \chi^2 = 4.75, P < 0.05 \). The use of the "natural" diet of whole wheat and whole dried milk, diet 100, resulted in a further increment of susceptibility to the 90 per cent level. These differences in susceptibility, nutritionally arranged, were not reflected in the usual criterion of nutritional adequacy, gain in body weight, for all the diets used were adequate in this sense as indicated by the close correspondence in body weights achieved during the 4 weeks the weanling mice were being reared to young adulthood.

The severity of the neurological lesions produced paralleled, in general, the frequency of susceptibility produced, i.e., when susceptibility was reduced to a frequency of 21 per cent by diet 191 only 4 of 19 mice showed any neurological lesions and these were all scored as "mild"; whereas on stock diet 1157, with a susceptibility frequency of 100 per cent, only 3 of 17 were scored as "mild" and the remaining 14 were scored as "moderate" or "marked."

The Effect on Susceptibility of Single Additions of Biotin, Folic Acid, and Vitamin \textit{B}_{12} to the Synthetic Basal Diet

The finding that a multiple supplement of biotin, folic acid, and vitamin \textit{B}_{12} was capable of partially restoring the reduced susceptibility produced by a synthetic diet to the 100 per cent susceptibility supported by a stock diet now raised several problems. Since the restoration of susceptibility was only partial the possibility arose that this was a reflection of the quantitative amounts supplied in the multiple supplement, and that increasing these amounts might further increase the degree of restoration of susceptibility. A failure in the latter instance would suggest that possibly other qualitative items might be needed further to restore susceptibility to the 100 per cent level. Another consideration was the possibility that the action of the multiple supplement was analyzable in terms of its three members, the effect being attributable to one or two of these three, or to combinations thereof. Consequently, the following experiment was performed to examine the effect of enhancement of susceptibility by (a) increasing tenfold the supplement to diet 191 of biotin, folic acid, and vitamin \textit{B}_{12}, and (b) supplying these three vitamins singly to diet 191 at the new high level. The experiment was expanded to include, as a technical aspect, a test of the extension of the prefeeding period from 4 to 8 weeks as a
means of increasing the effect of the synthetic diet, diet 191, in its failure to support phenotypic susceptibility. In this experiment the test for susceptibility to ADE was shifted from the SC₄ method to the IC₄.

**TABLE III**

*Effect of Single and Combined Supplements of Biotin, Folic Acid, and Vitamin B₁₂ on the Diminished Susceptibility of BSVS Mice Fed a Simplified Synthetic Diet*

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet Description</th>
<th>No. of mice</th>
<th>Avg. weight at test (g)</th>
<th>Pasteurization deaths</th>
<th>Lesion score</th>
<th>Total susceptible per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F♂</td>
<td>F♀</td>
<td></td>
<td>F♂</td>
<td>F♀</td>
</tr>
<tr>
<td>1</td>
<td>Synthetic, basal</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td>22.1</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>Basal plus Bi, biotin, folic acid, (low)</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td>23.3</td>
<td>19.8</td>
</tr>
<tr>
<td>3</td>
<td>Basal plus Bi, Bi, biotin, folic acid, (10 X low)</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td>22.8</td>
<td>19.6</td>
</tr>
<tr>
<td>4</td>
<td>Basal plus folic acid, 10 X (100 mg./kilo)</td>
<td>4</td>
<td>10</td>
<td>9</td>
<td>22.7</td>
<td>18.0</td>
</tr>
<tr>
<td>5</td>
<td>Basal plus Bi, 10 X (500 μg./kilo)</td>
<td>4</td>
<td>20</td>
<td>9</td>
<td>22.8</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Basal plus biotin, 10 X (50 mg. /kilo)</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td>22.8</td>
<td>19.5</td>
</tr>
<tr>
<td>7</td>
<td>Fox chow, bread, milk</td>
<td>4</td>
<td>9</td>
<td>10</td>
<td>24.0</td>
<td>20.3</td>
</tr>
<tr>
<td>8</td>
<td>Synthetic, basal</td>
<td>8</td>
<td>101</td>
<td>102</td>
<td>24.2</td>
<td>19.8</td>
</tr>
<tr>
<td>9</td>
<td>Fox chow, bread, milk</td>
<td>8</td>
<td>101</td>
<td>102</td>
<td>25.4</td>
<td>21.1</td>
</tr>
</tbody>
</table>

*All males in this group by technical error. The 2 survivors failed to show ADE.

§ 5 of each 10 treated with penicillin (see text).

‡ Composed of 5 untreated and 3 treated.

178 weanling BSVS mice were divided, by litter and sex, into nine groups as indicated in Table III. Each mouse was caged individually and the following diets were supplied: Group 1, diet 191, synthetic basal diet; Group 2, diet 1000, basal plus, per kilogram, 1 mg. biotin, 10 mg. folic acid, and 50 μg. crystalline vitamin B₁₂; Group 3, diet 1153, basal plus, per kilogram, 10 mg. biotin, 100 mg. folic acid, and 500 μg. crystalline vitamin B₁₂; Group 4, diet 1154, basal plus 100 mg. folic acid per kilogram; Group 5, diet 1155, basal plus 500 μg. crystalline vitamin B₁₂ per kilogram; Group 6, diet 1156, basal plus 10 mg. biotin per kilogram; Group
7, diet 1157, fox chow plus bread and milk; Group 8, diet 191, synthetic basal; and Group 9, diet 1157, fox chow, bread, and milk. Groups 1 to 7 were fed the diets for a period of 4 weeks, groups 8 and 9 for a period of 8 weeks. All animals were weighed weekly. At the end of the preparative feeding period, with the experimental diets being continued, the mice were tested for susceptibility to ADE by the IC\textsubscript{2} method. The experiment was terminated 21 days after the second intracutaneous injection of proteolipide and histopathological examination of brains followed with lesions being scored as previously. Results are presented in Table III.

A Digression: The Incitement of Fatal Pasteurlosis from Latency by the Manipulations of the IC\textsubscript{2} Method, with Diet, Sex, Latent Infection, and the Inoculation Event as Determinants.

In performing the last experiment an unanticipated event occurred which, when analyzed, illuminated a hazard latent in the dietary analysis when pursued by the IC\textsubscript{2} method of testing for susceptibility to ADE, and since this unexpected experience involved the incitement of an infectious disease in somewhat novel circumstances, it seems worthwhile to report it here. Beginning on the 2nd day after the first intracutaneous injection of proteolipide emulsion a wave of deaths occurred which was confined exclusively to male mice on the simplified synthetic diets. These deaths reached their peak during the 3rd to 7th postinjection day and by the 10th day, when the second injection was scheduled, of 70 males on the synthetic diets 65, or 93 per cent, died. In sharp contrast with this, females remained unaffected. Male mice on the stock diet were markedly less affected with only 2 dead out of 8. That these events were directly connected with the injections in some manner was indicated by the remaining 40 uninjected mice being fed for an additional 4 weeks in this experiment, housed in identical and adjacent cages, and which remained completely unaffected during this period. Several hundred other mice in the same room also remained unaffected.

When death occurred it was preceded by only a short period, of perhaps a day, during which the animal showed lassitude, anorexia, and a ruffled coat. Conjunctival exudate which glued the eyelids together was frequently seen. At autopsy the only noteworthy finding was a mottling of the lungs with frequently frank areas of reddish consolidation. From lungs, spleen, and occasionally from heart's blood a short coccoid bipolar Gram-negative rod-like organism was cultivated which was tentatively identified as a species of Pasteurella. This encounter with Pasteurella was a repetition of previous episodes (17) in other investigations in this laboratory in which deaths due to apparently latent Pasteurella infection have been brought about in mice by the simple operation of an intraperitoneal injection. These previous episodes have had an irregular periodicity, occurring usually in the winter months during a period of several weeks and disappearing spontaneously. Severity of the incited disease has varied from year to year, and appeared only by elicitation by means of some experimental procedure. No outbreaks among the stock animals or among untreated mice on a great variety of diets have ever been observed. On balance it would appear that the outbreak of respiratory disease observed in the present instance, as well as previous episodes, was an instance of infection with Pasteurella pneumotropica as described by Jawetz (18). (See also footnote 2.) Interestingly this author has implicated “sensitivity” in some of the infection phenomena observed by him (19). In the present experiment it would seem that the well known sensitizing effects of H. pertussis vaccine were implicated in the explosive outbreak of pneumonic disease.

When Groups 8 and 9 in the present experiment, after a feeding period of an additional 4 weeks, were tested for susceptibility to ADE by the same IC\textsubscript{2} method an attempt was made to test the practicability of controlling the incidence of Pasteurella infection by intramuscular injection of 2 doses of 0.05 ml. of ticillin (Wyeth) penicillin = 30,000 units, at the time the H. pertussis vaccine was introduced intraperitoneally, and 11 days later. Half the number of
the mice were thus treated, the remainder serving as a control. The results (Table III) indicated that latent *Pasteurella* was still present. All 5 of the untreated males on the synthetic diet of Group 8 died of pasteurellosis following the first injection of proteolipide. Again, the 5 untreated females on the same diet escaped the infection. Of the 5 penicillin-treated males, 3 died and 2 survived, indicating the unsatisfactory nature of the attempted prophylaxis. On the stock diet all mice escaped pasteurellosis. Whether other antibiotics would be more successful has remained untested. From the work of Jawetz and Baker (19) streptomycin would appear a more hopeful choice.

In summing up this digression it would appear that latent *Pasteurella* can be incited to frank and fatal pneumonic disease in BSVS mice subjected to the manipulations of the IC2 method of testing for susceptibility to ADE. The provocation of the latent infection by this means is restricted to mice which (a) are on a synthetic diet, (b) are males, (c) are carriers of *Pasteurella*, and (d) come under the stress of inoculation. Stated in another way it can be said that the natural resistance of mice to a pasteurellosis incited from the latent state is markedly conditioned by (a) sex, with males more susceptible, and (b) by diet, with mice on a synthetic diet being more susceptible.

**Completion of ADE Susceptibility Tests**

In view of the outbreak of pasteurellosis among the male mice of the experiment the completion of the susceptibility test was confined to the females, as indicated in Table III. These results clearly reveal that on diet 191 susceptibility to ADE fell to very low levels (0 to 10 per cent) compared to the 100 per cent susceptibility supported by the stock diet of fox chow, bread, and milk. This dietary effect was elicited by the IC2 method thereby confirming the previous observation (Table II) in which the SC4 method was used. The shift to the IC2 method as a technique in pursuing the nutritional analysis of the dietary effect on susceptibility was thus supported. Extension of the preparatory feeding period from 4 to 8 weeks (Groups 1 and 7 vs. 8 and 9) offered no advantage.

Again in confirmation of previous experience (cf. Table II) supplementation of the basal synthetic diet (diet 191) with biotin, folic acid, and vitamin B12 resulted in a partial (60 per cent) restoration of susceptibility to ADE. Multiplying the supplement tenfold had no additional effect on this partial restoration of susceptibility (Group 2 vs. Group 3, P > 0.4). The nutritional gap between diet 191 and the stock diet is thus only partially explained by the effect of the three vitamin supplement. The suggestion consequently offered itself that still other qualitative nutritional entities remain to be identified as supplements to diet 191 before susceptibility can be restored to the 100 per cent level.

The analysis of the effects of the individual members of the triple vitamin supplement revealed that folic acid alone was as effective in restoring susceptibility as the combination (Group 4 vs. Group 3). The single supplementation
with biotin seemed not to support this restoration of susceptibility to the same degree (Group 6 vs. Group 3) but the small size of the samples left this issue in doubt ($P > 0.3$). The vitamin $B_{12}$ comparison was lost, of course, because of the initial error of failing to provide females in this group, a blunder which proved catastrophic upon the appearance of the "male-pasteurellosis" phenomenon.

In order to cope with the statistical necessities indicated by the preceding experience and to test the feasibility of circumventing the "male-pasteurellosis" problem inherent in these experiments, a second and similar experiment was performed.

In this new experiment, only female mice were used and the size of the group receiving vitamin $B_{12}$ as a supplement was increased to compensate for its loss in the previous trial. As a test of the continued endemicity of *Pasteurella* in the mice, 2 months after the previous experiment, December, 1955, males were included in the groups receiving the synthetic and the stock diet.

Specifically, 80 female weanling BSVS mice were litter-divided into 6 groups of 10 each and one group of 20. To 2 of these 7 groups a further addition was made of 9 and 10 males respectively. All mice were individually caged and fed the experimental diets for a preparatory period of 4 weeks as follows: (The number identifying each diet and the composition of each was the same as in the preceding experiment.) Group 1, 9 males, 10 females, diet 191; Group 2, 10 females, diet 1000; Group 3, 10 females, diet 1153; Group 4, 10 females, diet 1154; Group 5, 20 females, diet 1155; Group 6, 10 females, diet 1156; Group 7, 10 males, 10 females, diet 1157. All animals were weighed weekly. At the end of the 4 week preparative feeding period, with the experimental diets being continued, the mice were tested for susceptibility to ADE by the IC method. The experiment was terminated 21 days after the second intracutaneous injection of proteolipide and histopathological examination of brains followed with lesions being scored as previously. Results are presented in Table IV.

As the results presented in Table IV indicate, the use of female BSVS mice in the susceptibility test was successful in providing test animals which were not lost from the experiment by pasteurellosis. As the control males showed, latent *Pasteurella* infection was still present at the time of the experiment, and once again male mice on a synthetic diet had a high death rate attributable to the incitement of the latent infection to frank and fatal disease. The effect of diet in this infectious disease was again evident for no deaths occurred among the males on the stock diet. Until other, tested methods are available to eliminate this "male-pasteurellosis" problem as encountered in these mice, it is evident that the use of females in the susceptibility test permits a by-pass and furnishes animals adequate for the continuation of the analysis begun here.

To confine comparisons to the females at test: Diet 191 again failed to support the phenotypic susceptibility of BSVS mice (30 per cent) compared to the 100 per cent susceptibility exhibited by mice receiving the stock diet of fox chow, bread and milk ($P < 0.005$). Supplementation of diet 191 with biotin, folic acid, and vitamin $B_{12}$ resulted again in a partial restoration of susceptibility
### TABLE IV

Repeat Test of the Effect of Single and Combined Supplements of Biotin, Folic Acid and Vitamin B12 on the Diminished Susceptibility of BSVS Mice Fed a Simplified Synthetic Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>No. of Mice</th>
<th>4 wks., Av. weight (gm.)</th>
<th>Pasteur- losis Lesion score</th>
<th>Female for test</th>
<th>Total susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>191</td>
<td>9 10</td>
<td>23.0 18.9</td>
<td>0 3 0</td>
<td>3 3 0</td>
<td>30 per cent</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>10 10</td>
<td>18.6</td>
<td>0 10 3</td>
<td>2 3 8</td>
<td>80 per cent</td>
</tr>
<tr>
<td>3</td>
<td>1153</td>
<td>10 10</td>
<td>18.4</td>
<td>0 10 3</td>
<td>2 5 10</td>
<td>100 per cent</td>
</tr>
<tr>
<td>4</td>
<td>1154</td>
<td>10 10</td>
<td>19.1</td>
<td>0 10 2</td>
<td>3 3 8</td>
<td>80 per cent</td>
</tr>
<tr>
<td>5</td>
<td>1155</td>
<td>20 20</td>
<td>18.1</td>
<td>0 20 7</td>
<td>4 5 16</td>
<td>80 per cent</td>
</tr>
<tr>
<td>6</td>
<td>1156</td>
<td>10 10</td>
<td>19.1</td>
<td>0 10 2</td>
<td>2 2 8</td>
<td>80 per cent</td>
</tr>
<tr>
<td>7</td>
<td>1157</td>
<td>10 10</td>
<td>22.1 20.1</td>
<td>0 10 2</td>
<td>4 4 10</td>
<td>100 per cent</td>
</tr>
</tbody>
</table>

### TABLE V

Dietary Effects on Susceptibility to ADE of Female BSVS Mice (Tables III and IV, combined)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Susceptible</th>
<th>Probability that difference from diet 191 group arose by chance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Supplements to synthetic basal</td>
<td>Total tested</td>
</tr>
<tr>
<td>191</td>
<td>None</td>
<td>3/20</td>
</tr>
<tr>
<td>1000</td>
<td>Folic acid, 10 mg./kilo B12, 50γ/kilo</td>
<td>14/20</td>
</tr>
<tr>
<td></td>
<td>Biotin, 1 mg./kilo</td>
<td></td>
</tr>
<tr>
<td>1153</td>
<td>Above, X 10</td>
<td>14/20</td>
</tr>
<tr>
<td>1154</td>
<td>Folic acid, 100 mg./kilo</td>
<td>12/19</td>
</tr>
<tr>
<td>1155</td>
<td>B12, 500 γ/kilo</td>
<td>16/20</td>
</tr>
<tr>
<td>1156</td>
<td>Biotin, 10 mg./kilo</td>
<td>9/20</td>
</tr>
<tr>
<td>1157</td>
<td>Fox chow, bread, milk</td>
<td>20/20</td>
</tr>
</tbody>
</table>
to the 80 per cent level, and multiplying the supplements tenfold resulted in 100 per cent susceptibility. Considering the sample sizes this achievement of complete susceptibility is probably more attributable to chance than to any real effect of increasing supplement concentration. Indeed, in the previous experiment (Table III) the trend was reversed.

When the three vitamins were fed as single supplements it was found that each resulted in a partial restoration of susceptibility and these rises were all equally to the 80 per cent level. Whether this ostensible equivalence is real or is only a chance effect cannot be determined by samples of this size. It can be concluded, however, that each of these vitamin supplements tended to restore the diminished susceptibility arranged by diet 191. That these effects of single vitamin supplements were not of equal magnitude was suggested, but not proved, by combining the results obtained in these last two experiments, as presented in Table V.

The statistical analysis of this total experience indicates that the partial restoration of susceptibility by the three vitamin supplement is a real effect and that increasing the concentration of these supplements tenfold does not result in any further increase in susceptibility. Folic acid and vitamin B₁₂ are apparently equal to each other and to the combined supplement in their effect, while the suggestion arises that biotin has a slighter effect than either of these two vitamins.

**DISCUSSION**

It will be recalled that the experiences recorded here had their beginning in an attempt to round out a genetic analysis of susceptibility to ADE in the mouse. The hypothetical case of a nutritional influence on the degree of penetrance of the dominant allele, resistance to ADE, remains unexamined, and must be deferred until certain primary phenomena have been analyzed and brought under control. These antecedent matters have to do with the nature of susceptibility to ADE, for, as the experiments above have demonstrated, if the phenomenon of susceptibility is itself nutritionally dependent in homozygous BSVS mice then this nutritional dependency must first be analyzed and brought under control before other nutritional manipulations can be entered upon in a study of the consequences of the introduction of genetic factors for resistance. All this is to say that what is first needed is a nutritional benchmark, a definition of the nutritional environment in which the BSVS mouse genotype achieves, as a phenotype, a susceptibility of 100 per cent. That this is an attainable goal is certified by the 100 per cent susceptibility invariably observed when BSVS mice are on the stock regimen of commercial fox chow, bread, and milk. In the present experiments this goal of definition has not been completely achieved for the defined simplified diet with its three additional vitamin supplements reaches a susceptibility of only 70 per cent.
This would suggest that there remain other unidentified nutrients, supplied by the stock regimen, which are needed as further supplements. What their nature might be is not known, but must remain as objects for further investigation.

What is to be made of the role of folic acid, vitamin B₁₂, and, to a lesser extent, biotin in the present experiments? In the first place the basal synthetic diet is fully capable of supporting the growth of the mice and mice thus fed seem to have no overt need of these three vitamins, either because of innate, small but adequate biosynthesis, or by supply from imperfectly purified materials such as the "vitamin-free" casein, or by intestinal synthesis due to the bacterial flora. But whatever may be the case it is clear that the pathological events set in train by the attempt to produce ADE pose an additional and crucial requirement in order that the lesion which we seek to incite can be brought into being. For this end the BSVS mouse needs dietary supplements, of which we have been able to identify three. The identification of these three is, in a sense, an historical accident, for when, in 1940, the basal synthetic diet was formulated for use in this laboratory (14) these three items were not available for inclusion. Their inclusion here as pure chemical entities to be tested was a direct consequence of the historical development of the science of nutrition in the intervening years. Indeed, folic acid, biotin, and vitamin B₁₂ may have no unique connection with susceptibility to ADE but, as may well turn out, other vitamins and other nutrients now included in the basal synthetic diet may be similarly necessary for the susceptibility of BSVS mice to ADE. This possibility is now under examination.

Returning to the information now directly in hand it is interesting that folic acid and vitamin B₁₂ are found once again in close association, so close, indeed, as to be mutually replaceable. It is tempting to speculate that the dependency of susceptibility to ADE on folic acid or vitamin B₁₂ rests, at the metabolic level, on the well known participation of these vitamins in the formation and transfer of one-carbon intermediates, and in the synthesis of nucleic acids. This may, however, be somewhat extravagant; rather more justifiable, seemingly, is the more direct connection which is indicated in the recent nutritional investigations on the problem of antibody formation. This connection can now be elaborated as follows:

There are significant indications (20) that acute disseminated encephalomyelitis can perhaps be regarded as a specific manifestation of hypersensitivity. In this view brain tissue or proteolipide as incitants of ADE are regarded as antigens in the susceptible host as an antibody-forming system. The pathological lesions of ADE in the CNS are hence the sequelae of the antigen-antibody reaction. It follows that to be susceptible the host must be an antibody producer. Now, mainly through the work of Axelrod and Pruzansky (21), it has been shown that the fabrication of antibodies is quantitatively dependent on
certain vitamins in the nutritional environment. Specific deficiency results in impaired antibody production, and it has been shown that the various vitamins range from trivial to pronounced effects in this connection. Further, any single vitamin, which may have a pronounced effect in the antibody response to one antigen may have a relatively trivial effect in the response to a second antigen. It should occasion no surprise, therefore, that in the present experiments two vitamins have emerged as important supporters of susceptibility to ADE and a third vitamin be less effective in this connection. The harmony of these results with the more generalized analysis of Axelrod and Pruzansky on antibody production as influenced by nutrition can be regarded as one more piece of evidence in support of antigen-antibody, or hypersensitivity, as the mechanism of the pathological process in ADE.

**Summary**

The susceptibility of homozygous BSVS mice to acute disseminated encephalomyelitis (ADE) has been found to be nutritionally dependent. On a laboratory stock regimen of commercial fox chow pellets, whole wheat bread, and milk this genotype is 100 per cent susceptible to the disease. On a "synthetic" diet, containing a minimal list of vitamins adequate for growth and maintenance, susceptibility was found to be reduced to 15 per cent. Supplementation of the "synthetic" diet with biotin, folic acid, and vitamin B₁₂ restored susceptibility to a frequency of 70 per cent. Increasing the supplements tenfold had no further effect in restoring susceptibility frequencies to the 100 per cent level.

In the restoration of susceptibility, folic acid and vitamin B₁₂ were equally effective as single supplements and equivalent to the triple vitamin supplement. The effect of single biotin supplementation was less.

An outbreak of fatal pasteurellosis among BSVS mice latently infected with *Pasteurella* and used in an ADE susceptibility test has been described. The fatal pasteurellosis has been ascribed to a constellation of determinants including (a) diet, (b) sex, (c) inoculation events, and (d) latent infection with *Pasteurella*. With males the susceptible sex it was possible to avert the fatal pasteurellosis and continue the nutritional experiments by using females exclusively.

**Bibliography**

20. For literature on this problem in mice and other animals see references 10 to 12.

EXPLANATION OF PLATE 28

Brains deriving from 2 BSVS mice reared on natural diet 100, and exposed intracutaneously to proteolipide A + B. Stained with hematoxylin-eosin.

Fig. 1. Cerebellum, showing chiefly the infiltrated vascular lesions; vessel partly in granular layer exhibits a thrombus. × 105.

Fig. 2. Subependymal area of lateral ventricle, showing the massed infiltrations apart from vascular lesions. × 315.

Litter mates similarly exposed to proteolipide A + B, but reared on synthetic diet 191, revealed no changes in the CNS.
(Schneider et al.: Nutrition and disseminated encephalomyelitis)