acids are absent or present in comparatively small amounts in the hydrolysate under analysis, the addition of known quantities of this amino acid to the hydrolysate will serve as an internal standard.

When 25 replicate chromatograms are run, the average per cent error for a mixture of 14 amino acids in proportions simulating β-lactoglobulin, was −2% in one series and −4% in a second. The maximum variations in individual amino acids ranged from −12 to + 9%. This error, however, can be appreciably reduced by increasing the number of chromatograms.

Cystine is largely or entirely destroyed on two-dimensional chromatograms using S. & S. No. 596 or Whatman No. 4 paper under the conditions described above. It is best determined by a combination of the platinic iodide[9] and Fisher[3] procedures. Proline, hydroxyproline and tryptophan are likewise best estimated by special methods, the first two by the isatin method of Fromageot,[10] the last by a combination of the Voisenet-Rhode p-dimethylaminobenzaldehyde reaction (*cf.*[6]) as applied to paper chromatograms and the area[3] or area-density[4] methods.

Methionine,† histidine and tyrosine, which are reasonably accurately estimated by specific chromatographic procedures (*cf.* above) have proven to be valuable for the quantitative estimation of amino acids by the ninhydrin method on two-dimensional chromatograms.

Results. The values given in Table III show the color ratios and approximate quantities of arginine, histidine, lysine, tyrosine, phenylalanine, methionine, serine, threonine, leucines, valine, glycine, alanine, glutamic acid and aspartic acid obtained on less than 0.3 mg of casein N. The figures given in the adjoining column (Table III) are those calculated from casein by conventional chemical and microbiological methods.[6]

Summary. Two methods are described which permit the estimation of colored substances on one-dimensional and two-dimensional paper chromatograms with a minimum expenditure of time and material.

* Experiments which are underway indicate that the concentration of each amino acid on the chromatograms may be estimated within approximately 15% by the maximum color density method, when the color value is read off the appropriate "standard curve" prepared from two dimensional chromatograms which are run *simultaneously* with the unknown solution. As in all colorimetry, it is important to have the color density of the standard as nearly equal as possible to that of the unknown.

9 Winegard, H. M., Toennies, G., and Block, R. J., *Science*, 1948, **108**, 506.

10 Fromageot, C., private communication, May 1949.

† Cystine and methionine have been determined in casein, lactalbumin and human hair hydrolysates by the area method[3] using platinic iodide[9] as the color reagent and water saturated n-butanol-acetic acid as the developing solvent. The following amounts of cystine and methionine, in grams of amino acid per 16.0 g of nitrogen, were found: casein, 0.3; 3.4, lactalbumin 3.0; 2.6, hair 16.0; 0.6.

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Prophylactic and Therapeutic Effect of Para-Aminobenzoic Acid and Sodium Salicylate on Experimental Allergic Encephalomyelitis.* (17426)

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That encephalomyelitis in experimental animals can be produced by the injection of homologous brain tissue was shown by Rivers, Sprunt, and Berry[1] and confirmed by Ferraro

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† Helen Hay Whitney Foundation, fellow in rheumatic fever.

1 Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

and Jervis.[2] Studies by Schwentker and Rivers[3] and Kopeloff and Kopeloff[4] demonstrated the association of this disease with the production of specific anti-brain antibodies. Recent studies[5-10] using Freund's adjuvants to potentiate and hasten the development of encephalomyelitis against homologous or heterologous brain tissue have provided a working laboratory model of acute disseminated encephalomyelitis. Almost without exception investigators studying this disease of laboratory animals have supported the allergic theory of its pathogenesis.[11] That the sensitizing agent is concentrated in the white matter of the nervous system, but also present in the gray matter seems clear[12,13] Alvord's[14] studies have, further, implicated certain purified phospholipids extracted from brain tissue as at least one of the sensitizing hapten groups capable of producing encephalomyelitis, and Bell *et al.*,[15] have recently shown that calcium acetate precipitation of aqueous suspensions of central nervous tissue removes their encephalogenic properties.

In spite of the apparent relation of experimental encephalomyelitis to a group of human diseases (acute disseminated encephalomyelitis, multiple sclerosis) for which effective therapy is lacking, little work has been reported of attempts to find therapeutic or prophylactic agents capable of interrupting the cycle resulting in allergic central nervous system disease in animals. The long recognized clinical usefulness of salicylates in the treatment of the exudative phase of rheumatic fever has led, however, to a series of investigations demonstrating a pronounced effect of large doses of salicylate on the course of antibody production[16-19] and allergic phenomena resulting from antigen-antibody reactions,[20,21] while Coburn and Kapp[22] showed that salicylate could prevent the *in vitro* precipitation of antigen with specific antibody. Suppression of rheumatic symptomatology with para-aminobenzoic acid has been reported from several clinics and the combination of para-aminobenzoic acid with salicylates has been suggested for the treatment of rheumatic fever. Recent studies indicate that both para-aminobenzoic acid and salicylates are protective against certain allergic or hypersensitive states in rabbits[20] and several rather poorly controlled clinical studies indicate that salicylates might be helpful in certain human encephalitides.[23-25]

In an attempt to prevent the development of the acute allergic encephalomyelitis due to immunization against homologous brain tissue, the following 3 experiments were carried out.

In the first experiment 112 guinea pigs weighing 250 to 325 g each were used. All animals were fed balanced guinea pig rations

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24 Behague, P., *Rev. neurol.*, 1942, **74**, 159.

25 Ferraz, A. J., *Rev. neurol. e. psiquiret. de São Paulo*, 1944, **10**, 1.

with supplemental vitamin C. Sodium salicylate medications were given by subcutaneous injection of a sterile 5% solution in distilled water. The para-aminobenzoic acid was administered orally with the drinking water in a concentration of 2.5 mg/cc. Sensitization to homologous brain tissue was carried out by subcutaneous injection of a brain-adjuvant (water in oil) emulsion prepared according to our modification of the method of Freund. Ten grams of sterile fresh guinea pig brain were mixed with 100 cc of normal saline by agitation in a Waring Blendor. Two parts of this brain emulsion were added to two parts of Bayol F (a light paraffin oil) containing 7.5 mg of heat killed *Mycobacterium butericum* per cc and one part Falba (a Pfaltz and Bauer product containing a mixture of hydroscopic lipids from lanolin.) The final mixture was then agitated with a Waring Blendor for 4 periods of 5 minutes each. In this way a stable emulsion was produced, each cc of which contained 2.5 mg of heat killed *Mycobacterium buterium* and 30 mg guinea pig brain. The plan of the first experiment was to begin all medications 8 days prior to the sensitizing injections and to continue the medications for 60 days after the injection of brain and adjuvant. Ten guinea pigs were given para-aminobenzoic acid alone in dosages of .5 g per kilo per day. Twenty were given moderate dosages of salicylate, (.2 g per kilo in one subcutaneous injection each day.) Ten were given larger dosages of salicylate, (.3 g per kilo in divided dosage given 2 times daily), while an additional 20 were treated with a combination of para-aminobenzoic acid (.5 g/K/D) and sodium salicylate (.2 g/K/D). Control groups comprising guinea pigs injected with brain and adjuvants, brain alone, adjuvants alone and adjuvants in addition to salicylate and para-aminobenzoic acid medication were included in the study. Simultaneously, 10 normal untreated guinea pigs fed the same type of rations and given the same routine care as the experimental animals served as additional controls.

In an attempt to determine the therapeutic effect, if any, of sodium salicylate and para-aminobenzoic acid on experimental disseminated encephalomyelitis the second experiment was carried out. Twelve guinea pigs from among 20 weighing 300 to 500 g, which had been inoculated with the brain-adjuvant emulsion previously described, were selected for treatment after the onset of unequivocal central nervous system disease. Four of these diseased animals were treated with sodium salicylate alone in a dosage of .2 g/K/D, and eight were placed on the combined para-aminobenzoic acid and salicylate regimen described for the animals of experiment 1.

In an attempt to establish the time at which para-aminobenzoic acid and salicylate became effective in the prophylaxis of acute disseminated encephalomyelitis, the third experiment was carried out. Forty guinea pigs weighing 425 to 600 g were studied. Ten of these animals injected with the brain adjuvant emulsion and given no further treatment served as controls, while one of the remaining guinea pigs was started on treatment with the para-aminobenzoic acid and salicylate combination previously mentioned on each successive day beginning eight days prior to the injection of all the animals with the brain adjuvant emulsion and continuing for 22 days thereafter.

Results. The results of Experiment 1 are summarized in Fig. 1. Approximately 90% of 22 guinea pigs receiving injections of guinea pig brain and adjuvants developed symptoms of central nervous system disease between 13 and 27 days after the injection. Most frequently the disease began with spasticity of the hind legs followed within a few days by flaccid paralysis and encephalitis. Tremors, nystagmus, and convulsions were prominent symptoms in the late stages of the disease. Seventeen of these control animals died from encephalomyelitis between 19 and 42 days after the initial injection of the water in oil emulsion of guinea pig brain. The duration and course of the disease after onset were variable with exacerbations and remissions being noted occasionally. Once unequivocal evidence of central nervous system involvement became apparent, the animals usually died. In several guinea pigs, however, the disease became stationary for long periods. A few animals recovered, some with sequellae,

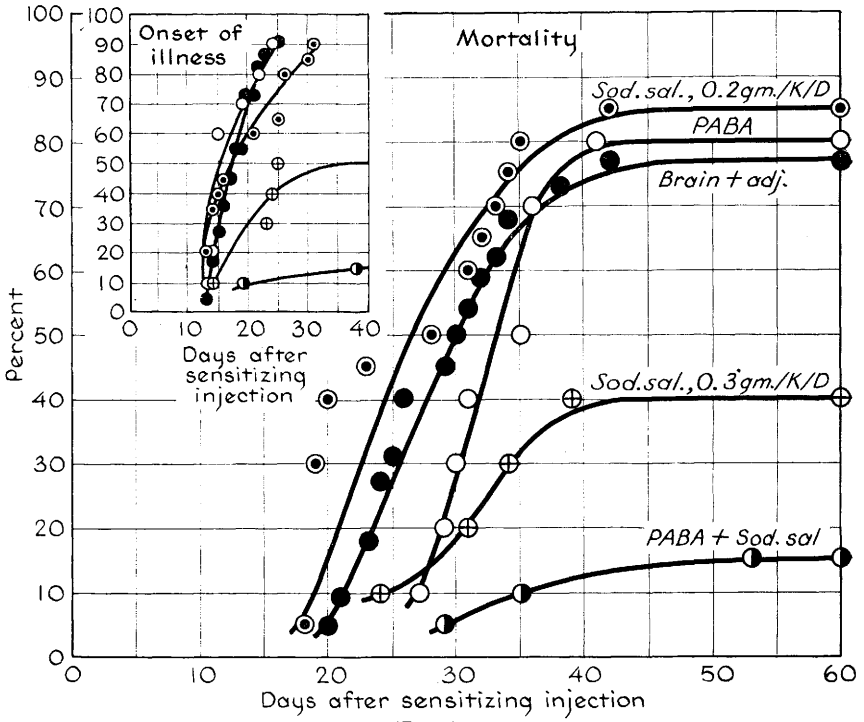

FIG. 1.

Table of mortality curves of 112 guinea pigs of experiments I and II. Insert shows onset of illness in same series. See text.

others with none. Cultures of the central nervous tissue removed with careful aseptic precautions from moribund, paralysed, encephalitic animals showed no growth on blood agar plates. Neither were attempts to demonstrate a virus in the involved nervous tissue by intracranial and intraperitoneal mouse passage, intracranial and intraperitoneal guinea pig passage, nor corneal and intraocular rabbit passage successful.

While premedication and medication with moderate doses of either sodium salicylate or para-aminobenzoic alone failed to alter the incidence or the course of the central nervous system disease, larger total dosages of sodium salicylate seemed to delay the time of onset and reduce both the incidence and mortality rate. Even more striking were the results obtained when a combination of para-aminobenzoic acid and sodium salicylate was used for the prophylaxis and treatment of this experimental disease. Fifteen per cent of the guinea pigs treated in this way developed symptoms and signs of encephalomyelitis after injection with brain and adjuvant and only 10% died. Injection of either adjuvants or homologous brain alone was well tolerated by the controls. During the period of the experimental study, 10 normal guinea pigs remained in good health showing substantial growth on the same dietary regimen provided for the experimental animals.

The therapeutic effectiveness of sodium salicylate and para-aminobenzoic acid was tested on a group of 12 guinea pigs selected on the first day of central nervous system involvement following 14-30 days after the injection of brain and adjuvants. No alteration in the course of the disease nor apparent amelioration of symptoms was produced in any of the animals treated with sodium salicylate or combined sodium salicylate and para-aminobenzoic acid. One guinea pig in the group treated with para-aminobenzoic acid and salicylate showed several remissions followed by exacerbations and ultimately survived. The remaining seven guinea pigs of this group and all 4 treated with sodium

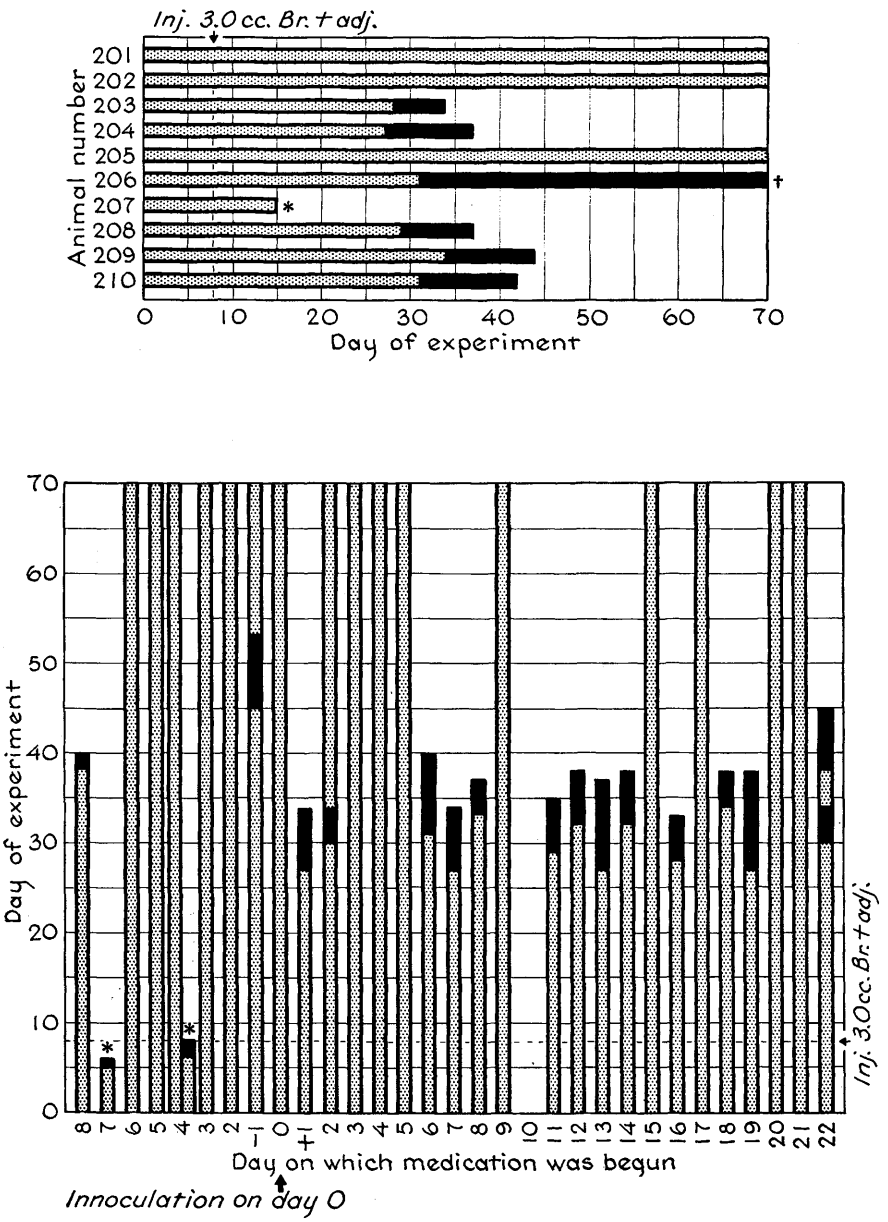

FIG. 2.

Chart showing individual clinical histories of 10 control animals and 30 which commenced medication at various days, before and after the sensitizing injection. Stippled columns indicate disease-free period, solid columns represent period of illness. Termination of the columns indicates death of animal. Asterisks indicate death of animals from causes other than the experimental disease. Animal 206, long sick, was killed at the termination of the experiment.

salicylate alone died between 4 and 18 days after the initial symptoms and beginning of treatment. This result was not significantly different from that occurring among the control guinea pigs.

The results obtained in Experiment 3 are indicated in Fig. 2. Here again evidence of a protecting effect of combined para-aminobenzoic acid and salicylate prophylaxis is apparent. The control group (Fig. 2) which

received brain plus adjuvant but no medication, showed 5 out of 9 dying (55%) with an incidence of 6 out of 9 acquiring the disease (67%). In the treated series, little morbidity was seen in the group in which medication was commenced before the fifth day following injection. Past that point, however, the incidence of symptoms and death appear not significantly different from the control. These results suggest that protection against experimental allergic encephalomyelitis is effective when sodium salicylate and para-aminobenzoic acid medication are begun early. It would seem from these data that if medication is delayed for several days following the injection of the brain and adjuvant no protecting effect can be demonstrated. In this series significant protection was apparent among the guinea pigs whose treatment was initiated prior to the fifth day following injection with brain and adjuvant, while those whose treatment was begun later were not protected.

Although the pathologic studies of the series of animals will be the subject of another report, a brief mention of the results obtained are of interest here. The central nervous system changes include scattered foci of inflammatory reaction with subependymal and perivascular concentrations being characteristic. The marked plasmacellular component of the inflammatory reaction previously noted by others[8-10] was also apparent in our material and is in keeping with an hypothesis previously expressed[26-28] that local plasma cell accumulation in the central nervous tissue, as well as elsewhere, indicates the operation of antigen-antibody reactions.

Discussion. Many problems are posed by these results. Although in these experiments serum antibodies were not studied, we assume that the disease with which we are dealing is an expression of the specific antibrain hypersensitivity. A number of investigators have demonstrated the association of the nervous disease produced in this way with the development of antibrain antibodies.[3,4,32] Because of our previous experience[29,30] with the activation of latent virus in the central nervous system by antigen-antibody reaction and because the symptoms of nervous system involvement in this disease simulate in certain respects known virus disease of guinea pigs, mechanisms involving virus invasion were suspected of operating in this situation. It seems reasonably certain, however, that our futile search for virus in the tissues of these animals as well as negative searches for virus in the central nervous tissues of similar animals by others render this possibility very unlikely. The constant two to four week latent period following injection of antigen is compatible with the allergic hypothesis of the pathogenesis of this encephalomyelitis.

The protection afforded by the combination of salicylates and para-aminobenzoic acid can probably be explained through operation of known actions of these drugs. An *in vivo* as well as *in vitro* effect of salicylates in blocking antigen-antibody reactions seems well established. In addition, the weight of the evidence would indicate that large doses of salicylates act in some way to reduce antibody formation. That moderate dosages of salicylates do not affect antibody response to the streptococcus and its products, was pointed out by Rantz, Boisvert, and Spink[31] while the effect of larger dosages of salicylate on the antibody response to a variety of antigens has been demonstrated. This relationship might help to explain the lack of protection against acute allergic encephalitis noted when sodium salicylate in a dosage of .2 g/K/D was used while protection was obtained with larger dosage of salicylate as well as when the smaller salicylate dosage was combined with para-aminobenzoic acid.

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Para-aminobenzoic acid may by itself have anti-anaphylactic properties[20] and the effect noted here might be an expression of a synergistic antianaphylactic action. It is entirely possible, however, that the decreased elimination of sodium salicylate in the urine in the presence of para-aminobenzoic acid[33] results in higher, more effective, blood levels of salicylate than when this drug is given alone. If so, other acid salts, like ammonium chloride, might also result in a comparable enhancement of the salicylate effect. While toxic reactions to salicylates and para-aminobenzoic acid may preclude the safe attainment of effective levels in the treatment of human disease, further studies are indicated to elucidate the underlying mechanisms of this protecting effect. There is reason from this and other studies to believe that a less toxic drug with antianaphylactic properties in common with salicylates might be an important therapeutic and prophylactic agent in human disease.

The now extensive studies of auto-immunization phenomena in diseases of experimental animals demand further attempts to establish the operation of such mechanisms in the pathogenesis of human disease in spite of the technical difficulties involved. It seems obvious that this phenomenon could be at work in post infectious states, the pathogenic mechanisms of which remain obscure. Further studies on rheumatic fever, post infectious leukoencephalitis, multiple sclerosis, sympathetic ophthalmia, and glomerulonephritis might, indeed, reveal that autosensitization is a common denominator for many otherwise unrelated diseases.

Summary and conclusions. 1. Progressive acute disseminated encephalomyelitis was produced in 90% of a group of guinea pigs by the subcutaneous injection of water in oil emulsions of homologous brain tissue plus adjuvants.

2. While prophylaxis and treatment with moderate dosages of either para-aminobenzoic acid or sodium salicylate alone provided no protection of guinea pigs against experimental allergic encephalomyelitis, the use of larger dosages of salicylate as well as combined salicylate and para-aminobenzoic acid therapy were effective in preventing this disease.

3. Effective prophylaxis was demonstrated only when the medication was started before or shortly after the sensitizing injection of brain tissue was given.

4. No therapeutic effect of sodium salicylate alone or combination of para-aminobenzoic acid with sodium salicylate could be shown.

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The Initial Uptake of C^{14}-Labelled Methadone by Rat Tissues.* (17427)

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Recently it has been shown in this laboratory that within one hour after a subcutaneous injection of 10 mg/kg of C^{-14} labelled methadone (methadone*) into rats, the concentration of the drug in the brain is relatively low.[1] A fairly rapid mobilization of the drug from the site of injection is indicated by the work of Eisenbrandt, *et al.*[2] who found that within 10 minutes after such an injection,

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