N-acetylserotonin, and 0.1 μc of C^{14}-methyl-*S*-adenosylmethionine (3.7 mμmole, 10,000 count/min) were incubated at 37°C for 1 hour. The C^{14}-melatonin formed was extracted with 8 ml of chloroform, which was washed twice with borate buffer, *p*H 10.0. A 6-ml portion of the chloroform extract was evaporated to dryness, and counted in a liquid scintillation spectrophotometer after the addition of ethanol and phosphor.

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Differences in the Amino Acid Composition of a Third Rabbit Antibody

Abstract. *Antibody directed against a neutral hapten,* p-*amino-β-phenyl lactoside, has been purified, and its amino acid composition has been determined. When the results were compared with the previous analyses of two rabbit antibodies, small but significant differences were found. The demonstration of a third unique antibody composition supports the hypothesis that the amino acid differences are related to specificity rather than to the heterogeneity of γ-globulin.*

In recent studies (*1*) a few small but significant differences have been found in the amino acid compositions of two rabbit antibodies. The antibody directed against a negatively charged haptenic group, *p*-aminobenzenearsonic acid, had a higher arginine and isoleucine content whereas the one directed against a positively charged hapten, *p*-aminophenyltrimethylammonium ion, had a higher aspartic acid and leucine content. The remaining amino acid contents were strikingly similar.

One interpretation of these results is that antibody synthesis is under genetic control, and the differences represent changes in the amino acid residues at or near the active site. An alternative interpretation is that the differences represent pools of differently charged γ-globulin molecules in which the antibody activities are segregated. The latter possibility was minimized by isolating both antibodies from individual rabbits which were homozygous with respect to their γ-globulin production and by finding the same differences in amino acid composition (*1*). Further evidence to resolve these alternative hypotheses would be obtained by the analyses of other rabbit antibodies, particularly those prepared against uncharged haptenic groups. The first of such studies, a comparison between the amino acid compositions of antibody to *p*-azo-β-phenyl lactoside and antibody to *p*-azobenzenearsonic acid is given in this report.

The immunizing antigens were synthesized by coupling at *p*H 9.0 (i) the diazonium salt of *p*-aminobenzenearsonic acid to bovine γ-globulin and (ii) the diazonium salt of *p*-amino-β-phenyl lactoside (*2*) to either bovine γ-globulin or bovine serum albumin. The amounts of diazotized hapten added were twice the molar concentration of tyrosine present in the protein carrier. After dialysis to remove excess reagent, equal quantities of each antigen were precipitated with alum (*3*), and the mixture was injected into New Zealand white rabbits in increasing doses over a period of 4 weeks.

Each animal received a total of 120 mg of antigen and was killed 5 days after the last injection. One-half of the animals received the two haptens conjugated to the same protein carrier while the other half received the two haptens conjugated to different protein carriers. Five of the rabbits used were γ-globulin homozygotes kindly provided by the National Institutes of Health; the remaining rabbits were heterozygotes at the "*a*" locus.

The isolation of purified arsonic antibody has been described fully (*4*, *5*). Similar methods were employed in the purification of the lac antibody (*4*). First, antibody formed against the protein component of the azoantigen and complement were removed from the antiserum by the addition of 20 μg of bovine serum albumin nitrogen per milliliter. The antihapten antibody remaining in the supernatant was then precipitated with concentrations of antigen ranging from 8 to 12 μg of nitrogen per milliliter. The antigen used consisted of carboxymethylated human fibrinogen, of which 10 μmole were coupled to 6 mmole of diazotized β-phenyl lactoside. The washed immune precipitate was dissolved in 0.05*M* lactose and the resulting solution was applied to a diethylaminoethyl-cellulose column equilibrated with 0.02*M* phosphate buffer, *p*H 7.2. The azofibrinogen was retained on the resin because of the increase in net negative charge achieved by the preliminary carboxymethylation. The antibody, mixed with hapten, appeared in the eluate as a single peak at column volume. The hapten was then removed by dialysis against 0.075*M* phosphate buffer, *p*H 7.2 and 0.1*M* in NaCl.

The efficiency of the separation procedure was assayed by use of radioactive reagents. When the azofibrinogen was labeled with I^{131} (*6*), only negligible amounts of radioactivity could be detected in the eluted antibody solution. The antigen contaminant was calculated to be less than 0.02 percent on a molar basis. When the hapten concentration was measured with C^{14} lactose (*7*), it was found that 0.5 to 0.7 mole remained per mole of antibody after the usual 48 hours of dialysis. A further threefold reduction was achieved in the trichloroacetic acid precipitation of the antibody prior to hydrolysis. This amount of lactose contaminant did not have a significant effect on the amino acid recoveries since identical values were obtained when the lactose present in the sample was deliberately increased by a factor of five.

Amino acid analyses were carried out on nine pairs of antibodies isolated from

Table 1. Average amino acid recoveries after acid hydrolysis of two purified antibodies (arsonic and lac) from the same rabbit. Hydrolysis time, 20 hours.

Amino acid *	Antibody			
	Residues per 160,000*g*		Standard error of a single determination	
	Arsonic †	Lac †	Arsonic	Lac
Lysine	70.0	70.8	0.41	0.84
Histidine	16.5	16.9	0.45	0.29
Arginine	44.6	44.6	0.53	0.82
Aspartic acid	105	112	1.41	1.37
Threonine	161	163	2.55	1.77
Serine	148	143	1.90	1.94
Glutamic acid	122	121	2.24	2.35
Proline	110	110	1.27	1.77
Glycine	109	109	1.06	2.03
Alanine	79.8	77.3	1.46	2.21
Valine	128	129	1.00	1.73
Methionine	13.5	13.6	0.21	0.30
Isoleucine ‡	48.1	47.2	0.80	1.17
Leucine	89	89		
Tyrosine	56.3	50.9	0.49	0.55
Phenylalanine	44.2	44.5	0.35	0.61

* Tryptophan + ½ cystine residues were not determined. † Average value from the analyses of 9 different preparations. ‡ Sum of isoleucine and alloisoleucine.

the serums of single rabbits. The assays were performed under carefully controlled conditions; an analysis of an amino acid standard was alternated with each analysis of an antibody hydrolysate and a single pipette was used for the application of the samples on the long and short columns of the analyzer. The average recoveries obtained for each antibody after 20 hours of acid hydrolysis and the calculated errors of an individual determination are given in Table 1. The data have been expressed as moles of residue per mole of antibody and normalized to a leucine content of 89. The value of 89 represents the average recovery of leucine per mole of antibody as calculated from nitrogen analyses of the antibody hydrolysates, a nitrogen content of 16 percent and a molecular weight of 160,000 (*1*).

The average amino acid composition of arsonic antibody (column 1) was identical to that previously obtained when arsonic and ammonium (*4*) antibodies were prepared in the same rabbit (*1*). These results permitted the comparison of the lac antibody with the previously published data since they demonstrated that the amino acid composition of any one antibody was not detectably affected by the simultaneous production of another antibody. Furthermore, these results provided additional evidence that the amino acid compositions were independent of the γ-globulin allotypy since the antibody preparations used were isolated from both heterozygous and homozygous animals.

The average values for the lac antibody (column 2) were in most cases strikingly similar to those of its arsonic control. However, a few small but significant differences appeared. The lac antibody was characterized by a higher aspartic acid content while the arsonic antibody had a higher tyrosine content. These differences were shown by standard statistical methods to have a 99.9 percent probability of being outside experimental error. In addition, the serine value was 3 percent lower in the lac antibody, but whether this recovery reflected a lower initial content or a slightly increased lability to acid hydrolysis could not be determined without further measurements of the rate of serine destruction.

The standard deviations of an individual determination listed in columns 3 and 4 were very similar for both antibodies despite the fact that the arsonic antibody was always prepared against a hapten-bovine γ-globulin complex whereas the lac antibody was prepared against hapten conjugated either to bovine γ-globulin or to bovine serum albumin. Thus, the observed differences in amino acid compositions could not be related to the protein carrier used in the immunization.

Since ammonium antibody has been shown to differ from arsonic antibody in its aspartic acid, arginine, leucine, and isoleucine content (*1*), while lac antibody differs only in its aspartic acid and tyrosine contents, these combined data established that each of the three antibodies has a unique amino acid composition. It was conceivable that differences in amino acid content of two antibodies could be explained by the heterogeneity of γ-globulin especially since two pools of globulin which differ slightly in their amide and charged amino acid content have been found in studies of the univalent fragments liberated by papain digestion (*8*). This hypothesis becomes much more improbable with the demonstration of a third unique antibody composition because it would require not only the existence of three different globulin pools in all the animals used, but also the distribution of one antibody in each pool. A more probable explanation is that the observed differences in amino acid composition are directly related to the one property which distinguishes these antibodies from each other, the specificity of their reaction with respective antigen.

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Nerve Fibers and Terminals: Electron Microscopy after Nauta Staining

Abstract. *Sections of cat spinal cord and rat mammillary body in which degenerating nerve fibers were stained by the Nauta silver method have been examined with the electron microscope. Silver granules were present in the axoplasm of some myelinated fibers and in some of the axon terminals.*

The Nauta method stains degenerating nerve fibers selectively, and shows the localized swellings and discontinuities that occur along the degenerating fibers (Fig. 1). The method has been widely used for tracing fiber pathways in the central nervous system, but there is disagreement about its suitability for studying synaptic relationships (*1*, *2*). Whereas the Glees silver method stains some synaptic boutons as readily recognizable rings or clubs, the Nauta method either does not stain the boutons at all or stains them as swellings not clearly distinguishable from the swellings that occur all along the course of degenerating axons (Fig. 1).

This study was undertaken to determine whether the Nauta method can stain terminal boutons and also to find

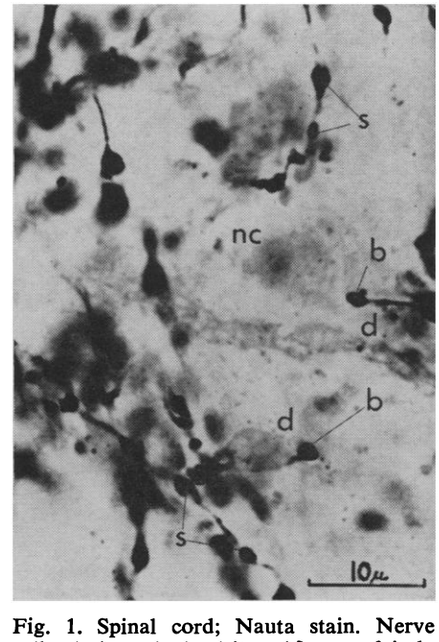

Fig. 1. Spinal cord; Nauta stain. Nerve cells (*nc*) and dendrites (*d*) are faintly stained. The degenerating fibers are dark. Two appear to end as boutons (*b*), but these boutons cannot be easily distinguished from the swellings (*s*) that occur along the course of the degenerating fibers.