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A RADIOAUTOGRAPHIC AND HISTOLOGICAL STUDY OF
PROTEOLYTIC SPECIFICITY ON WHITE RAT
BRAIN STEM MOTONEURONS

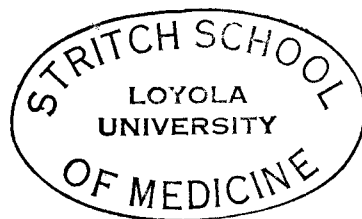
by

Jack T. Beuttas

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

February

1961



LIFE

Jack T. Beuttas was born in Chicago, Illinois, February 4, 1925.

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I. INTRODUCTION

It has long been known that the biological catalysts known as enzymes are capable of certain actions on specific substances by virtue of their chemical composition and configuration. However, as far as could be determined, only relatively little work has been done to demonstrate enzyme specificity as a result of the enzyme's action on nerve cells.

The present study was motivated by a desire to supplement this paucity of information by providing a more adequate understanding of proteolytic specificity through the use of enzymes of both plant and animal origin. Wang et al. (1955, 1957) used this approach to determine enzymic specificity on beef muscles, which are in reality dead but unfixed tissue, and found distinct differences as to their action on the collagenous and elastic tissues of muscle, as well as on the muscle cells proper. More recently, Wang (1960) employed the same approach and obtained evidence of enzyme-substrate specificities on living *Paramecia*.

It is the more particular objective of this thesis to investigate the proteolysis of fixed mammalian nerve cells, as depicted by post-digestion staining techniques. The large moto-

neurones of the lower brain stem were found to be an especially suitable enzyme substrate because of the discreteness and exceptional size of their components. The Nissl bodies, neurofibrils, and nuclei were particularly important in this respect. Li (1958) employed spinal motoneurones in a comparative histochemical study in which he pointed out essential differences between the susceptibilities of the various cellular components to the three animal proteases, pepsin, trypsin, and chymotrypsin. His results and those of the present study are in substantial agreement. Mazia and Yeager (1939) made use of pepsin and trypsin to demonstrate specific proteolysis of the giant chromosomes of the fruit fly. Their work showed that trypsin digested the basic nucleoproteins, while pepsin failed to seriously affect chromosomal integrity.

II. MATERIALS AND METHODS

A. Use of Proteolytic Enzymes

Sections of normal white mouse brain tissue were subjected to the action of proteolytic enzymes and then stained by various techniques for viewing under the microscope.

Anesthetised adult animals were first perfused with ice-cold saline for five minutes and neutral saline-formalin for one hour to ensure an equal and thorough fixation of the brain. The tissue was then allowed to remain in formalin for an additional twenty-four hours, and was washed in running water for eighteen. Although Wolman (1955) has provided an excellent review of fixation, it is worthy of mention that formalin has proved to be the best fixation for all components in terms of this study. Others have been recommended, even by originators of the staining methods used herein, but all have proved less favorable than formalin itself. Normal histological processing followed. In order to prevent loss of sections from the slides, Gooch crucibles were used to hold the sections during deparaffinisation, digestion, washing, and staining. Care must be exercised when the crucibles are removed from the fluids to prevent loss of sections through the holes at the bottom. At the end of all processing, a section lifter may prove helpful when brushing the sections onto microscopic slides.

The enzymes and their optimal conditions of use are shown in the following table. In order to determine the effects of the solutions in which the enzymes were dissolved, experimental controls were subjected to the identical conditions, but without enzymes, while the normal controls were only stained.

It can be seen that the enzymes were incubated at varying temperatures and pH values, but in every case for 2.5 hours, except where otherwise stated.

Table I
Conditions of Use for Enzymes.

Enzyme	Concentrations	Solutions	pH	Temp.
<u>A. Animal</u>				
1. Pepsin, powdered; from stomach	0.1, 0.5, 1.0% 1, 2, 3% (1/2 hr.)	0.02 N HCl	1.8	37°
2. Trypsin, powdered, also crystalline; from pancreas	0.1, 0.5, 1.0% 1, 2, 3% (1/2 hr.) 1 mgm%	PO ₄ buffer	7.6	37°
		PO ₄ buffer	7.6	37°
<u>B. Plant</u>				
1. Bromelin, powdered; from the pineapple	0.1, 0.5, 1.0%	0.01 M urea+ 0.01 M NaHSO ₃		40°
2. Ficin, powdered; from the fig tree	0.1, 0.5, 1.0%	0.01 M urea+ 0.01 M NaHSO ₃		40°
3. Papain, powdered; from the papaya	0.1, 0.5, 1.0%	0.01 M urea+ 0.01 M NaHSO ₃		60°
<u>C. Plant, Microbial (species unknown)</u>				
1. EB - 21, powdered	2, 3, 4%	PO ₄ buffer	7.2	40°
2. MT - 7820, powdered	2, 3, 4%	PO ₄ buffer	5.2	37°
3. A - 4	3, 5%	PO ₄ buffer	8.0	50°
4. P - 11	1, 2, 3, 4, 5%	PO ₄ buffer	7.3	50°
5. W - 15	3, 5%	PO ₄ buffer	7.0	60°

B. Staining Techniques.

In order to show the results of digestion on the structural components, several different histological stains were employed.

Bodian's (1936) silver protargol method was used to demonstrate the neurofibrils. His paper has also supplied an excellent account of perfusion technique, except that 10 per cent neutral formalin was used for the present enzyme studies, instead of 80 per cent alcohol.

Bodian's technique allows uniform and specific staining of the nervous elements, such as neurofibrillae, myelinated and unmyelinated fibers, and the nuclear membrane and nucleolus.

Luxol fast blue, as a phthalocyanin dye, stains myelin, and has been very aptly described by Klüver and Barrera (1953). This stain is actually a copper derivative, which Pearse has shown has a strong affinity for phospholipids.

Formalin perfusion fixed material is satisfactory, and again preferred.

Myelinated tracts stain a brilliant blue which contrasts very well with the protargol impregnated axon cylinders and neurones.

A procedure for staining the tigroid substance of the nerve cell was first developed by Nissl (1896), who used his stain to

differentiate certain cytoplasmic components. These are today probably most commonly referred to as the "Nissl substance". Nissl originally used methylene blue, although later workers introduced toluidine blue, thionin, cresyl violet, and gallocyanin, which belong to the oxazines and thiazines, to achieve the same results.

It is with the two latter stains that the present study is concerned. Two Nissl stains have been used in order to ensure that post-digestion staining results of only one stain cannot be construed as an artifact.

Cresyl violet is prepared and used according to the method prescribed by Klüver and Barrera (loc. cit.)*

The second stain is known as gallocyanin-chromealum, and this technique was developed and described especially for central nervous system use largely by Einarson (1955). The stain is exquisitely progressive and no differentiation is needed. The reasons for this are best given here in order fully to account for the lack of the need for differentiation.

The dye and the metal salt, as a mordant, unite to form an inner-complex compound, the staining lake, which then becomes selectively and firmly attached to the nucleo-proteins of the fixed basophilic cell structures by reacting with the phosphoric acid groups of the nucleic acid molecule. In short,

1. Dye and metal salt (mordant) = dye-lake

2. Dye-lake and tissue = stained tissue.

The phosphoric moieties become progressively occupied by the lake-cations of the stain, and when maximal, no further staining can take place, according to Einarson (loc. cit.), and because of this stable chemical combination, the stain resists treatment by the ordinary dehydration agents. Gallocyanin can thus be considered an extremely useful adjunct to almost any histological or cytological study in which changes in Nissl substance are expected.

The last staining technique used in this study is the ninhydrin Schiff histochemical method for protein, as presented by Yasuma and Ichikawa (1955).

Several methods are available for the identification of tissue protein, but like Millon's reaction, they may damage the tissue, or fade, like the Sakaguchi test for arginine.

This procedure, however, is not so damaging, nor does it fade easily.

When ninhydrin reacts with protein, it deaminates free amino groups to aldehydes. These are then coupled with Schiff's reagent to give the well known red color of Feulgen staining.

A red or purple color indicates the site of distribution of protein or peptides. The authors claim a high protein specificity for the method, and document this by pepsin digestion and other means.

C. Autoradiography.

The autoradiograph affords a means of investigating the relative extent of the incorporation of radioactive metabolites into the animal organism. Thus, the use of a labeled amino acid provides an additional method of demonstrating enzyme specificities.

For this investigation, a 30 gm mouse was injected with 2.5 microcuries of uniformly labeled C^{14} L-leucine in 0.2 ml of saline. After one hour, to allow for incorporation, the animal was subjected to the same histological procedure as previously, and again with identical tryptic and peptic digestions.

The dipping technique of Messier and Leblond (1957) was used to coat the slides with Ilford G 5 nuclear emulsion. For convenience, their method was modified by placing the slides into numbered slide boxes, and exposed in a light-tight container, such as a painted, enclosed, vacuum desiccator, for introduction of the dry, inert gas, nitrogen, and kept at 4° C. These conditions are necessary, since air and humidity decrease the density of the autoradiograph, and heat and light increase the background fogging of the photographic emulsion.

The further processing of developing and fixing was conducted after the method of the two previous authors (loc. cit.). The slides were then stained in the normal manner with hema-

toxylin and eosin, cleared in xylol, and mounted.

Disintegrating C^{14} atoms emit beta rays of 0.154 Mev energy. When these beta ray electrons strike the silver halide molecules in the emulsion, a latent image is formed. The silver in these molecules is then reduced by the developer to form grains of 0.1 to 0.5 micron, while that portion of the halide without a latent image is removed by the fixer.

Those areas of tissues or cells which have assimilated the most labeled leucine will also show the greatest silver grain density. These grains must be counted, or determined photometrically, in order to make any valid comparisons. Proteolytic digestion, utilising only trypsin and pepsin, naturally removes, in a specific fashion, the peptide chains of which the C^{14} leucine has become a part.

Counting observations were made as follows. The slides were viewed under phase contrast, for superior resolution, at 400 X. A finely marked ocular reticle was used to divide the microscopic field into squares measuring 729 square microns.

Subdivided squares were placed over a particular cell or area to be counted, and the count recorded with a hand counter. The advantage of the subdivided squares was simply to avoid double or non-counting of grains.

Inclusion of emulsion background counts was necessary because latent images not due to tissue radioactivity may be pre-

sent. Sources of background fog are heat, mechanical disturbances, and cosmic rays. These events of reduction can take place over tissue areas as well as elsewhere.

III. OBSERVATIONS AND RESULTS

A. As Shown by Nissl, Protargol, and Ninhydrin Staining

The results of this enzymic hydrolysis study demonstrate that each enzyme affects the nerve cell substrate in a fashion according to the enzyme's specific capabilities. This means that the chemical interaction between the individual enzyme and each structural component of the cell leads to qualitative specificity. Table II thus affords a direct comparison of each proteolytic entity and its effects on the neurons. The gradient effect of each enzyme is also presented as a means of indicating that an increase in concentration will generally lead to a quantitatively greater proteolysis of each structure concerned.

Fig. 13, as the normal control, shows the neurofibrils to be fine, individual fibrils. Their course as a cytoplasmic constituent goes from one cell process to another.

0.02 N HCl, pH 1.8, was used as the experimental control solution. This treatment did not change any feature of the protargol staining of the neurofibrils, and Fig. 13 is also to be regarded as the result of this acidic incubation at 37°. The length of exposure played no role, as shown by a comparison of controls.

Pepsin, however, specifically digests the neurofibrils.

Time does play a role here. Fig. 14 shows cells after incubation in 3 per cent pepsin for 0.5 hour. The neurofibrils have been disintegrated. In their place is a photographic density slightly greater than the surrounding cytoplasm. This density is triangular with its base at the nuclear membrane, and probably marks the former site of the neurofibrils. It is likely that just enough molecular structure remained to reduce a small amount of silver. The nucleoli, however, seem unaffected.

Fig. 18 demonstrates the effect of 0.1 per cent pepsin after 2.5 hours. The neurofibrils are no longer so clear and heavily impregnated. The surrounding tissue also shows gross changes. The cells in Fig. 19 are examples of the destructive effects of 0.5 per cent pepsin, after 2.5 hours. Neurofibrils are scarcely in evidence, but the continuing presence of the nucleolus denotes a greater resistance to pepsin than that of the neurofibrils. 1.0 per cent pepsin completely removed all cells, and is not shown.

Specific digestion of neurofibrils after only 0.5 hour, even though in a 3 per cent solution, seems to point to a high liability of this protein to enzymatic attack.

This progressivity did not extend to the Nissl bodies. These components in all pepsin digested sections (not illustrated) and experimental controls (not illustrated) remained the same as in the normal control (Fig. 1). Digested sections may show broken

cell walls and shrinkage, but these conditions are also found in the completely untreated normal material (Fig. 1). The results were the same with respect to times and concentrations. The nucleoli show a slight diminution in staining after 0.5 hour in 3 per cent (not illustrated).

In addition to pepsin's tissue degradation as shown by protargol stained slides, however, it did noticeably reduce the intensity of the staining with the Ninhydrin-Schiff technique. Although the pepsin series of ninhydrin slides was not quite as dark as that of MT 7820, digested sections definitely became progressively lighter. This may indicate proteolysis by the long incubation in a pH 1.8 medium, in comparison to pH 5.2 (Figs. 11 and 12).

The results of pepsin's digestion of radioactive sections are discussed separately.

Trypsin, in its turn, is also highly specific. There were two different sources of this enzyme, and each will be differentiated as to its effects.

The first trypsin used for proteolysis was of a bulk type. It was available as a very fine powder. After only one half hour of digestion in 1, 2, and 3 per cent (as in Fig. 13), these slides showed no neurofibrillar degradation, and the control slide also showed good Bodian staining.

Crystalline trypsin, in 1 mgm per cent concentration also

failed to attack the neurofibrils, although this concentration was entirely sufficient to remove the Nissl substance as discussed later. The bulk product, also used in concentrations of 0.1 per cent (as in Fig. 18), 0.5 per cent (as in Fig. 19), and 1.0 per cent (not illustrated) caused gross digestion in gradient fashion, but only after 2.5 hours. The gradient effect of the powdered bulk product was similar to that shown by pepsin. Again, 1.0 per cent bulk trypsin completely removed all cells.

Trypsin was definitely effective in removing Nissl bodies in both cresyl violet and gallocyanin stained sections. A definite gradient is present in going to higher concentrations. Many cells were still available for photography as in Fig. 4, but the number was sharply reduced in Figs. 5 and 6. The digestion gradient of the nucleoli is also shown in this series. Bulk trypsin's gradient of 0.1 (as in Fig. 4), 0.5 (Fig. 5), and 1.0 per cent (Fig. 6), was necessary, however, to achieve the same effect as crystalline trypsin's 1 mgm per cent (Fig. 8). Although glia cells were apparently not attacked, neurons were definitely devoid of Nissl bodies after digestion with the crystalline product. Only faint nucleoli and cell membranes remained, demonstrating high specificity for this nucleoprotein. By means of the cell membrane, the former outline of the cells could easily be determined. There were simply no more inclusions.

3 per cent powdered trypsin had an effect on the Nissl bo-

dies comparable to that shown in Fig. 10. There was no widespread, complete breakdown of Nissl substance, but a beginning dissolution in about half the cells. The nucleoli were also attacked in the same fashion.

The fact that the more crude product would first affect the Nissl body as shown by 3 per cent trypsin for 0.5 hour (Fig. 10) and not cause changes in the neurofibril, and that the highly concentrated crystalline enzyme would completely remove the Nissl body alone, is a strong indication of trypsin's specificity.

Ninhydrin stained sections also showed decrease in intensity after trypsin digestion, again with the greatest decrease after exposure to 1.0 per cent of the bulk product. The action is similar to that shown in Fig. 11 for the control solution, pH 7.6, and Fig. 12 for 1 per cent trypsin.

Of the plant enzymes, papain displayed a definite similarity to trypsin's specificity for the basophilic substance. While papain's experimental control sections (as in Fig. 1) were equivalent to the untreated normal controls, papain also caused hydrolysis of the Nissl protein. Galloccyanin and cresyl violet staining show the same results: at 0.1 per cent (Fig. 4) only the very chromophilic cells remain unattacked, while other, smaller cells, have lost much Nissl substance from the cytoplasm, and their nucleoli are shrunken. The 0.5 per cent digestion

(Fig. 7) has removed all Nissl substance except that from the highly chromophilic cells. Only the nuclear membrane and shrunken nucleoli remain in the 1.0 per cent papain treated (as in Fig. 8) sections, although, as with trypsin, the outlines of many cells are still visible.

Bodian stained sections of papain digested material show neurofibrils in a few cells of the 0.1 per cent papain (Fig. 20) series, with a total lack of cells in sections digested by higher concentrations of papain. Unfortunately, plant enzyme activator solutions themselves caused as much damage as could be ascertained from the sections digested in 0.1 per cent papain. The controls (as in Fig. 20) themselves have been seriously affected in their stainability by the solution necessary for papain's activation. Nevertheless, there is evidence for papain's hydrolysis of the basic protein of the Nissl substance, as well as the degradation of neurofibrils. Digestion by higher concentrations is equivalent to Fig. 21 for 0.5 per cent papain, and Fig. 23, for 1 per cent.

Ficin shows a truly peculiar effect on nerve cells. Although its hydrolysis of Nissl substance is minimal, even at 1.0 per cent (similar to Fig. 1), it succeeded in specifically removing the nucleolus from Bodian stained sections, and left only an eye-like vacuole in the nucleus (Fig. 22). The cells were largely still integral in the most dilute solution of 0.1 per cent,

although definability of the neurofibrils was already lost. Increasing the concentrations, however, left progressively fewer cells discernible (Fig. 23).

Bromelin is an enzyme which showed effects similar to papain's on the neurofibrils (Figs. 20 and 21). The activating solution again caused much damage to the cells and tissue, although the 0.1 per cent bromelin treatment left no doubt that the enzyme had an additionally destructive effect on the neurofibrillar and silver stainable nucleolar components. Comparison of 0.1 per cent with 0.5 and 1.0 per cent proves this.

Fig. 2 demonstrates that bromelin's specificity for the Nissl bodies is much lower than trypsin's and papain's. This was only a 0.1 per cent mixture. The digestion caused by 0.1 per cent solutions of the other two should be borne in mind for comparison purposes (Fig. 4). 0.5 per cent bromelin did, however, cause moderately severe (Fig. 3) damage to Nissl bodies. The nucleoli were removed. Bromelin's effects did not go beyond those shown in Fig. 3. 1.0 per cent bromelin led to only the same amount of reduction of Nissl substance (not illustrated).

Consideration of these results points to a specificity of bromelin intermediate between that of trypsin and pepsin.

The fungal proteases showed distinct specificities in their effects on nerve cells, and the delineation of these differences is definitely enhanced by the use of various stains.

Prolase MT 7820 showed a gradient of digestion of the nucleoli. There was first a slight diminution of staining intensity after hydrolysis in the 2 per cent solution (not illustrated), which progressed to a moderate faintness of staining, as shown with cresyl violet. The Nissl bodies and cellular and nuclear membranes remained largely intact, although at the highest concentration of 4 per cent, the Nissl bodies assumed a less particulate appearance, (Fig. 9).

The ninhydrin protein stain also showed definite decreases in staining with increasing enzyme concentrations. At the lowest concentration of 2 per cent (not illustrated), the sections are almost on a par with the controls (Fig. 11). The highest, 4 per cent (Fig. 12), shows a very apparent progression of proteolysis. It becomes difficult to even find the former sites of neurons. The nucleolus, as always with this method of protein staining, remains consistently darker than the cytoplasm, but only in relation to the cytoplasm. Although the glia cells usually resist all forms of digestion, they evince here a decrease in intensity. Had these latter sections been stained for the Nissl substance instead, they would also appear as in Fig. 9. MT 7820 causes hydrolysis of the Schiff staining groups in the cellular proteins, but not of the cresyl violet staining component.

This enzyme caused its least change in the Bodian silver stained sections (not illustrated). They remained comparable to

the controls in every way, with the cytoplasm, nuclei, and neurofibrils apparent throughout.

Hydrolysis by EB 21 of cresyl violet stained components of cells (not illustrated) did not proceed as far as that of MT 7820. The nucleoli seem somewhat lighter, but not to the previous extent, and a breakdown of the Nissl substance did not occur at all.

There was a progression of digestion of the protein of the cell, corresponding to the enzyme concentrations, as shown by the ninhydrin-Schiff technique, but the intensity was little different in the sections treated with the highest concentration than the intermediate, although less than with the lowest (not illustrated).

Changes in cellular structures stainable with protargol were greatest after the use of this enzyme, in comparison with control sections (not illustrated). The lowest concentration, 2 per cent (Fig. 15), shows minimal attack on the neurofibrils. Higher concentrations, 3 per cent (Fig. 16), and 4 per cent (Fig. 17), show less and less evidence of fibrils. The nucleoli become lighter, although the nuclear membrane remains, and the cytoplasm appears to have been cleanly divested of neurofibrils. Contrary to the results with most of the other enzymes, the fibers and processes seem relatively to have retained much of their integrity.

Results of digestion with Rohm & Haas enzymes can only be described as disappointing. Only one, A-4 (Fig. 10), seems to

have non-specifically attacked the Nissl bodies. The formerly large, stained granules are broken down and diminished in size, and staining in many cells is less intense. Some cells still appear normal.

Even less change seems to have taken place with P-11 and W-15, although the latter may have caused a slight what can only be described as fuzziness of the Nissl bodies in some cells (not illustrated).

Bodian silver stainability in all experimental sections has been reduced to a mere staining of the nuclei and cell membrane (not illustrated). The fibrous processes have also lost their sharpness. This indicates that digestion has taken place.

Because of the unique situation presented by the two fungal proteases and the Rohm & Haas products, it is deemed advisable to provide a separate discussion of the results obtained. Since neither the amino acid composition nor the mechanism of action for any of these is known, only the merest inferences can be made partly on the basis of other results.

Nucleolar digestion by MT 7820 and its concomitant partial attack on the Nissl bodies point to a mild trypsin-like action. Both of these structures are high in their content of nucleohistones, with high percentages of diamino acids. Why the nucleolus should be so specifically removed, when it is more resistant

to the action of trypsin and papain, is unknown, particularly in view of the darker nucleolus as shown with ninhydrin staining. It may simply be that the moiety which stains with cresyl violet was more easily lost, while the total protein was hydrolysed at a rate more consistent with that of the cytoplasm. The ninhydrin histochemical stain in any case demonstrates the effect of increasing concentrations of enzyme on cellular proteins.

The failure of this enzyme to change the stainability of the neurofibrils seems to parallel the disparity of action between trypsin and pepsin, for EB 21, in its highly progressive attack on the silver staining components, largely eschewed the digestion of the basic nucleohistones.

Rohm & Haas Co., in their advertising brochure, claim proteolytic action for these three proteases, to the extent that A-4 readily hydrolyses casein to amino acids, W-15 primarily solubilises it to peptides, while P-11 is intermediate in its action. This may explain why A-4 showed any difference at all. The larger break-down molecules after P-11 and W-15 may still have offered better opportunities for combination with the stain. Furthermore, in a communication from their Special Products Department, P-11 and W-15 are listed as having 90 per cent salt as a standardisation, while A-4 contains up to only 75 per cent corn starch and diastase.

B. As Shown by Autoradiography.

The autoradiographic observations which were made by counting the silver grains in Table III are to be considered primarily as concentration gradient phenomena. Qualitative differences can be seen, however, between the two enzymes, pepsin and trypsin, in that definability of trypsin digested cells is again extremely poor (see Table I), while the definability and staining of the pepsin treated material does not change in comparison with controls. Column (1), table III, shows the conditions of treatment. Column (2) lists the grain counts over each individual neuron, in averages per section. This figure is divided by the average cell area for the corresponding section in column (3), and multiplied by 729 to give the counts per 729 square microns. From this is subtracted the background count in column (7).

The net count is then obtained in column (4). The figures in this column diminish perceptibly from the nearly neutral trypsin control solution, to the acidic pepsin control, and the succeeding pepsin concentrations.

This principle is also true for the non-cellular or fibrous white matter areas. Figures in column (5) minus the background in column (7) are reflected in column (6). These net counts in control sections are equivalent to each other. 0.1 per cent trypsin causes a much greater decrease than 0.1 per cent pepsin,

however. Remaining concentrations of pepsin do not cause a significant further loss in protein radioactivity.

The accompanying graph depicts these changes as described.

TABLE II

INDIVIDUAL ENZYME EFFECTS ON CELLULAR COMPONENTS

	<u>Neurofibril</u>	<u>Nissl Body</u>	<u>Nucleolus</u>		<u>Ninhyd.</u>
	silver protargol	cresyl viol. gallocyanin	silver protarg.	Nissl stain	protein stain
1. Pepsin					
2.5 control	0	0	0	0	0
hr. 0.1%	++	0	+	0	+
0.5	+++	0	++	0	++
1.0	++++	0	++++	+	+++
0.5					
hr. 1.0%	0	0	0	0	
2.0	++	0	0	0	
3.0	++++	0	0	+	
2. Trypsin					
2.5 control	0	0	0	0	0
hr. 0.1%	++	+	++	0	+
0.5	+++	+++	+++	++	++
1.0	++++	++++	++++	++++	+++
0.5					
hr. 1.0%	0	0	0	0	
2.0	0	+	0	0	
3.0	0	++	0	+	
Crystall					
2.5					
hr. 1 mgm%	0	++++	0	++	
3. Papain					
control	++	0	++	0	
0.1%	+++	+	+++	+	
0.5	++++	+++	++++	++	
1.0	++++	++++	++++	++	
4. Ficin					
control	++	0	++	0	
0.1%	+++	0	++++	0	
0.5	++++	0	++++	0	
1.0	++++	+	++++	+	

TABLE II (continued)

INDIVIDUAL ENZYME EFFECTS ON CELLULAR COMPONENTS

		<u>Neurofibril</u>	<u>Nissl Body</u>	<u>Nucleolus</u>		<u>Ninhyd.</u>
		silver protargol	cresyl viol. galloccyanin	silver protarg.	Nissl stain	protein stain
5. Bromelin						
	control	++	0	++	0	
	0.1%	+++	0	+++	0	
	0.5	++++	++	++++	+++	
	1.0	++++	++	++++	+++	
6. EB 21						
	control	0	0	0	0	0
	2.0%	+	0	0	0	0
	3.0	++	0	0	+	+
	4.0	++++	+	0	++	++
7. MT 7820						
	control	0	0	0	+	0
	2.0%	0	0	0	+	+
	3.0	0	+	0	++	+++
	4.0	0	++	0	+++	++++
8. A-4						
	control	0	0	0	0	
	3.0%	0	+	0	+	
	5.0	0	++	0	++	
9. P-11						
	control	0	0	0	0	
	3.0%	0	0	0	0	
	5.0	0	0	0	0	
10. W-15						
	control	0	0	0	0	
	3.0%	0	0	0	0	
	5.0	0	0	0	0	

TABLE II (continued)

INDIVIDUAL ENZYME EFFECTS ON CELLULAR COMPONENTS

	<u>Autoradiograph</u>			
	H & E stain		unstained	
	1	2	1	2
1. Pepsin				
2.5 control	0	+	++	+
hr. 0.1%	0	++	++++	++
0.5	0	+++		
1.0	0	++++		
2. Trypsin				
2.5 control	0	0	++	0
hr. 0.1%	++++		++++	++
0.5	++++		++++	+++
1.0	++++		++++	++++

1 = definability

2 = grain density

0 denotes no effect in comparison with the normal control

+ = mild damage to component

++ = moderate damage to component

+++ = severe damage to component

++++ = total disintegration

TABLE III

COUNTING DATA OF RADIOAUTOGRAPHS

All observations were made under phase contrast at 400 X.
Each figure is the average of 20 separate counts.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
I. Trypsin digestion	Grain counts over each neuron	Reticule estimates of each cell area in square microns	Net count (cell minus background) per 729 square microns	Noncellular or fibrous areas, where each count represents 1 square of 729 square microns	Net count of reticles (square minus background) per 729 square microns	Emulsion background where each count represents 1 square of 729 square microns
A. Control sections, treated in Sørensen PO ₄ buffer, pH 7.6, for 2.5 hr., at 40°.						
1. Hematoxylin and eosin stained (Fig. 24)						
Section 1	145.9	650	141.0	80.0	56.9	23.0
Section 2	125.7	600	134.0	55.0	57.0	22.0
2. Unstained control						
Section 1				58.3	23.8	34.5
Section 2				69.0	37.2	37.2
Section 3				88.2	57.4	30.8
B. 0.1% trypsin in control solution.						
1. Unstained						
Section 1				50.1	9.8	40.3
Section 2				46.8	7.6	39.2
2. Stained section (Fig. 25)		(Emulsion has been lost)				

TABLE II I(Continued)
COUNTING DATA OF RADIOAUTOGRAPHS

(1)	(2)	(3)	(4)	(5)	(6)	(7)
II. Pepsin digestion	Grain counts over each neuron	Reticule estimates of each cell area in square microns	Net count (cell minus background) per 729 square microns	Noncellular or fibrous areas, where each count represents 1 square of 729 square microns	Net count of reticles (square minus background) per 729 square microns	Emulsion background where each count represents 1 square of 729 square microns
A. Control sections, treated in 0.02 N HCl, pH 1.8, for 2.5 hr., at 40°.						
1. Hematoxylin and eosin stained (Fig. 26)						
Section 1	116.8	560	106.3	76.5	30.8	45.7
Section 2	79.1	399	104.0	90.3	49.8	40.5
2. Unstained control						
Section 1	138.6	690	97.5	93.6	43.8	48.8
Section 2	200.5	885	131.6	75.0	42.6	33.4
B. 0.1% pepsin in control solution.						
1. Hematoxylin and eosin stained (Fig. 27)						
Section 1	112.6	790	77.5	65.8	26.4	39.4
Section 2	56.8	440	86.0	47.5	8.3	39.2

TABLE III(Continued)

COUNTING DATA OF RADIOAUTOGRAPHS

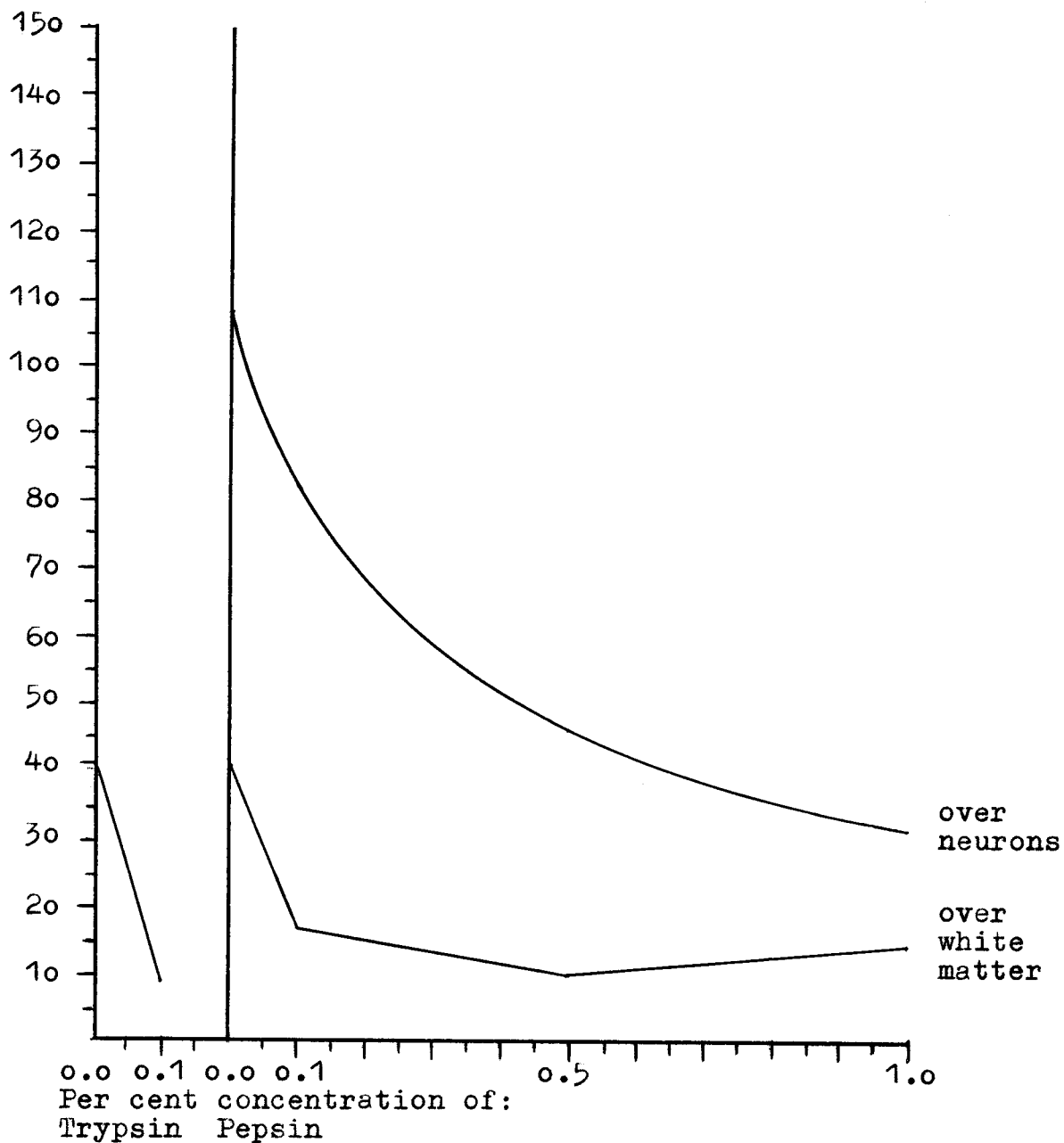
(1)	(2)	(3)	(4)	(5)	(6)	(7)
II. Pepsin digestion (Continued)	Grain counts over each neuron	Reticle estimates of each cell area in square microns	Net count (cell minus background) per 729 square microns	Noncellular or fibrous areas, where each count represents 1 square of 729 square microns	Net count of reticles (square minus back- ground) per 729 square microns	Emulsion background where each count rep- resents 1 square of 729 square microns
C. 0.5% pepsin in control solution						
1. Hematoxylin and eosin stained (Fig. 28)						
Section 1	68.2	600	50.6	43.4	11.2	32.2
Section 2	72.8	620	42.0	46.0	2.0	44.0
2. Unstained section						
Section 1				59.1	17.5	41.6
D. 1.0% pepsin in control solution						
1. Hematoxylin and eosin stained (Fig. 29)						
Section 1	42.9	460	33.0	38.0	2.6	35.4
Section 2	42.4	550	29.7	43.8	17.6	26.2
2. Unstained						
Section 1				54.1	22.1	32.0

Grain
counts

31

trypsin

pepsin



TEXT FIGURE 1

RADIOAUTOGRAPHIC COMPARISON OF THE PROTEOLYTIC EFFECTS OF
TRYPSIN AND PEPSIN ON THE RADIOACTIVITY OF NEURONS

It can be seen from the foregoing results that concentration and time are important factors in enzymic action. It would seem that a longer time and a lower concentration are more effective in causing extensive cellular damage. High concentrations may lead to a substrate surface saturation, and a certain proportion of the enzyme molecules are thus prevented from acting on the substrate. Self-digestion of the enzyme is perhaps also more likely to occur.

In view of these possibilities, a concentration of 3 per cent may have been equivalent to one much lower, and the shorter time of action proved itself advantageous by more sharply limiting the effects to its most specific action. This is particularly true for the animal enzymes in powdered form. The fungal proteases appear to be in a much less refined state, and a concentration of 4 or 5 per cent appears to have been necessary in any case.

It is also clearly evident that qualitative results take precedence over quantitative, for unless an enzyme possesses a chemical specificity for the molecular structure of a given component, it will be inadequate to cause any breakdown changes of that component. It is obvious, however, that the more molecular particles of enzyme are present, the greater the chance will be of such breakdown in a given time.

Particularly pointed is pepsin's failure to digest Nissl bodies, regardless of concentration, as a qualitative effect. On the other hand, papain aptly demonstrates its high specificity for Nissl bodies also in a quantitative fashion, and EB 21 acts similarly on the neurofibrils.

It is important to emphasise that none of the three categories of enzymes, animal, plant, or microbial possesses exclusively a proteolytic specificity for any cell component. Trypsin and papain have a similar effect on the Nissl bodies, and yet, they are derived from different kingdoms. Secondly, papain's need of an activator connotes at least a slightly different mechanism of scission of the arginin-peptide bond. The animal pepsin and microbial EB 21 both act very restrictedly on the neurofibrils, while the Nissl bodies continue to stain normally. However, the very fact that the former is effective only at a highly acid pH, and the latter near neutrality, indicates that all organisms of origin have a need of certain, and similar, end effects with respect to proteolysis in order to maintain their metabolism. The mechanism in each case is an example of evolutionary adaptation and biological specificity.

IV. DISCUSSION

A. The Neurofibril

One of the most significant works with regard to the neurofibril is that of Weiss and Wang (1936). They succeeded in showing the progressive elaboration of the neurofibrils in chick embryo sensory neurons. Hild (1959) and Reiser (1959) have written separately very extensive reviews of the literature. The consensus of all the papers cited by them is that the neurofibril has risen from the status of an artifact to that of an integrated and functional part of the nerve cell. Several theories of function are presented, including that of conduction of the nerve impulse, a role in cellular metabolism, and even involvement in the memory trace.

None of the theories of function, unfortunately, has been able to throw any light on the composition of the neurofibril. According to Reiser (loc. cit.), this matter is still practically untouched.

Maxfield and Hartley (1957) and Maxfield (1950-51) isolated a fibrous protein from the giant nerve fibers of the squid. This protein is believed to be the chief constituent of the neurofibrils. Koechlin (1955), working in the same group, has investigated the chemical composition of the axoplasm from which the protein was derived. Squid axoplasm total "base", as represented

by ionic potassium and sodium, is balanced largely by organic acids. Analysis showed high percentages of aspartic and glutamic acids. These two together almost equaled the sum of all other amino acids, both in regard to free amino acids and total axoplasm of wet tissue.

Although a high content of di-carboxylic amino-acids may be present, which would aid the peptic digestion of this fibrous protein, nothing is said of the aromatic amino acids, tyrosine and phenylalanine, which are a first prerequisite, or of the amino acid sequence, which is so important.

Schmitt (1950, 1957), as a colleague of the previous three authors, reiterates that the fibrous constituent of the axoplasm is visualized as the neurofibrils. Since the neurofibrils of the soma are continuous with those of the processes, what is characteristic of one would most likely be characteristic of the other. They are also almost universally histologically demonstrable. He states finally, however, that there is still no clear knowledge of their function, structure, or composition. There is simply the possibility of continuous polypeptide chains along the fibril's length.

The present observations show that 1 mgm per cent of crystalline trypsin does not digest the neurofibrils, while removing the Nissl bodies completely. The non-crystalline tryptic degradation

of the neurofibrils may therefore point to pancreatic contaminants of proteolytic nature. In view of the possibly acidic nature of the squid axoplasm protein, and if it be true that this acidity can be extended to mammalian neurofibrils, then it is quite conceivable that the free carboxylic groups of aspartic and glutamic acids attenuated action by the trypsin molecule.

B. The Nissl Body and The Nucleohistones

The basophilic components of the nerve cells were first discovered and objectively considered by Nissl in 1889 and 1894. This cellular material has thus received the name of Nissl substance or Nissl body, or tigroid or chromatophilic substance, and is easily discernible as cytoplasmic particulates, which may reach a size of three to four micra in large motoneurons.

Hild and Reiser (loc. cit.) again provide excellent reviews of all available material pertaining to the subject. They present evidence from phase contrast, ultra-violet absorption, centrifugation, and micro-incineration studies and demonstrate by means of fixation, staining, and photographic techniques the pre-existence of Nissl bodies in the living neuron. Held (1895) studied the chemical composition of the Nissl body and first determined its resistance to pepsin. This was confirmed by MacCallum and Scott (1898), working independently, who also found that the Nissl substance was susceptible to trypsin.

Work very pertinent to this thesis has been done by Li Ch'ao-T'eh (loc. cit.). The fundamental method of investigation was a histochemical approach. Localisation of the three amino acids tryptophane, tyrosine, and histidine, by the tetrazone reaction after enzymic proteolysis was used to localise these three and thereby demonstrate the properties of proteins of indi-

vidual organelles of the neuron. These experiments made it possible to construct a map of protein distribution in the cell. Thus, the proteins of the achromatic network of the nucleus are rich in acidic and aromatic residues, while the chromatin consists of deoxyribonucleic acid and of proteins with both acidic and basic amino acids. Digestion of the Nissl bodies indicates that the constituent ribonucleic acid is located at the terminal position of the protein molecule in direct relation to the basic amino acids. These latter are specifically split off by trypsin, and the ribonucleic acid comes with them. Ribonuclease treatment leaves the neuron proteins unchanged. The remaining protein components are trypsin resistant, but apparently sensitive to chymotrypsin and pepsin. This means a high content of dicarboxylic amino acids with many aromatic groups. This includes the neurofibrils. The cell membrane is the most resistant to chymotrypsin, which bespeaks the predominance of peptide linkages formed by non-aromatic amino acid radicals. These findings are in agreement with those of this study.

The principal constituent of the Nissl body is the conjugated protein nucleohistone. It is supplemented by acid and basic proteins. The nucleohistone consists of a nucleic acid, which in turn consists of multiples of nucleotides, each containing a purine or pyrimidine base, the sugar ribose, and three phosphoric

acid molecules, while the histone portion is a basic protein. Histones have a high content of the di-basic amino acids arginine and lysine, ranging up to 17-19 per cent nitrogen, markedly more than the usual protein's 14-15 per cent. All types of histones show a great predominance of cationic over anionic groups, as a result of their arginine and lysine content.

Trypsin and papain have high specificities for these two basic amino acids, and their digestion of the Nissl bodies is explained on this basis. The failure of peptic digestion can also be explained on this basis, in that the high basicity inhibited pepsin's action even though the proper combinations of amino acids to permit peptic digestion may be present.

C. The Nucleus and Nucleolus

E. and E. Stedman (1949) have contributed a paper on the chemical nature of the cell nuclei of several vertebrates. Unfortunately, they did not use any nerve cells, but they have made some important findings.

The discovery of histone nucleates and preparations of nucleoproteins and isolated nuclei have lead to the conclusion that all cell nuclei are similarly composed. It was shown that there are three main components: nucleic acid, a histone, and a second protein known as chromosin. The histone was typically basic, with a positive Millon's tyrosine and a negative tryptophane reaction. Chromosin was non-basic, with a strong tryptophane reaction. This protein accounted for the remainder of the nuclear contents.

The Stedmans have made the generalisation that all animal nuclei contain arginine to the extent of about one quarter of the total nitrogen content of the histoprotein. The nucleic acid and the histone form a conjugated protein in the nucleus for a role in cell metabolism and protein synthesis. Less is known about chromosin. It is admitted that it is hard to obtain it free of nucleic acid. Its function is also unknown.

Hild indicates, however, that by far the greatest part of the ribose nucleic acid is to be found in the nucleolus. The ultraviolet absorption method has proved that significant amounts

are present, and moreover the basophilia is removed through the use of ribonuclease.

This study has shown by enzyme hydrolysis and staining that if an enzyme can attack the Nissl body, as trypsin and papain, then it can also remove the histone of the nucleolus, by reasons of the nucleolus' high arginine content.

The peculiar effect of ficin on the nucleolus is hard to explain. This enzyme, although it affected the cresyl violet and gallocyanin stainability very little, caused a complete vacuolisation of the nucleolus as shown by the Bodian stain. Some protargol stainable component of this organelle was digested out, and which was either not a nucleohistone protein, may have been a part of this histone's molecule not concerned in Nissl type staining, or may have been chromosin itself.

D. Enzyme Specificities

The specificity of trypsin has been widely documented by Bergman, Fruton, and Pollok (1939), Green and Neurath (1954), Hofmann and Bergmann (1941), Sumner and Myrbäck (1950), and as far as proteins are concerned, there are only two types of chemical bond which trypsin attacks.

Trypsin requires the presence of basic diamino acids for its action. There must be either the free basic epsilon-amino group of lysine or the delta-guanido group of arginine in the side chain immediately adjacent to the site of hydrolysis. Again, nucleohistones contain high percentages of these amino acids.

Papain is an enzyme with proteolytic properties from the latex of the papaya plant. This enzyme must be activated by agents such as cyanide ion, hydrogen sulfide, cysteine, glutathione, or ascorbic acid. In this particular study, activation was by sodium sulfite and urea which is recommended by Weiner (1956), and also by Lennox (1953).

Although papain shows a very wide range of specificity toward synthetic substrates, which leads to suspecting the presence of more than one active component, the highest specificity is toward the arginine amide, Stockell and Smith (1957). This is shown by the table accompanying the latter work. Sub-

strates with other amino acids showed far less specificity.

Hofmann and Bergmann (loc. cit.) have shown a very interesting comparison of trypsin's and papain's specificities for the basic amino acids of the Nissl body. The sensitivity of the substrate to the enzyme, or the specificity of the enzyme, are dependent on the nature and position of the amino acids in the substrate's peptide chain.

It is to be noted that bromelin's intermediate specificity for the Nissl bodies, described in the section on results, is in accordance with the experimental data discussed in the aforementioned literature, Bergmann, Fruton, and Pollok (loc. cit.).

Unfortunately, no information is available on its mechanism. Data on ficin are also lacking.

Pepsin shows an entirely different specificity, almost diametrically opposed to that of trypsin.

As shown by Fruton and Bergmann (1939), synthetic substrates are amenable to the use of the enzyme pepsin, and their study effectively demonstrates a high specificity. The most important prerequisite for this hydrolysis is the presence of one of the aromatic amino acids, tyrosine or phenylalanine. Substitution of other amino acids for the aromatic groups resulted in resistance to peptic action. The most sensitive substrate was shown to be carbobenzoxy-l-glutamyl-l-tyrosine. Substitution of

the dicarboxylic acid indicated hydrolysis at lower rates. These findings seem to point to a preference of pepsin for a highly acidic peptide bond, both in terms of the phenolic group of tyrosine, and the free carboxylic group of glutamic acid. However, sensitivity toward pepsin is dependent not only upon the nature of the participating amino acids, but on still other structural details.

This favorable influence of the free carboxyl group suggests inhibition of pepsin's action in the vicinity of free amino groups. 1-glutamyl-1-tyrosine is completely resistant. A large number of substrates with the diamino groups of lysine and arginine, as well as histidine, was also tested, and all proved resistant. Pepsin does rapidly and extensively hydrolyse proteins with high percentages of aminodicarboxylic acids, plus the aromatic groups. This may help to explain the digestion of the neurofibril. Protamins and histones, with much lysine and arginine, are resistant.

E. Interpretation of Autoradiographs

The results obtained from the autoradiographs confirm the preceding findings. Histological processing and washing are known to remove, almost quantitatively, the non-protein components which have taken up the labeled amino acids. In addition, leucine is utilised primarily for protein metabolism, particularly when the animal is killed only one hour after injection. Therefore, whatever radioactivity is present, as shown by the grain density, is due almost solely to proteins.

Tryptic digestion of sections has proved to be the most efficient in removing protein from tissues. This is strongly indicated both by the degradation of cells, shown by the hematoxylin and eosin (Fig. 24) stained sections, and by the grain counts. Comparison of control counts with those of the digested sections points to a hydrolysis which, by cleaving proteins at the previously discussed arginine and lysine sites, also succeeded in removing a large portion of the remaining unmetabolised leucine from the tissues (Fig. 25). In view of trypsin's specificity, it comes as no surprise that cell counts could not be made, since the scission of the linkages of these two basic diamino acids in the nucleohistone of the Nissl bodies caused breaking off of numerous peptide chains in which the leucine was included.

Although the trypsin control section counts were high for the cells, no counts could be made for digested sections because the emulsion was lost from the slides, and also, as could be seen from the stained sections which remained on the slides, the cells except the Glia and such as half digested nucleoli and the remnants of cell walls, had been digested out (Fig. 25). Trypsin also caused a large drop in the counts over non-cellular areas, as seen in the unstained slides. Had the autoradiographic emulsion remained on the sections, it is a practical certainty that the radioactivity would have been found to be almost quantitatively removed.

The lesser extent of the peptic digestion is also explainable on the basis of specificity. The high content of lysine and arginine inhibited the action of pepsin to the extent that the protein of the Nissl bodies remained relatively intact. Hydrolysis could only take place at those sites where this basicity could not interfere with the breaking of the aromatic amino acid bond. What digestion did take place, and that it did is shown by the diminution of the counts from the control to 0.1 per cent (Fig. 27), 0.5 per cent (Fig. 28), and 1.0 per cent (Fig. 29) pepsin solutions, was probably greatly limited to other proteins of the cell. The neurofibrils, if they truly possess a more acidic protein, would also be more susceptible to pepsin, although

this component very likely assumes a lesser role in the total protein picture.

Pepsin in itself has shown interesting results. The pepsin controls (Fig. 26), strongly acidic, show definitely lower counts than those of trypsin in their neutral buffer solution. This would apparently indicate acidic hydrolysis, or a decrease, through acidic effects, of the absorption of the labeled substance, of protein. The one deviate high count among the pepsin controls comes largely from a group of giant motoneurons which may have had a relatively higher uptake of the labeled amino acid. It is also possible that cellular protein is more labile than the protein of the nerve processes. The similarity of counts from sections from both control solutions points to this. Even the counts of digested sections are similar. Increasing concentrations of pepsin do not seem to very greatly change the counts in comparison to minimal digestion with 0.1 per cent.

The decrease in the number of grains after digestion with successively higher concentrations of pepsin is not to be underestimated, however. The change in the appearance of the neurofibrils from the excellent results in the controls to the remnants in the 0.1 per cent and the complete lack of fibrils in the 1.0 per cent can also only point to the above interpretation.

Pepsin's graded reduction of the Ninhydrin-Schiff protein

staining is also an indication of this, and agrees with the radioautographic data. It appears that whatever radioactivity remained was retained by the nucleohistones.

V. SUMMARY AND CONCLUSIONS

1. Peptic digestion showed its greatest effects on the neurofibrils progressing from the lowest concentration of 0.1 per cent through 0.5 per cent and 1.0 per cent. The nucleolus also shows increasing degradation after Bodian staining in the 2.5 hour series. Incubation in 3.0 per cent pepsin for only 0.5 hour showed specific disintegration of the neurofibrils. Cresyl violet and gallocyanin staining showed no change in either Nissl bodies or the nucleoli after peptic action. The Ninhydrin-Schiff protein stain showed a progressive decrease in intensity.

The autoradiograph also showed a progressive decrease in grain counts with increasing concentration, and although there was no evident change in the appearance of the stained cells from the controls, it became increasingly difficult to find unstained cells with phase contrast. Counts over non-cellular areas were diminished.
2. Powdered bulk trypsin's effects on the neurofibrils was similar to pepsin's, with a progressivity due to concentration increase. Bulk trypsin removed Nissl bodies, also in gradient fashion, together with most of the remainder of the cell, as well. Only an occasional cell could still be found.

Trypsin's effect on protein staining with the ninhydrin method was similar to pepsin's. The autoradiograph showed higher grain counts in the trypsin control solution than in the 0.02 N HCl control for pepsin, but the nuclear emulsion of the stained sections was lost. The grain density could thus not be counted. Nevertheless, these sections also lacked evidence of cells, as above. Grain counts over non-cellular areas were less, as with pepsin. Crystalline trypsin, in the amount of 1 mgm per cent, completely removed Nissl substance, without affecting the cell membrane or nuclei. The entire outline of the cell remained visible. Crystalline trypsin did not affect the neurofibrils.

3. Papain's effects on cellular components were similar to those of bulk trypsin. Control sections stained with protargol showed degradation effects of the activator solution, but digested sections were degraded even more.
4. Bromelin caused similar effects on neurofibrils, as shown by all previous non-crystalline enzymes. Its effect on the Nissl bodies was only moderate. Cell shrinkage and loss of Nissl substance were still in evidence.
5. Ficin's effects on neurofibrils were similar to those of the previous enzymes. In addition, there was a vacuolisation of the nucleolus, although increasing concentrations left fewer

of these cells to be seen. Changes in the Nissl bodies and nucleoli after Nissl staining were minimal.

6. Prolase MT 7820 caused the least change in the Bodian silver stained sections. All components remained visible. Nissl bodies and cellular and nuclear membranes remained largely intact, although the 4.0 per cent solution attacked the integrity of the Nissl bodies. The nucleoli, as shown by Nissl staining, have been digested in gradient fashion, and are extremely faint after 4.0 per cent digestion. The ninhydrin technique also shows a definite gradient. Cells are no longer visible after 4.0 per cent.
7. 2.0 per cent EB 21 showed a minimal attack on the neurofibrils. The highest concentration (4.0 per cent) caused a loss of all silver staining cytoplasmic constituents. The nucleoli after silver staining also became lighter. Cresyl violet staining showed fewer changes than after MT 7820 digestion. The nucleoli became somewhat lighter, but Nissl changes were not present. The Ninhydrin-Schiff reaction showed the greatest difference after digestion with 4.0 per cent.
8. Of three Rohm & Haas enzymes, A-4, P-11, and W-15, only A-4 can be described as having had a slight effect on the Nissl bodies. Silver stainability in all experimental sections ap-

peared in the cell membrane and nucleoli only.

After histological processing, ten micron sections of the lower brain stem of white rats were subjected to the action of several enzymes from animal, plant, and microbial sources. A total loss of Nissl stainability resulted after incubation in trypsin and papain, with lesser differences following exposure to bromelin, MT 7820, and A-4. On the other hand, treatment by pepsin and EB 21 specifically eliminated the argyrophilia of the neurofibrils. Bromelin, ficin, papain, and powdered trypsin completely disintegrated the cells in silverstained sections. Ficin also had the peculiar effect of causing the loss of silver stainability in the nucleolus. The significance of these results has been discussed.

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VII. PHOTOMICROGRAPHS

All Nissl Sections were Stained with Cresyl Violet

Fig. 1. Normal control sections for Nissl bodies. No treatment was instituted, although shrinkage is present. All experimental control sections presented the same picture. High power view.

Fig. 2. 0.1 per cent bromelin in a sulfite/urea activator, 2.5 hours. This concentration has caused no change. Low power aspect of Nissl bodies.

Fig. 3. 0.5 per cent bromelin in a sulfite/urea activator, 2.5 hours. Moderate damage to the Nissl bodies is present. The nucleoli have disappeared. This was also the extent of the damage in the 1.0 per cent solution. High power view.

Fig. 4. 0.1 per cent papain in a sulfite/urea activator, 2.5 hours. Moderately extensive damage to Nissl substance and nucleoli can be seen. 0.1 per cent trypsin (powdered form) presented a very similar picture (not illustrated). Low power view.

Fig. 5. 0.5 per cent trypsin (powdered) in pH 7.6 buffer, 2.5 hours. Almost complete dissolution of the Nissl bodies, with a still resistant nucleolus. Only a few such cells could be found. High power view.

Fig. 6. 1.0 per cent trypsin (powdered) in pH 7.6 buffer, 2.5 hours. Here, the nucleolus has also disintegrated, and the cell body is even further damaged. Only a very few cells remained. High power view.

PLATE I

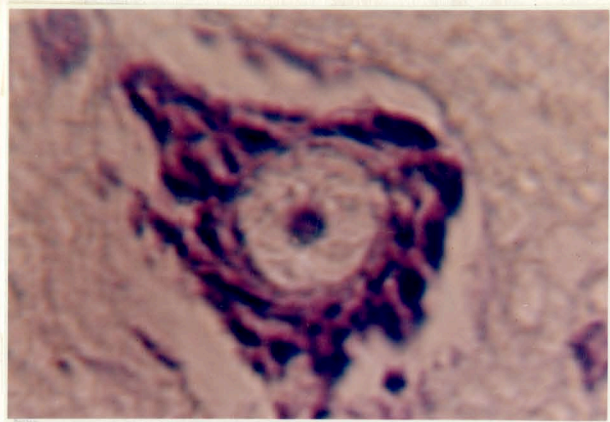


Fig. 1

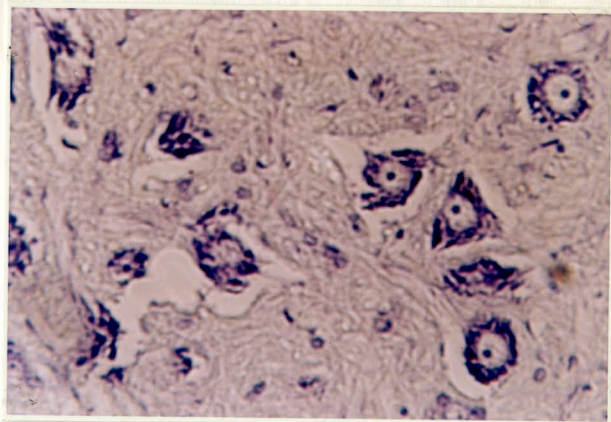


Fig. 2

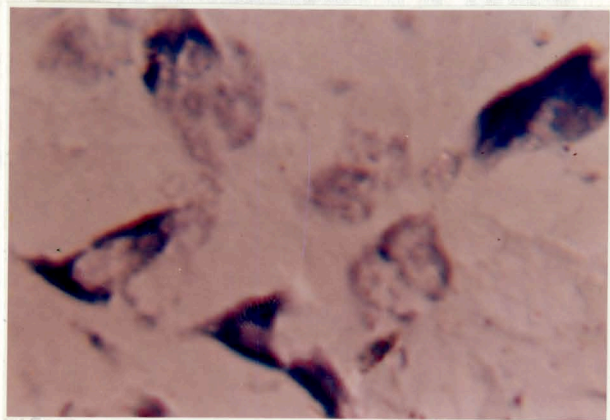


Fig. 3

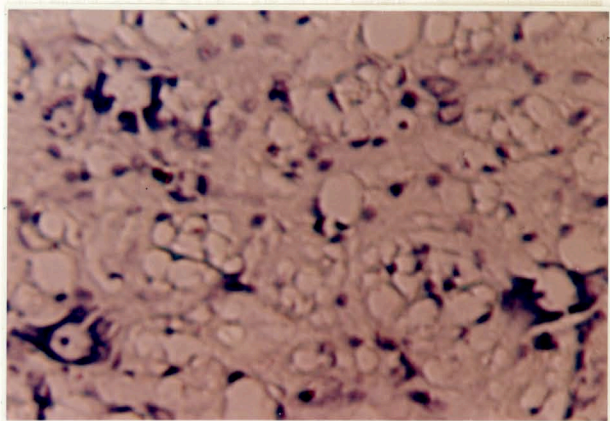


Fig. 4

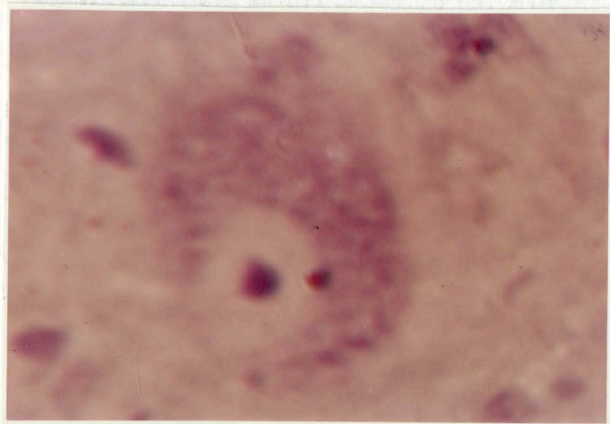


Fig. 5

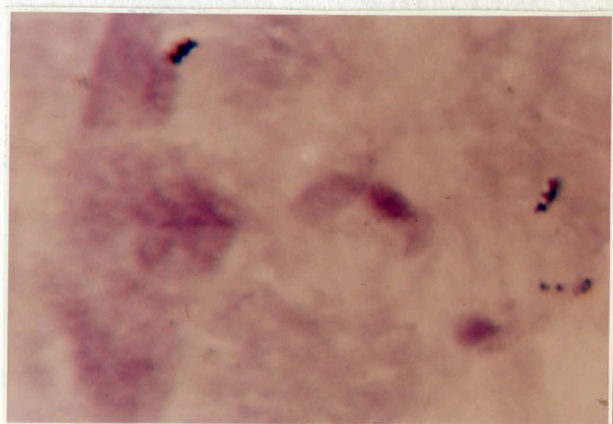


Fig. 6

Fig. 7. 0.5 per cent papain in a sulfite/urea activator, 2.5 hours. Damage to cells is similarly far advanced, but there were more still to be seen. Low power view.

Fig. 9. 4.0 per cent MT 7820 in pH 5.2 buffer, 2.5 hours. Mild Nissl changes are visible, with specific nucleolar damage. Low power view.

Fig. 11. Control solution in pH 5.2 buffer, 2.5 hours. Ninhydrin-Schiff protein stain. This picture shows the cytoplasm to be considerably richer in protein than the nucleus. The nucleolus is also seen as a dark spot.

Fig. 8. 1.0 mgm per cent trypsin (crystalline) in pH 7.6 buffer, 2.5 hours. The Nissl bodies have been specifically eliminated, but nuclei, nucleoli, and cell outlines are still present, to which arrows point for reference. Glia cells are highly resistant. This view is very similar to that of 1.0 per cent papain (not illustrated). Low power view. A = cell membrane; B = nucleus; C = Glia cells.

Fig. 10. 5.0 per cent A-4 in pH 8.0 buffer, 2.5 hours. The Nissl bodies and some nucleoli show extensive breakdown. This was the greatest effect shown by the Rohm & Haas enzymes, A-4, P-11, and W-15. This picture also depicts the effects of trypsin 3.0 per cent, 0.5 hour, on the Nissl bodies (otherwise not illustrated).

Fig. 12. 4.0 per cent MT 7820 in pH 5.2 buffer, 2.5 hours. Ninhydrin-Schiff protein stain. This picture resembles Fig. 8 in that only the nucleus and nucleolus are clearly visible, but the enzyme has totally removed the protein from the cytoplasm. Even the Glia cells have not been spared. In Figs. 8, 11, and 12, the following letters with arrows are equivalent: A = cell membrane; B = nucleus; C = Glia cells.

PLATE II

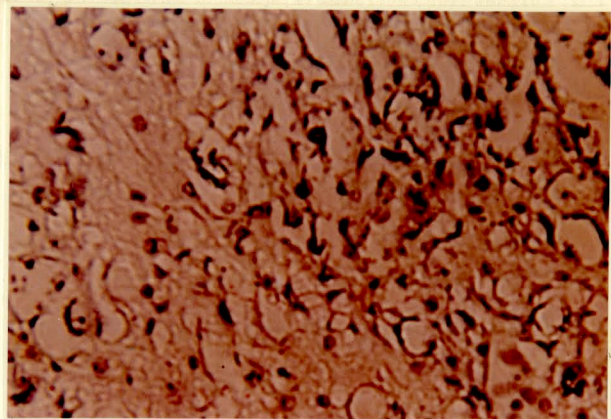


Fig. 7

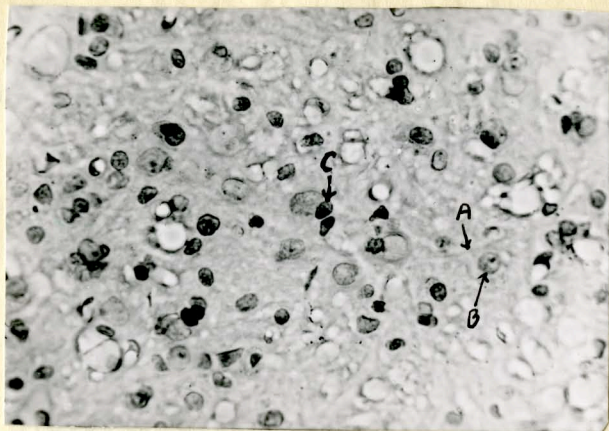


Fig. 8

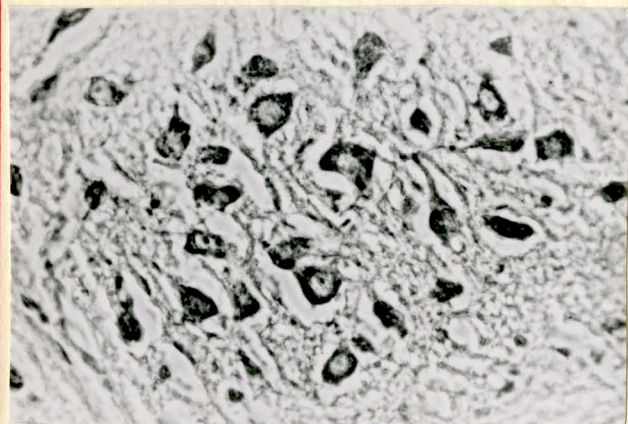


Fig. 9

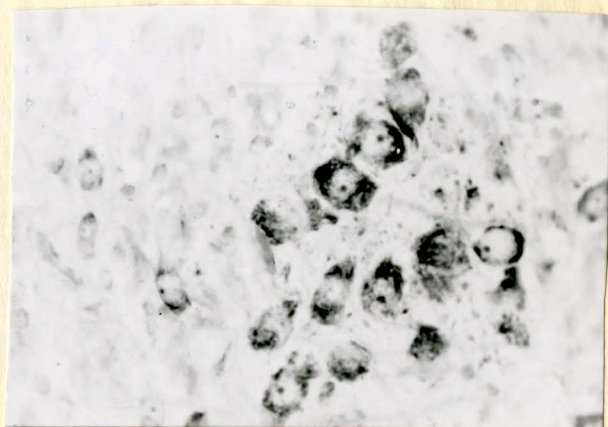


Fig. 10

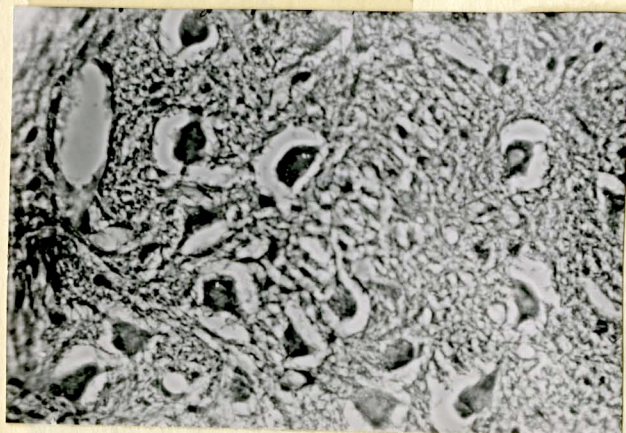


Fig. 11

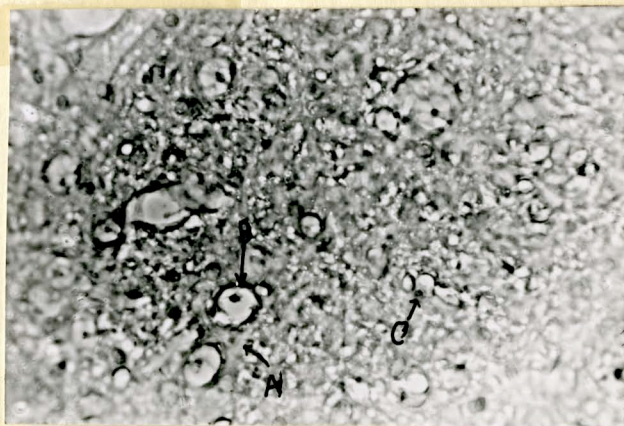


Fig. 12

All Neurofibril Sections were Stained with Silver
Protargol

Fig. 13. Normal control section for neurofibrils. No treatment was instituted, although all experimental control sections, except those for bromelin, ficin, and papain, presented the same picture. High power view.

Fig. 14. 3.0 per cent pepsin in pH 1.8 0.02 N HCl, 0.5 hour. Pepsin has specifically attacked the cytoplasmic neurofibrils without damaging the myelinated processes. Note that cell and nuclear outlines are still almost completely intact. This picture points out the effects of different time and concentration in comparison with Figs. 18 and 19. Low power view.

Fig. 15. 2.0 per cent EB 21 in pH 7.2 buffer, 2.5 hours. There is yet no neurofibrillar damage.

Fig. 16. 3.0 per cent EB 21 in pH 7.2 buffer, 2.5 hours. Only the more resistant cells have withstood enzymic attack. Cells devoid of neurofibrils are comparable to Fig. 14, as are the surrounding processes.

Fig. 17. 4.0 per cent EB 21 in pH 7.2 buffer, 2.5 hours. All cells have been grossly attacked. Only the nuclei are still resistant. Fibers have also suffered greatly. This series aptly demonstrates the concentration gradient effect.

Fig. 18. 0.1 per cent pepsin in pH 1.8 0.02 N HCl, 2.5 hours. This cell displays the initial effects of digestion. The neurofibrils are partially disintegrated, and the fibers have lost much definability.

PLATE III

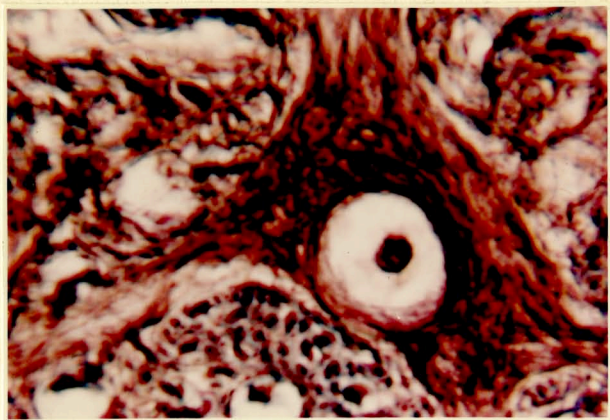


Fig. 13

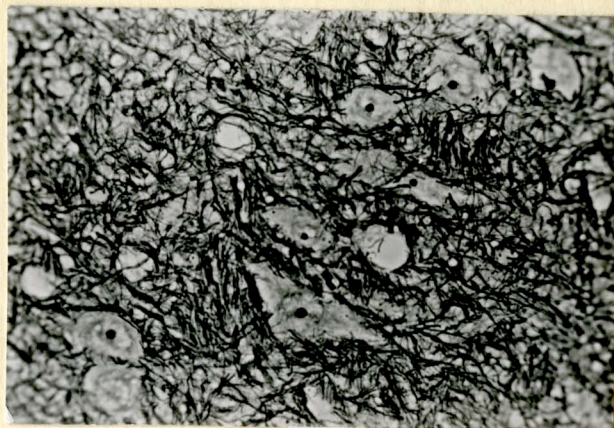


Fig. 14

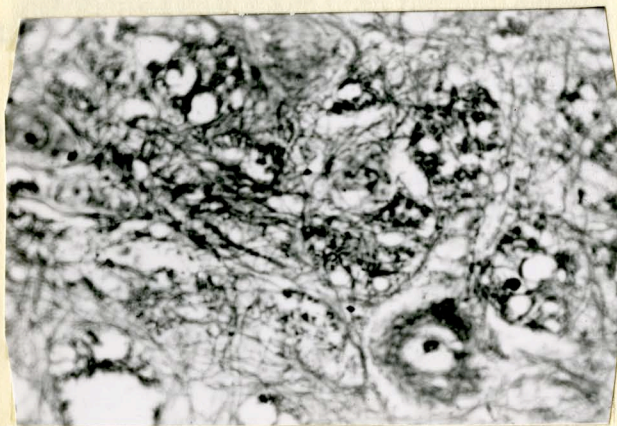


Fig. 15

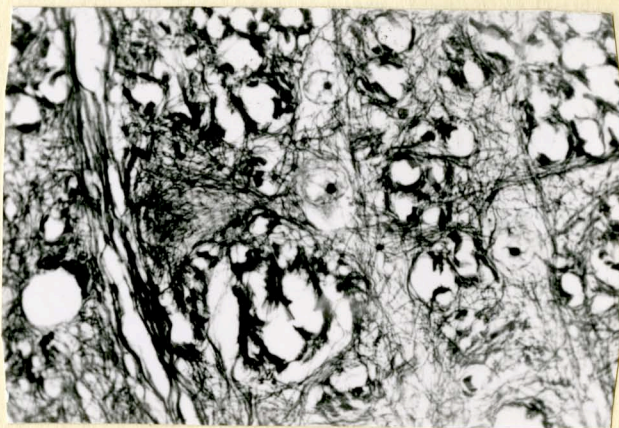


Fig. 16

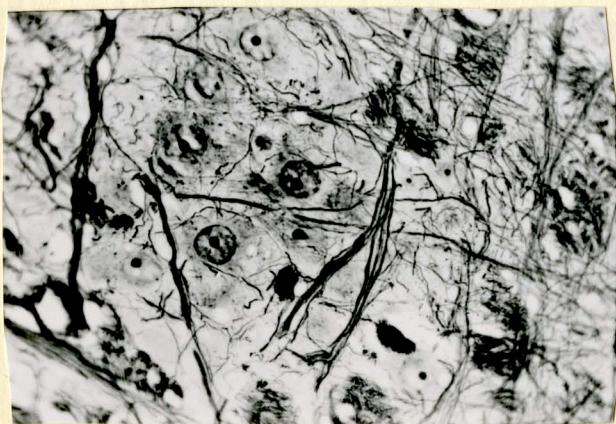


Fig. 17

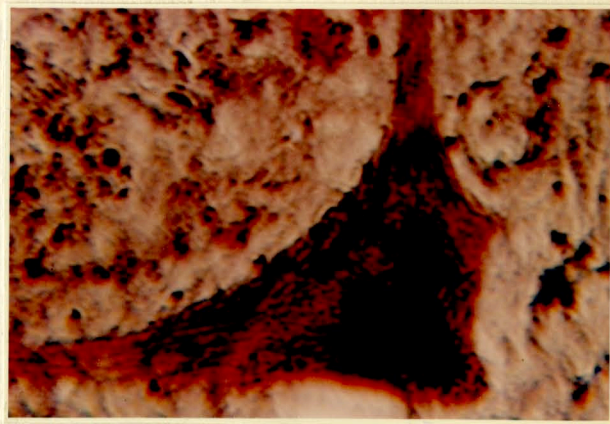


Fig. 18

Fig. 19. 0.5 per cent pepsin in pH 1.8 0.02 N HCl, 2.5 hours. There is a far greater degradation than previously, while the 1.0 per cent solution eliminated all cells, (not illustrated).

Fig. 20. 0.1 per cent papain in sulfite/urea activator, 2.5 hours. The activator solution caused similar damage. 0.1 per cent bromelin's effects were much like this (not illustrated).

Fig. 21. 0.5 per cent bromelin in sulfite/urea activator, 2.5 hours. Only a few dark cytoplasmic remnants are left, but no neurofibrils. 0.5 per cent papain's effects resembled these, (not illustrated).

Fig. 22. 0.1 per cent ficin in SO_3 /urea activator, 2.5 hours. The neurofibrils have been digested. This enzyme specifically digests out the silver staining nucleolar component, as well. 0.5 per cent ficin had the same effect, (not illustrated).

Fig. 23. 1.0 per cent ficin in SO_3 /urea activator, 2.5 hours. Gross digestion of all neurons is visible. This stage is also valid for 1.0 per cent papain and bromelin.

PLATE IV

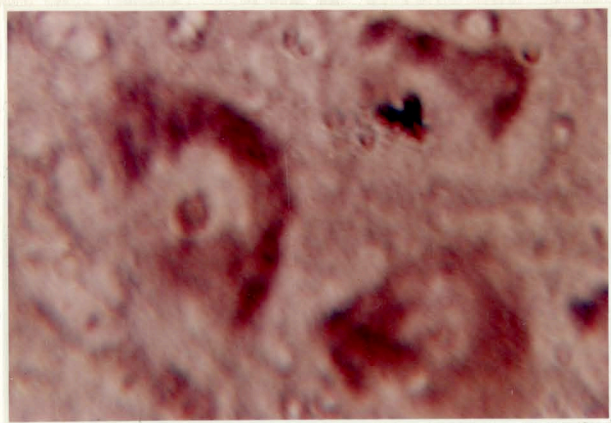


Fig. 19

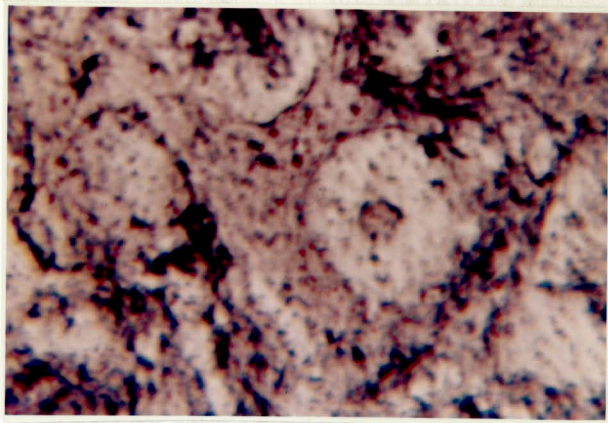


Fig. 20

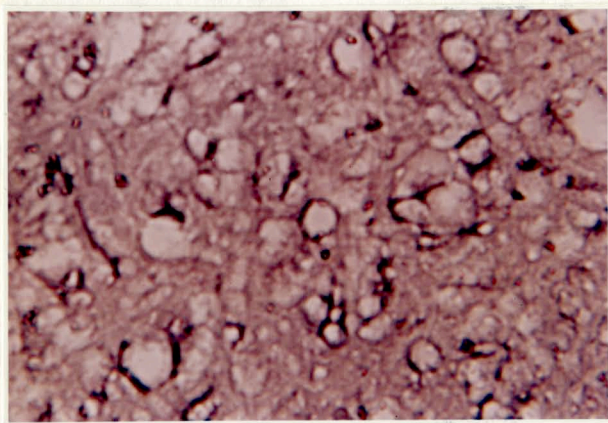


Fig. 21

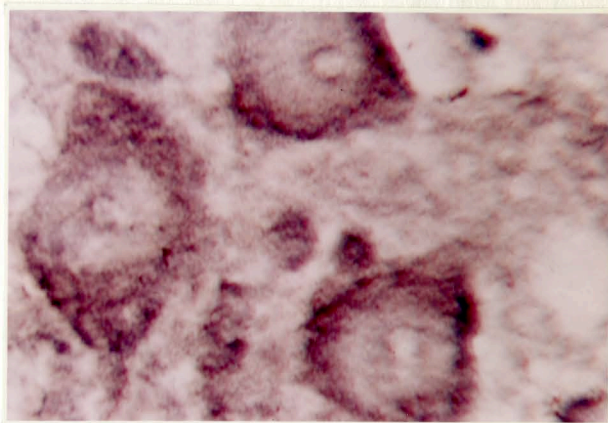


Fig. 22

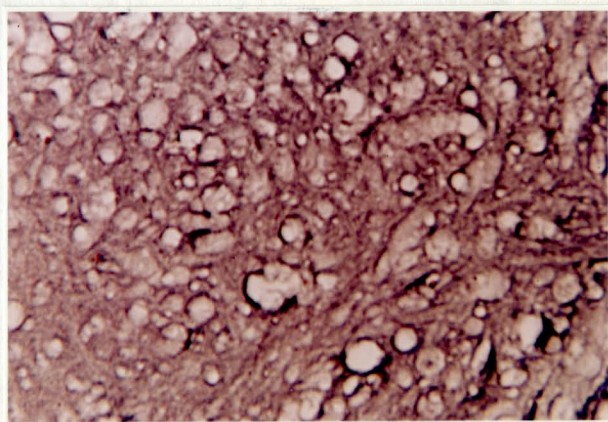


Fig. 23

All Following Figures Are of Hematoxylin and Eosin
Stained Autoradiographs, under Phase Contrast

Fig. 24. Experimental control in pH 7.6 buffer, 2.5 hours. Radioactivity is present and definability is better than in Fig. 26.

Fig. 25. 0.1 per cent trypsin in pH 7.6 buffer. Radioactivity and definability have decreased greatly. A = cell membrane; B = nucleus; C = Glia cells.

Fig. 26. Experimental control in pH 1.8 0.02 N HCl, 2.5 hours. Radioactivity is highly concentrated over the cells. The high acidity has decreased the definability, compared with Fig. 24.

Fig. 27. 0.1 per cent pepsin in pH 1.8 0.02 N HCl. Radioactivity has been decreased, but the stainability not greatly affected, compared with Fig. 26.

Fig. 28. 0.5 per cent pepsin in pH 1.8 0.02 N HCl, 2.5 hours. Radioactivity has been decreased even more.

Fig. 29. 1.0 per cent pepsin in pH 1.8 0.02 N HCl, 2.5 hours. Radioactivity is greatly decreased, but stainability still appears quite normal.

PLATE V

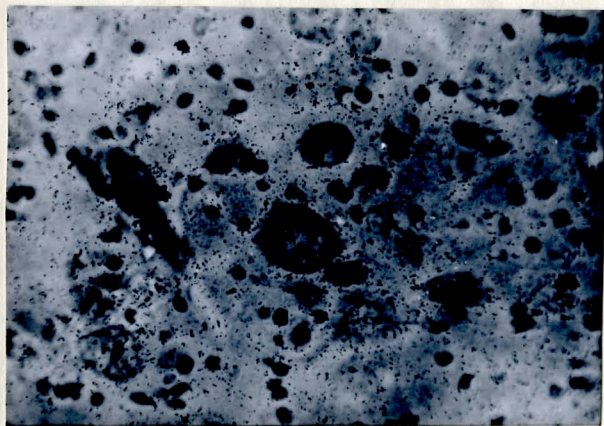


Fig. 24

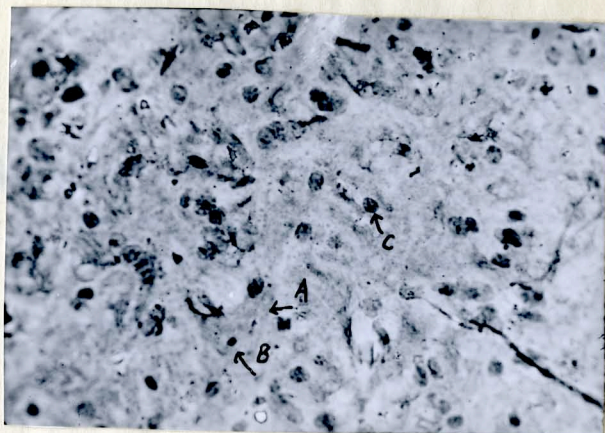


Fig. 25

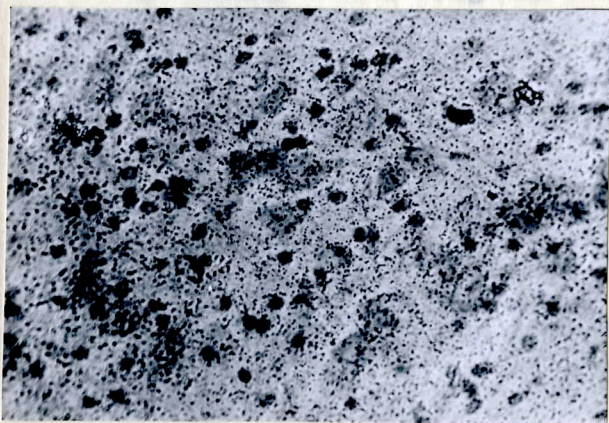


Fig. 26

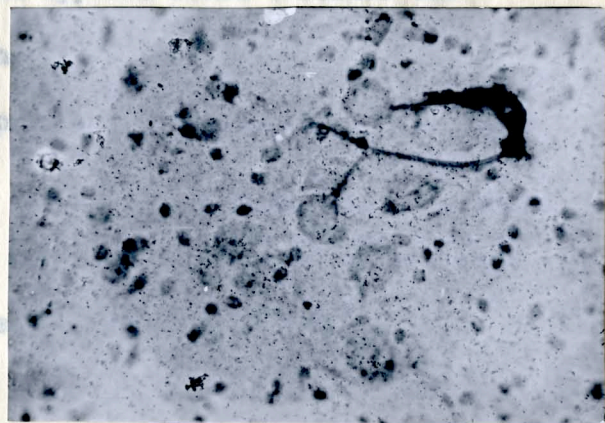


Fig. 27

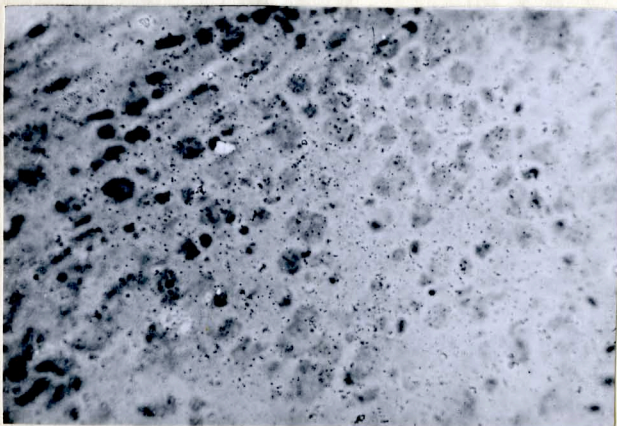


Fig. 28

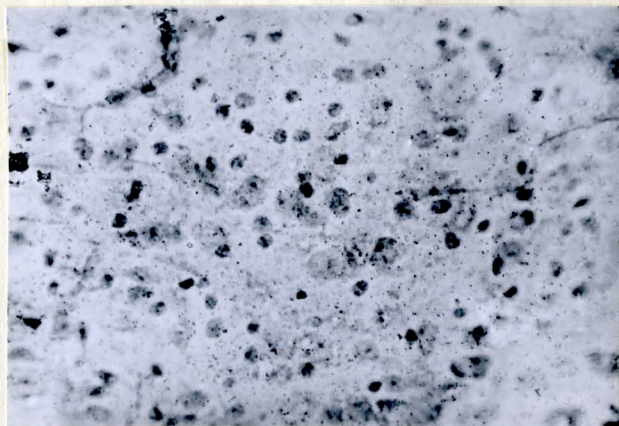


Fig. 29

APPROVAL SHEET

The thesis submitted by Jack T. Beuttas has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 17, 1961
Date

H. Wang
Signature of Advisor