Histochemical Demonstration of Protein-Bound Alpha-Acylamido Carboxyl Groups*

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ABSTRACT

A method has been developed to demonstrate the alpha-acylamido carboxyl groups of protein, taking advantage of the fact that acylamido carboxyl groups are converted to ketonic carbonyls by the action of acetic anhydride and absolute pyridine. The method utilizes deparaffinized sections of tissues fixed in a variety of fixatives. Following the conversion of carboxyls to the methyl ketones, the latter are stained with 2-hydroxy-3-naphthoic acid hydrazide.

Control experiments have indicated that methylation of carboxyls prevented staining, as did carbonyl reagents after the carboxyls were transformed to methyl ketones. Leucofuchsin did not stain the ketonic carbonyls, and only elastic tissue stained with 2-hydroxy-3-naphthoic acid hydrazide without the previous use of the catalyzed reaction with anhydride.

A brief survey of the reaction on various tissues of the albino rat was made, and the effects of various fixatives were assayed. Of particular interest were certain sites, such as acidophiles of the anterior pituitary gland, where an intense reaction occurred. The possibility exists that certain specific proteins rich in terminal acylamido carboxyl groups, by virtue of their protein side chains or low molecular weight, may be demonstrated by this method.

The free carboxyl groups of protein are the delta- and epsilon-carboxyl groups of aspartic and glutamic acids and those from any of the amino acids occurring at the ends of polypeptide chains. Most of the studies of carboxyl groups have been concerned with esterification with acid alcohol, epoxides, mustard gas, and diazo derivatives (1). The relationship of carboxyl groups to the bioactivity of proteins, outside of conferring amphoteric, solubility, and ionic exchange properties, is little understood, although it has been indicated that some protein hormones and a few enzymes require these groups free for activity (2). Much of the difficulty in dealing with the carboxyl groups of pure protein *per se* arises from the fact that, other than titration, there are no specific reactions for carboxyl groups. Esterification is used in most cases. These reactions are not specific, and the conditions of the reactions are somewhat drastic, leading to denaturation or alteration of the protein. In such circumstances, conclusions concerning the effect of esterification on bioactivity must be guarded.

The first prerequisite for the histochemical demonstration of carboxyl groups of protein is that the proteins be immobilized by denaturation or precipitation during fixation and preparation of tissue sections. This step can result in loss of bioactivity, but this is of no consequence from a histochemical point of view. A chromogenic reaction may then be applied for the localization of the groups in protein, provided the reagents are not destructive of tissue morphology.

The possibility of creating a specific method for

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the demonstration of carboxyl groups with an alpha-acylamido group was suggested by the work of Wiley (3), who showed that acylamido methyl ketones are formed by the base (pyridine) catalyzed reaction of acylamido acids and acetic anhydride. This reaction can be expected to occur with carboxylic acids containing an electrophilic nitrogen adjacent to the alpha-carbon. No reaction would be expected with the free delta- and epsilon-carboxyl groups of the dicarboxylic amino acids, because of the absence of an acylamido group alpha to such carboxyl groups. Since Wiley obtained this reaction with *N*-acetylbetaine, he has hypothesized that this reaction proceeds when an acylamido acid is first decarboxylated by the base (pyridine) to a carbanion (Equation 1), which then adds to a carbonyl group of acetic anhydride to give an intermediate anion (Equation 2), and this form subsequently loses acetate to form the methyl ketone (Equation 3).

$$CH_3CON(CH_3)CH_2COOH \rightarrow CH_3CON(CH_3)\overline{C}H_2 + CO_2 + H^+ \quad (1)$$

$$CH_3CON(CH_3)\overline{C}H_2 + (CH_3CO)_2O \rightarrow \left[CH_3CON(CH_3)CH_2\underset{\displaystyle CH_3}{\overset{\displaystyle O^-}{C}}OCOCH_3 \right] \quad (2)$$

$$CH_3CON(CH_3)CH_2\underset{\displaystyle CH_3}{\overset{\displaystyle O^-}{C}}OCOCH_3 \rightarrow CH_3CON(CH_3)CH_2COCH_3 + CH_3COO^- \quad (3)$$

Following the conversion of the carboxyl groups to methyl ketones (Text-Fig. 1, lines *A* and *B*), the latter were reacted with 2-hydroxy-3-naphthoic acid hydrazide, which had been used previously in developing a histochemical method for carbonyl groups of lipide (4). This reaction succeeds in linking a naphtholic group to protein by a hydrazone formation at the site of the ketonic groups (Text-Fig. 1, line *C*) so that a protein-linked azo dye may be developed by subsequent coupling with tetrazotized diorthoanisidine (diazo blue B) (Text-Fig. 1, line *D*). The remainder of this paper is concerned with the presentation and discussion of the method, certain control experiments, the effect of fixation, and the distribution of protein-bound carboxyl groups in selected rat tissues. The method described here was announced previously (5, 6).

Materials and Methods

Tissues were obtained from freshly killed albino rats and fixed from 2 to 16 hours in a variety of fixatives which will be discussed in a subsequent paragraph. These tissues were dehydrated, embedded in paraffin in the usual manner, and sectioned at 5 or 10 μ. Albuminization of slides for mounting was feasible, since smears of albumin alone did not develop a sufficient amount of color to be demonstrable and there was no difference in the degree or localization of staining for carboxyl groups in a series of sections mounted with and without albumin. After deparaffinization in xylene, the mounted sections were washed in several changes of absolute alcohol. They were stained by the following procedure for demonstrating protein-bound carboxyl groups.

Carboxyl Method:

(1) Incubate deparaffinized sections for 1 hour at 60°C. in a mixture of equal parts of acetic anhydride and anhydrous pyridine. A more rapid reaction occurs if the quantity of the anhydride exceeds that of the pyridine up to four of five parts to one part. The pyridine must be anhydrous (redistilled over barium oxide and stored over drierite) for the reaction to proceed effectively.

(2) Wash in absolute alcohol. All alcohols used should be aldehyde-free. The dilute alcohols used later should be freshly prepared from aldehyde-free absolute alcohol.

(3) Incubate for 2 hours at room temperature in 0.1 per cent 2-hydroxy-3-naphthoic acid hydrazide. This solution is prepared by dissolving 50 mg. of the hydrazide in 2.5 cc. of hot glacial acetic acid, to which is added 47.5 ml. of 50 per cent alcohol.

(4) Wash in 3 or 4 changes (10 minutes each) of 50 per cent alcohol.

(5) Incubate for ½ hour in 0.5 N hydrochloric acid at room temperature.

(6) Rinse in distilled water.

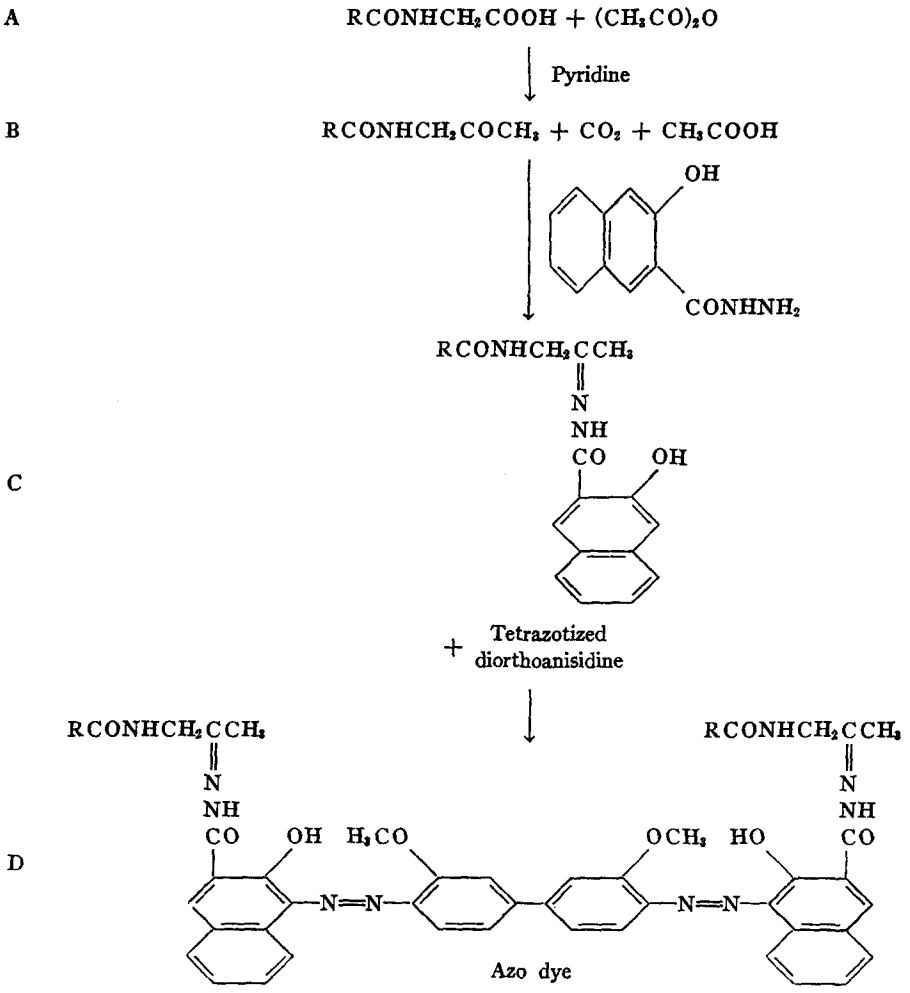
(7) Wash in several changes of 1 per cent sodium bicarbonate.

(8) Rinse in several changes of distilled water.

(9) Place slides in solution of 0.06 M Sorensen's phosphate buffer (pH 7.6), mixed with an equal volume of absolute alcohol, and stir in tetrazotized diorthoanisidine (1 mg./cc.). Allow color to develop, 2 to 5 minutes.

(10) Wash in several changes of distilled water.

(11) Mount in glycerin jelly or dehydrate in a series of alcohols to xylene and mount in permount.



TEXT-FIG. 1

RESULTS

Control Experiments:

If the carboxyl groups were esterified with acid-alcohol (incubation at 60°C. for 24 hours in 0.1 N hydrochloric acid in methanol (7)) prior to the anhydride-pyridine reaction, no staining could be developed with the naphthoic acid hydrazide method. After conversion of the alpha-acylamido carboxyl groups in tissue to ketonic carbonyls, the stain was completely prevented by treatment of the section with semicarbazide. Other tests dealing with the specificity of the reagent, 2-hydroxy-3-naphthoic acid hydrazide, for carbonyl groups are dealt with elsewhere (8). In addition, the ketonic carbonyl groups produced did not stain with leucofuchsin (Schiff's reagent), so that in deparaffinized sections, appropriately treated, only the free aldehydic carbonyls of elastic tissue stained with Schiff's reagent. Only the elastic tissue stained in deparaffinized sections reacted with 2-hydroxy-3-naphthoic acid hydrazide and diazonium salt without previous reaction with acetic anhydride and pyridine.

Effect of Fixatives:

A wide variety of fixatives were assayed with the above method. These fixatives, either alone or in combination, contained the following reagents: formalin, ethyl alcohol, acetone, chloroform, acetic acid, picric acid, mercuric chloride, potassium dichromate, osmium tetroxide. Regardless of the fixative used, a positive result was always ob-

tained, except in the case of osmium tetroxide. This is not surprising since carboxyls are less apt to be destroyed by fixatives than amino or sulfhydryl groups. Since some fixatives combine with carboxyl groups (mercuric chloride), this further emphasizes the fact that the reaction does not occur with the free carboxyl group alone, but depends on the alpha carbon bearing an electrophilic nitrogen.

It was clear from the beginning that the staining in many instances was proportional to the ability of the fixative to retain protein in the section. For example, the staining after sublimate-formol was generally more intense than after neutral 10 per cent formalin or 80 per cent ethyl alcohol. However, there were sites in tissues that will be described in subsequent paragraphs that stained more intensely than others, regardless of the fixative used. On the other hand, other sites were intensely reactive only when fixatives were used which were particularly effective protein precipitants. In the following paragraphs, we will review briefly the staining for protein-bound carboxyl groups of some rat tissue fixed predominantly by two means, with occasional reference to tissues fixed in other ways. The first fixative was Carnoy's fluid (absolute alcohol, chloroform, and glacial acetic acid). This fixative served as a base line for comparison and provided a generally unreactive fixative that retained cytoplasmic contents moderately well and nuclear contents very well. It is recognized, none the less, that some protein is lost from Carnoy-fixed sections, especially that which is initially denatured by the anhydrous reagents and becomes redissolved upon rehydration of the sections. The other fixative used was our modification of Romeis' fluid (85 ml. of physiological saline solution saturated with both mercuric chloride and picric acid, 10 ml. formaldehyde, and 5 ml. acetic acid), which we have found in previous experiments with *in vitro* testing to be an excellent and ordinarily irreversible protein precipitant.

Distribution of Protein-bound Acylamido Carboxyl Groups in Tissues:

The following description of selected tissues of the albino rat, illustrated with photomicrographs, serves as an example of the distribution of staining with this method. It should be recalled first that tetrazotized diorthoanisidine used in the method is a dicoupler, and two colors result when it monocouples (red) or dicouples (blue). In Text-fig. 1, dicoupling is illustrated. If monocoupling occurred, the azo dye would be linked to protein on one side only. When the protein-bound acylamido carboxyl groups were few or widely spaced, monocoupling resulted in a pink to red color, indicating a sparse to moderate amount of carboxyl groups. When the carboxyls were numerous and close together, dicoupling occurred, resulting in a blue color. An intermediate condition was obtained with mixed mono- and dicoupling, resulting in a reddish blue color.

Protein-bound carboxyl groups were widespread in distribution in rat tissues. All epithelial cells, muscle fibers, and the cells of the nervous system and connective tissue were reactive. The nuclei of all cells, excepting the nucleoli (Fig. 9) were less reactive than the cytoplasm. However, some structures were entirely unreactive; these included collagen fibers of connective tissue (Fig. 3), hair shafts, and mucus granules of intestinal goblet cells. Some organs like the stomach (Fig. 1) or the liver evinced a moderately positive reaction throughout; the reaction being homogeneous in all parts of the gastric pits or of the liver lobules. In the kidney cortex, the proximal convoluted tubules reacted more strongly than the distal ones (Fig. 7) or the collecting ducts. In the proximal tubules the reaction was most intense at the brush border and the basal parts of the cells, while in the distal tubules the reaction was practically confined to the basal parts of the cells (Fig. 7). In addition, it was especially clear with Romeis' fixed sections that the staining of the convoluted tubule cells was primarily due to carboxyl groups in mitochondrial proteins. Finally, basement membrane about the tubules, contrary to collagenous fibers, was moderately reactive. The glomeruli stained unusually intensely for a protein method, the major reactivity being due to the capillary endothelial cells (Fig. 7). In the medulla of the kidney (Fig. 8), the cells of the thick portions of Henle's loop reacted more intensely than the cells of the convoluted tubules; but the thin portions of Henle's loop and the collecting tubules reacted weakly.

In the pancreatic acinar cells a moderate reaction occurred in both the granular apical portions and in the bases. The islets stained less intensely than the rest of the pancreas, and there was no obvious difference between alpha and beta cells on the basis of the staining reaction. The apical portion of the epithelium of the pancreatic ducts was the most intensely staining element of the pan-

creas. In the submaxillary gland (Fig. 2) the acini stained like those of the pancreas, while the cells of the secretory and tubular portions of the duct system stained more intensely. Reaction of the cells of the duodenum and colon was not remarkable, and moderate reaction occurred throughout except for the mucus which was unstained. Smooth muscle fiber reacted more intensely than epithelial cells.

Sections of blocks taken from the neck region were of interest, particularly because of the number of different tissues included in such a block and the wide variety of staining results. In the esophageal epithelium (Fig. 3), the cytoplasm of basal cells stained more intensely than the rest of the cells in the stratum Malpighii, while the cornified cells of the stratum corneum were most reactive of all. However, the epithelium of the esophageal glands reacted more strongly than that of the mucous membrane. Striated fibers of the strap muscles of the neck were more reactive than epithelial cells. The A bands, particularly of contracted skeletal muscle, were more reactive with this method than the I bands (Fig. 12). This was determined by the presence of the Z band in the lighter stained region with the light microscope and was confirmed with the polarizing microscope.

In the trachea (Fig. 5), the pseudostratified epithelium was moderately positive, but the cilia reacted intensely. Contrary to the intestinal mucus, the mucoid droplets in the tracheal epithelium were moderately positive. The ground substance of the tracheal cartilage reacted the most intensely of all the tissues examined (Fig. 6). Although the reaction was not homogeneous, and consisted of islands of moderate staining amidst the intensely stained areas, it was clear that the area of interstitial substance immediately surrounding the chondrocytes, the cartilage capsule, was the most intensely reactive part. Chondrocytes reacted moderately. The follicle cells of the thyroid gland reacted moderately, while the colloid was somewhat more reactive.

In the central nervous system the Purkinje cells of the cerebellum reacted intensely, whereas the fibers in the molecular layer and the cells in the granular layer evinced a sparse reaction (Fig. 10). Somatic motor neurons in the brain stem were less reactive than Purkinje cells, but their nucleoli were more intensely reactive (Fig. 9). The epithelium of the choroid plexus (Fig. 4) was among the most reactive epithelia examined. In peripheral nerve both the axon and Schwann sheath were moderately positive.

Finally, in the anterior lobe of the pituitary gland, fixed with the most effective protein precipitants, Romeis' fluid or sublimate-formol, the granular cytoplasm of acidophiles reacted intensely, while those of basophiles and chromophobes reacted negligibly or weakly (Fig. 11). When the glands were fixed in reagents that were not as effective protein precipitants, the staining of all these cell types tended to be more equal.

DISCUSSION

Histochemical methods for the general identification of proteins include the Millon reaction (9), the diazonium reaction (10) and other methods for phenolic groups in protein (11), the Sakaguchi reaction (12) and various methods for amino (13, 14), and sulfhydryl and disulfide groups (15–17). The last two methods have been reviewed recently (18, 19) and demonstrate, in particular, the effectiveness of using naphthol-containing reagents for the demonstration of specific groups in protein. To this list can now be added the carboxyl method described here.

A point is to be made concerning group specific and non-specific protein reagents. The former are defined as those reagents which, under *suitable conditions*, react with only one type of functional group in protein. The number of such specific reagents is limited, whereas non-specific reagents are plentiful; and, although their reactions may be restricted to protein, they usually react with a number of groups. It should be noted that a specific reagent may become non-specific if the conditions for the specific reaction are not adhered to scrupulously. A good example of this difficulty is illustrated in the conditions required to limit the reaction of mercaptide-forming agents to sulfhydryls alone (16).

The acylamido carboxyl method, presented here, is a group-specific method that may be used as a general protein method. Its specificity derives from the fact that an acylamido methyl ketone is produced from carboxylic acids in a protein chain which contains an electrophilic nitrogen adjacent to the alpha carbon. This ketonic carbonyl reacts with 2-hydroxy-3-naphthoic acid hydrazide, but not with Schiff's reagent. The former reaction may be blocked by the usual carbonyl reagents (*e.g.*, semicarbazide). Moreover, in deparaffinized sections of tissue, there were no native free carbonyl groups

(except those in elastic tissue) to be confused with the ones produced in the reaction, since the carbonyls associated with lipides had all been extracted. Once alpha-acylamido methyl ketones are produced, reaction with 2-hydroxy-3-naphthoic acid hydrazide forms a hydrazone by which the chromogenic moiety (naphthol) is linked to protein. Subsequently a pigment of good quality and intensity and linked to protein is developed at these sites by coupling with a diazonium salt.

Although the histochemical distribution of acylamido carboxyl groups was found to be widespread, certain tissues are of interest because they reacted more intensely than the others, regardless of the fixative used, or after a fixation which precipitated and retained most of the protein. Some elements in these tissues are normally basophilic (*e.g.* cartilage matrix), while others are acidophilic (muscle fibers and pituitary acidophiles), and still others (*e.g.* basement membrane) do not stain with ordinary histological methods. This intense reaction is presumably due to the concentration of terminal acylamido carboxyl groups, including those of protein side chains. The second carboxylic acid group of a dicarboxylic amino acid cannot react, since it does not have an alpha-acylamido group.

A high concentration of low molecular protein would stain more intensely than an equal weight of high molecular weight protein. However, a negative reaction does not rule out the presence of acylamido carboxyl groups, since special steric factors may operate to block the conversion of carboxyl to ketone or the reaction of ketone with the hydrazide. On this basis, we feel that this method may be used to identify certain specific proteins rich in terminal amino acid groups or proteins of low molecular weight. Preliminary work on the acidophiles of the pituitary tends to substantiate this hypothesis. Only those fixatives which retain the simple protein hormones of the anterior pituitary are followed by the intense staining reaction, whereas a weaker stain is produced if one or more of the proteins are dissolved and removed. In addition, various physiological circumstances which alter the content of these hormones in the gland result in a parallel alteration in the intensity of staining for acylamido carboxyl groups of protein.

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EXPLANATION OF PLATES

All photomicrographs illustrate the results of the method for demonstrating carboxyl groups of protein. The tissues were taken from freshly killed albino rats and were fixed in either Carnoy's fluid (Figs. 1, 2, 4, 7 to 10) or Romeis' fluid (Figs. 3, 5, 6, 11, and 12).

PLATE 89

FIG. 1. Section of pyloric portion of stomach. A moderate reaction occurs in the cells of the gastric glands. Mucous neck cells and nuclei of all cells are unstained. Smooth muscle fibers (lower right) are reactive. × 125.

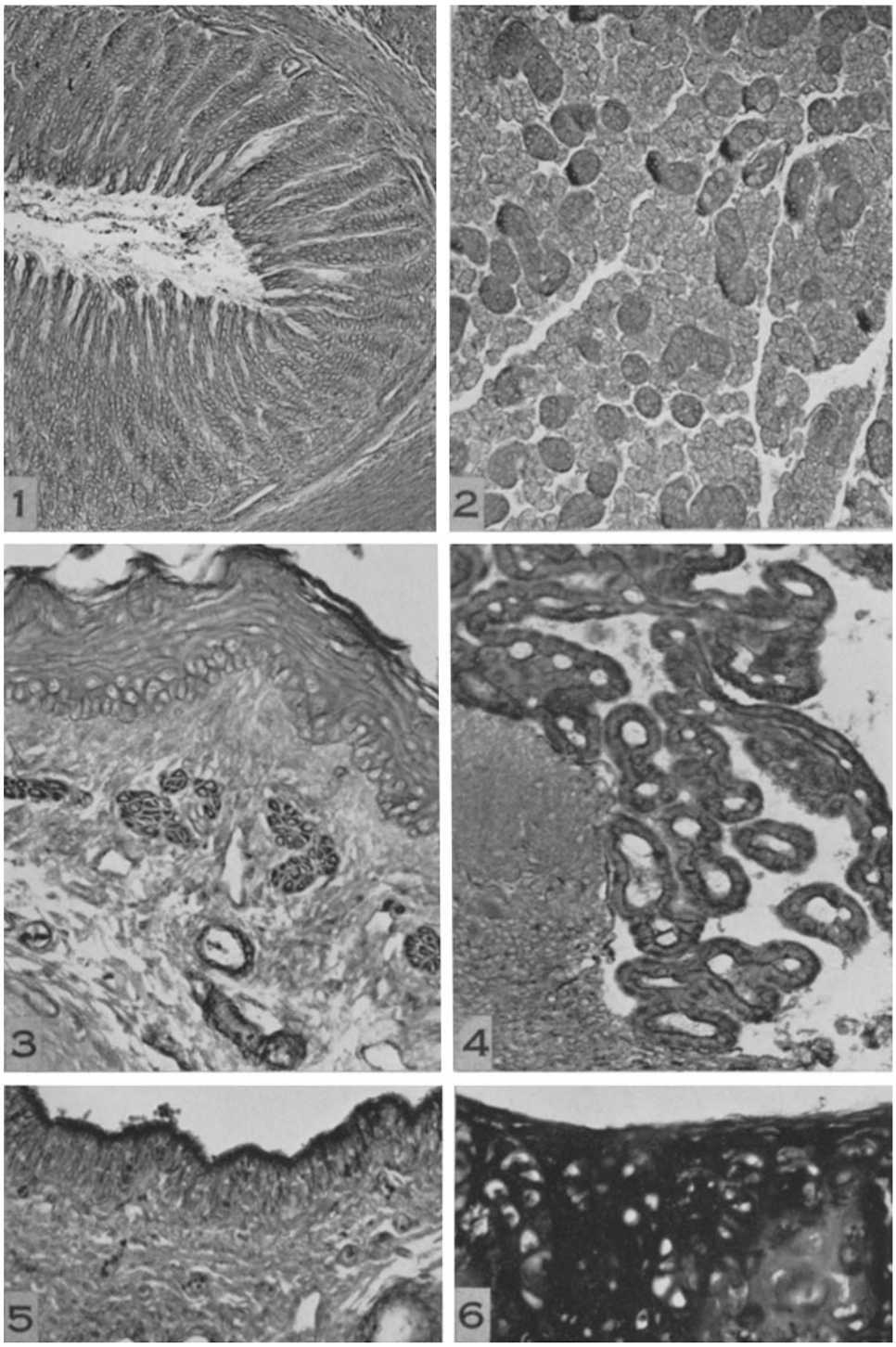
FIG. 2. Section of submaxillary gland. Acini are weakly reactive, while the secretory and tubular portions of the ducts react strongly. × 125.

FIG. 3. Section of esophagus. Outer scales of the stratum corneum of the mucous membrane are the most reactive part of the epithelium. Subepithelial esophageal glands are intensely reactive, but surrounding connective tissue fibers react poorly, if at all. The walls of two blood vessels in the connective tissue are also strongly reactive. × 225.

FIG. 4. Epithelial cells of the chorioid plexus are strongly reactive, especially their apical borders. × 250.

FIG. 5. Section of trachea showing an intense reaction at the apical surface of the tracheal epithelium, including cilia. Small reactive globules of mucoid substance are scattered throughout these cells, giving them a mottled appearance. Scattered cells in the lamina propria react weakly, while those of the tracheal glands (lower right) react moderately. × 225.

FIG. 6. Section through tracheal hyaline cartilage. The cartilage matrix was the most reactive structural element in the tissues surveyed. Reaction tended to be spotty with intensely and moderately reactive areas, and the most reactive areas were the cartilage capsules (the areas of interstitial substance immediately around the chondrocytes). × 225.



(Barrnett and Seligman: Demonstration of carboxyl groups)

PLATE 90

FIG. 7. Section of kidney cortex. Most of reactivity of the glomerulus (center) is confined to capillary endothelium. The proximal convoluted tubules which surround the glomerulus are reactive at the bases of the cells and at the brush border. The cells of distal convoluted tubules (lower left) are reactive only in the basal region. × 225.

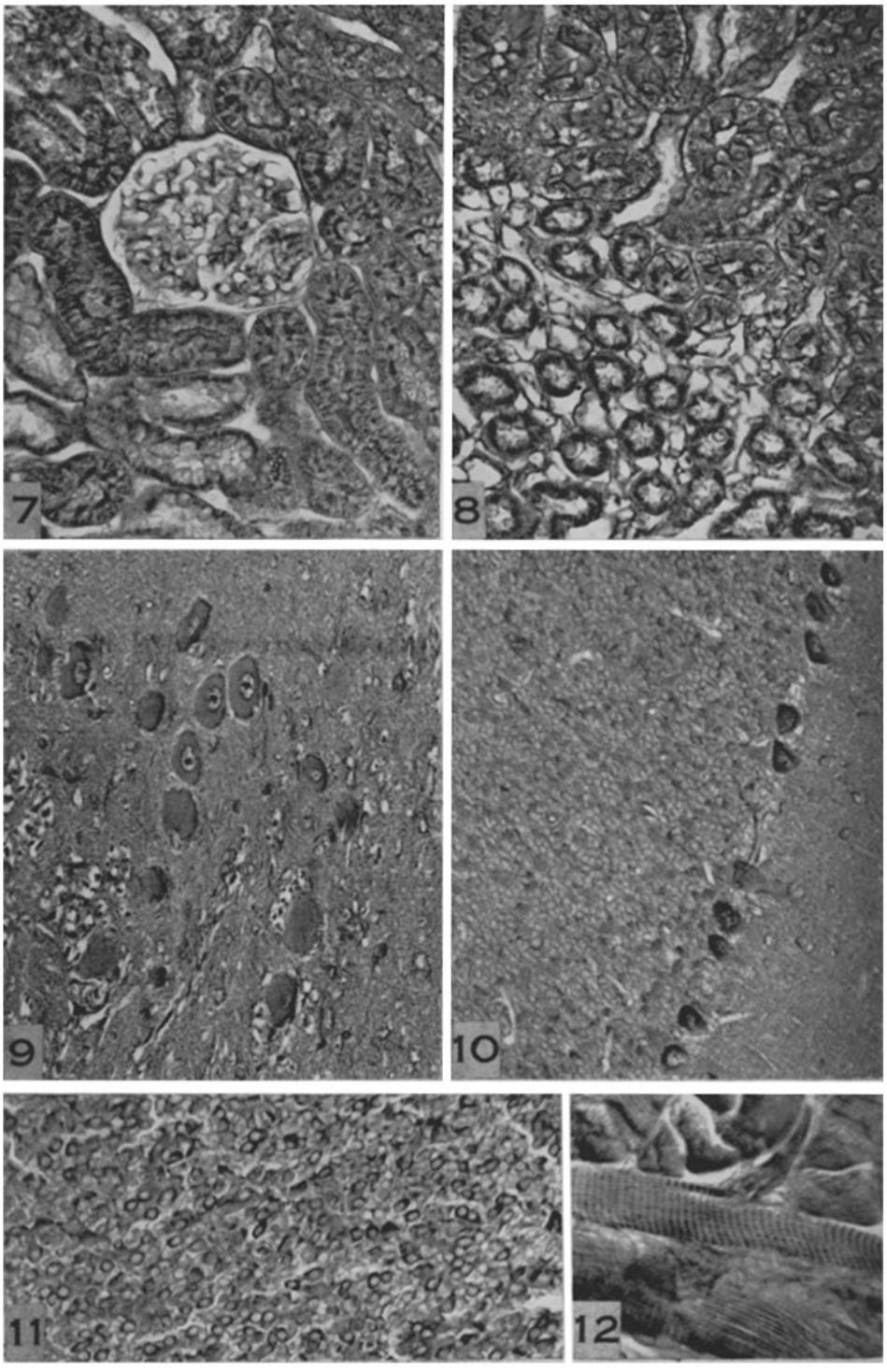
FIG. 8. Section of kidney through the junction of the cortex (upper part) and medulla (lower). The cytoplasm of the cells of the thick portion of Henle's loop in the medulla is more reactive than that of the convoluted tubules in the cortex. Note that the basement membrane surrounding the tubules of the cortex is reactive. × 225.

FIG. 9. Section through a motor nucleus of the brain stem. Cytoplasm of the large motor neurons is moderately reactive, and the nucleoli react intensely. The neuropil is weakly reactive. × 250.

FIG. 10. Section of cerebellum showing Purkinje cells with strongly reactive cytoplasm. Fibers in the molecular layer react weakly, whereas various elements in the granular layer react moderately. × 225.

FIG. 11. Section through the central part of the anterior pituitary gland. All the cells which contain intensely reactive, granular cytoplasm are acidophiles. × 225.

FIG. 12. Section of striated muscle fibers from strap muscles of neck. The center of the field is occupied by a contracted muscle fiber which shows that the A bands are more intensely stained than the I bands. × 500.



(Barrnett and Seligman: Demonstration of carboxyl groups)