A HISTOCHEMICAL METHOD FOR DISTINGUISHING BETWEEN SIDE-CHAIN AND TERMINAL (α-ACYLAMIDO) CARBOXYL GROUPS OF PROTEINS

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ABSTRACT

The specificity of the Barrnett-Seligman method for the histochemical demonstration of α-acylamido carboxyl groups (C terminal) of proteins is dependent on the conversion of such groups to ketones by the action of acetic anhydride and absolute pyridine. Studies on model compounds show that the side-chain carboxyl groups also react in the method and that most of the final color developed can be attributed to these carboxyls, rather than to the C terminal carboxyl groups. It is postulated that the side-chain carboxyls react by formation of mixed anhydrides in the presence of acetic anhydride and pyridine. This mixed anhydride then could link with a hydrazide to form a dihydrazide, which is capable of coupling with a diazo dye. Acetic anhydride treatment alone, without pyridine, also yields mixed anhydride. The mixed anhydride derived from the side-chain carboxyls can be destroyed by base, whereas the methyl ketone derived from the C terminal carboxyl is unaffected, and this treatment makes the method specific for C terminal carboxyl groups. Tissues treated in such a fashion demonstrate that all the color reaction obtained in the method is due to side-chain carboxyls, and that C terminal groups yield little or no staining as would be expected for "average" molecular weight proteins.

INTRODUCTION

Barrnett and Seligman (1) recently proposed a method for the histochemical demonstration of terminal α-acylamido carboxyl groups in proteins. The method was based on the work of Wiley (2), who had studied the base-catalyzed reaction of *N*-acylamino acids with acid anhydrides to form acylamido ketones. This reaction has been widely studied since 1928 when Dakin and West (3) first demonstrated the formation of ketones from amino acids. Many ketones have been obtained with *N*-acylamino acids (2–7), and the mechanism generally accepted proposes an azlactone as an intermediate (3–5). However, Wiley (2) also obtained a methyl ketone from acetylsarcosine when reacted with acetic anhydride in the presence of pyridine, under more drastic conditions, and claimed that substitution of N-CH_3 for N-H prevented formation of an azlactone from acetyl-

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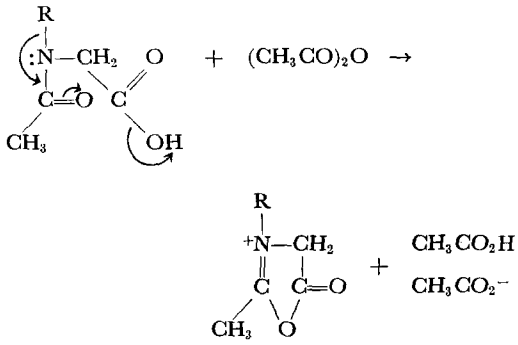
sarcosine. Consequently he proposed two alternate mechanisms, one involving decarboxylation due to the base, caused by the electrophilic nature of the α-amino group, and an attack of the carbanion thus formed with acetic anhydride. This doubtful hypothesis was challenged by two subsequent papers (8, 9). These two papers emphasized that it has been shown that the presence of a hydrogen atom on the α-carbon was the important factor (3, 5, 8–11) rather than the hydrogen on the nitrogen atom. A further example of the reaction of N-substituted amino acids was the conversion of α-dimethylaminophenylacetic acid to a ketone (10). Cornforth and Elliott (8) proposed the formation of a transitory oxazolonium cation as the intermediate, which would accomodate all known examples:—



This is then followed by acetylation on the α-carbon, ring opening and decarboxylation proceeding as normally. These authors further showed that heating acetylglycine in pyridine did not cause decarboxylation, as required by the Wiley (2) mechanism.

As a less likely alternative Wiley (2) suggested that the acylamido acid undergoes C acylation as an active methylene compound, a generalization of the previously stated azlactone mechanism.

Barrnett and Seligman (1) extrapolated the conclusions of Wiley (2) in the base-catalyzed conversion of acylamino acids to acylamido ketones, to the histochemical demonstration of C terminal carboxyl groups of proteins. They supposedly reacted only the methyl ketones produced from the terminal α-acylamido carboxyl groups with 2-hydroxy-3-naphthoic acid hydrazide with consequent hydrazone formation. A protein-linked azo dye was subsequently developed by coupling with tetrazotized diorthoanisidine (diazo blue B).

Barrnett and Seligman apparently accepted the first hypothesis offered by Wiley (2) for the mechanism of the base-catalyzed conversion of the C terminal carboxyl to a methyl ketone as being sufficient. Thus the specificity of their method is dependent on the validity of the hypothesis that only methyl ketones are produced from carboxylic acids in a protein chain in which there is an "electrophilic nitrogen" adjacent to the α-carbon. Thus the side-chain β- and γ-carboxyl groups of aspartic and glutamic acids would not be expected to react, because there is no "electrophilic nitrogen" adjacent to the carboxyl. As there are relatively few free carboxyls adjacent to so called "electrophilic nitrogen" groups in most tissue proteins because most of the free carboxyls are the β- and γ-carboxyls of the respective dicarboxylic acids, the development of a strongly positive reaction for α-acylamido carboxyls in tissue sections requires elucidation. Barrnett and Seligman have also postulated that the presence of an intense reaction can be attributed to a high concentration of reactive acylamido carboxyl groups, either due to a specific protein rich in such terminal groups or a high concentration of low molecular weight protein. We will present evidence that such a postulate is not necessarily valid.

Although Barrnett and Seligman assumed that the side-chain carboxyls of glutamic and aspartic acids would not react, and that only the C terminal carboxyl groups would give a ketone, they did not exclude the possibility that carboxyl groups, other than acylamido carboxyls, might yield reactive carbonyl groups in the presence of pyridine and acetic anhydride, by some mechanism *other* than that proposed by Wiley for C terminal carboxyl groups. The purpose of this paper is to demonstrate that this is indeed the case and that the original histochemical method is not specific for the C terminal carboxyl groups, but demonstrates the side-chain carboxyls of proteins as well. It will be further shown that the procedure may be adapted for the demonstration of C terminal carboxyl groups.

It might be emphasized at this point that although the Wiley mechanism for the base-catalyzed conversion of acylamino acids to acylamido ketones is unacceptable to the present authors, it is our main concern to show that the side-chain carboxyl groups of proteins will yield reactive carbonyl groups, presumably mixed anhydrides, in the presence of pyridine and acetic anhydride, which will further react with the hy-

drazide and subsequently couple with the diazo dye. It will also be shown that the C terminal carboxyls do yield reactive carbonyls, probably ketones *via* azlactone formation, but, in fact, this reaction is responsible for only a small proportion of the dye-coupling obtained. The major color reaction is due to the side-chain carboxyls yielding, in all probability, mixed anhydrides in the presence of acetic anhydride and pyridine. It will further be shown that the side-chain carboxyls also yield mixed anhydrides with acetic anhydride alone.

MATERIALS AND METHODS

A. Test Compounds: Powdered samples of the various compounds to be tested, weighing 10 mg., were placed in small centrifuge tubes and reacted in the protein-bound carboxyl method. The conditions were exactly those prescribed by Barrnett and Seligman (1) for tissue sections with one minor modification, namely that in those cases in which the polypeptide was somewhat soluble at neutral or alkaline pH, the alcohol and aqueous washes were kept slightly acid (pH 6.0). At every step of the procedure the compounds were thoroughly mixed with the added reagent, then centrifuged down, the supernatants discarded, and the next reagent added. The various controls and other additional procedures utilized are specified later.

B. Tissue Sections: Blocks of various tissues of the rat, calf nasal septum, rabbit ear, and from the neck region of the rabbit, rat, and guinea pig were taken for fixation. Blocks containing cartilage were selected because this tissue gives an intense reaction in the carboxyl method. The blocks from the neck region were especially useful in that they contain a number of different tissues. The tissues were fixed in buffered formalin (pH 7), 10 per cent formol-alcohol, and, as recommended by Barrnett and Seligman (1), a modified Susa fluid, containing acetic acid, and formalin in physiological saline saturated with both mercuric chloride and picric acid.

RESULTS

In order to test the specificity of the method for C terminal protein carboxyls, various model compounds were reacted in the method, as shown in Table I. The results obtained indicated that the side-chain as well as the C terminal carboxyl groups were reacting. Poly-α-L-glutamic acid, or its sodium salt, poly-α-L-sodium glutamate, which contain both C terminal carboxyl groups and side-chain carboxyls gave an intense color. When the side-chain γ-carboxyl groups of polyglutamic acid were blocked as benzyl esters, the C terminal carboxyl group, which was not blocked, gave only a slight reaction. Polyacrylic acid, in which there are no acylamido carboxyl groups, yielded an intense reaction. As polyacrylic acid is quite acidic, there was a possibility that the reaction obtained was due to non-specific ionic binding of the hydrazide. This was excluded as follows: after washing with 3 N HCl following the treatment with hydrazide, the coupling reaction remained strongly positive.

However, C terminal carboxyls do react, as was evidenced by the strong reaction of tetraglycine, which has no side-chain carboxyls, but provides numerous acylamido carboxyl groups. The slight reaction of poly-γ-benzyl-L-glutamate and poly-β-benzyl-L-aspartate is similarly due only to C terminal carboxyl groups.

The observation that glutamic acid was unreactive was anticipated, as Dakin and West (3) had shown that it did not react with acetic anhydride and pyridine to yield an acylamido ketone, because, under the conditions used, pyrrolidonecarboxylic acid is formed, which blocks the

TABLE I

Reaction of Model Compounds in the Histochemical Test for Protein Carboxyl Groups

Compound	Result*	Color‡
Poly-α-L-glutamic acid§ (mol. wt. 50,000)	+++	Blue
Poly-α-L-sodium glutamate§ (mol. wt. 50,000)	+++	Blue
Poly-γ-benzyl-L-glutamate§ (mol. wt. 64,000)	+	Slightly pink
Polyacrylic acid	++++	Blue
Tetraglycine (mol. wt. 266)	+++	Blue
Glutamic acid	—	—
Poly-α-L-aspartic acid‖ (mol. wt. 15,000)	+	Slightly pink
Poly-β-benzyl-L-aspartate‖ (mol. wt. 15,000)	+	Slightly pink

Controls: All compounds were reacted without prior anhydride and pyridine treatment. All controls were negative.

* Intensity of final color scored − to +4.

‡ Diazo blue B is capable of either mono- or di-coupling, yielding pink or blue colors respectively.

§ Blout, E. R., and Karlson, R. H., *J. Am. Chem. Soc.*, 1956, **78**, 941.

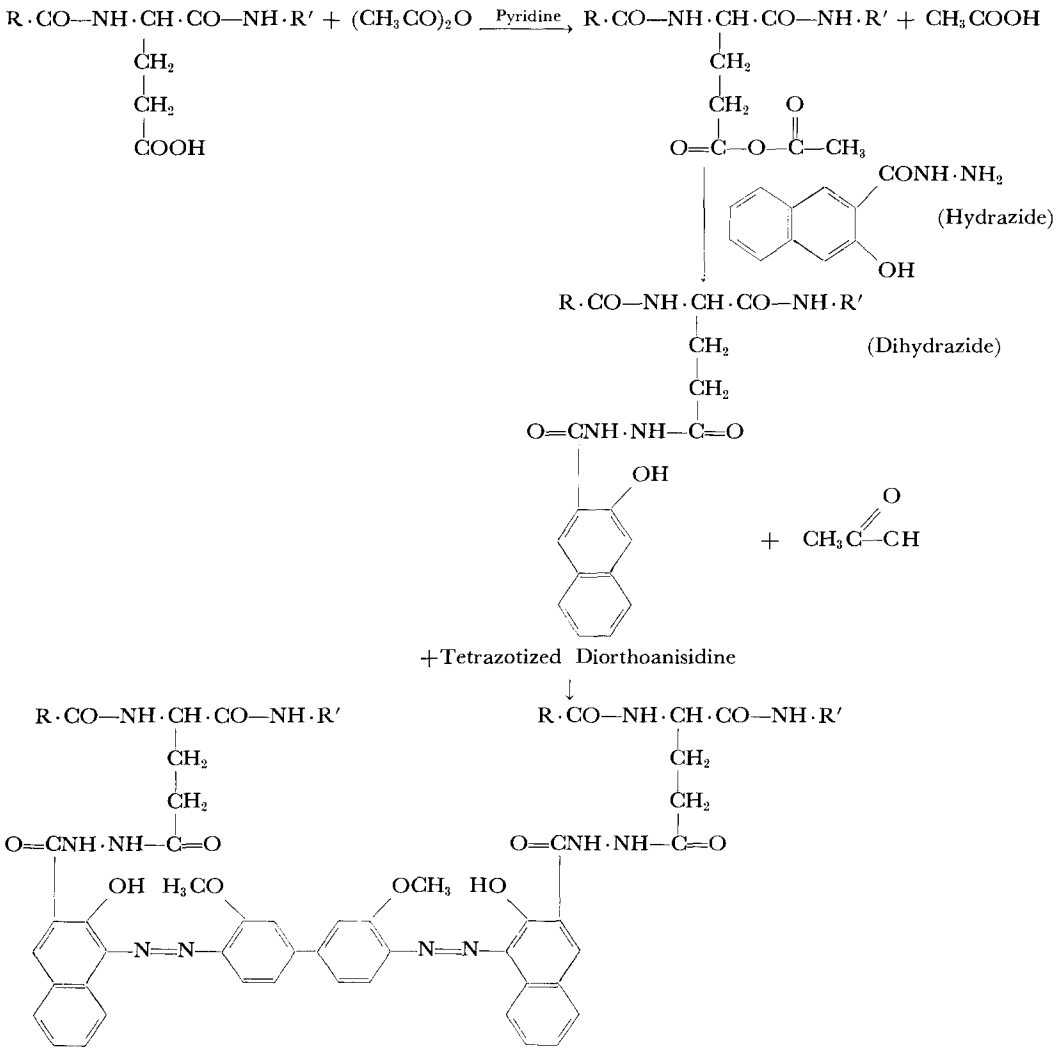
‖ Berger, A., and Katchalski, E., *J. Am. Chem. Soc.*, 1951, **73**, 4084.

azlactone formation. The failure of poly-α-L-aspartic acid to react strongly is, at present, inexplicable. Perhaps, due to stereochemical factors, the formation of mixed anhydrides is prevented. Nevertheless, both poly-β-benzyl-L-aspartate and poly-α-L-aspartic acid gave the same slight reaction, indicating that only the C terminal carboxyl group was reacting in each case.

Mechanism of Reaction of Side-Chain Carboxyl Groups

Accepting the azlactone mechanism for the reaction of C terminal carboxyls, it is obvious that the side-chain carboxyls cannot react by a similar scheme, as there is no α-nitrogen adjacent to the carboxyl group. Therefore, an alternative mechanism must be sought.

It was first determined that pyridine and other base treatment alone yielded negative results. It therefore appeared that acetic anhydride was necessary for the production of a reactive carbonyl group. The formation of a mixed anhydride is postulated: this could then link with the hydrazide to form a dihydrazide, which is capable of coupling with the diazo dye to yield a colored end-product. This scheme is shown below.



Scheme of reaction of side-chain carboxyls

If a mixed anhydride is so formed by the acetic anhydride and pyridine treatment, then it should be possible to destroy it by base, with consequent failure of formation of the dihydrazide, and negative color reaction on attempted dye-coupling. Such indeed was found to be the case. (See Table II.) Poly-α-L-glutamic acid gave only a very slight reaction after base or prolonged ethanol treatment, probably due to the methyl ketones derived from the C terminal carboxyl groups. Polyacrylic acid gave negative results after base treatment. On the other hand, tetraglycine gave no diminution of reaction after base or alcohol treatment. These results indicate that the C terminal carboxyl groups react in a different manner from side-chain carboxyls, with acetic anhydride and pyridine. Methyl ketones derived from C terminal carboxyls would not be destroyed by base treatment.

The reaction of the sodium salt of poly-α-L-glutamic acid might at first appear to contradict the postulate of anhydride formation. However, when it is recalled that a facile interchange between an anhydride and a salt,

Anhydride of A + Salt B
$\rightleftharpoons$ Salt of A + Anhydride of B,

can occur (12), this reaction fits well with the anhydride mechanism. It has also been shown, under comparable conditions, in the Perkin-Fittig reaction, that the anhydride is essential and the sodium salt of the carboxylic acid can be replaced by other bases such as pyridine (13). Indeed, we found that the sodium salt of poly-α-L-glutamic acid gave a strong reaction on treatment with acetic anhydride alone. On the other hand, tetraglycine and polyacrylic acid gave negative results with acetic anhydride alone, and positive results with acetic anhydride and pyridine, as would be expected.

Results with Tissue Sections

The results obtained with tissue sections corroborated those obtained with model compounds. Tissues reacted included rat uterus, spleen, lung, trachea, liver, heart, adrenal, kidney, thyroid, stomach, skin, striated muscle, ovary, anterior pituitary, and cerebral cortex. Treatment with 0.1 N NaOH at 22°C. for 15 minutes following the acetic anhydride and pyridine yielded completely negative staining in sites in which strongly positive staining is otherwise obtained *e.g.* hyaline cartilage, striated muscle, colloid of thyroid gland, acidophiles of pituitary. Further, treatment with acetic anhydride alone, omitting the pyridine, gave a reaction no different from that obtained with both acetic anhydride and pyridine treatment. This gives additional proof that it is mixed anhydride from the side-chain carboxyls rather than ketone formation which is responsible for coupling with the hydrazide.

TABLE II

*Effect of 0.1 N NaOH in 90 Per Cent Ethanol at 37°C. for 2 Hours following Acetic Anhydride-Pyridine Treatment in the Histochemical Test for Protein-COOH Groups**

	Base-treated	Controls in 90% ethanol‡
Poly-α-L-glutamic acid	+	+++
Tetraglycine	+++	+++
Polyacrylic acid	—	++++

* Intensity of final color scored — to +4.
‡ Prolonged ethanol treatment (16 hours) also partially decreases the final color obtained.

DISCUSSION

Barrnett and Seligman (1) contended that their histochemical reaction was specific on the *a priori* hypothesis that only those carboxyls which had an electrophilic group in the alpha position would yield reactive carbonyls in the presence of acetic anhydride and pyridine. Thus C terminal carboxyl groups would react, and side-chain carboxyls would not.

Setting aside the question of the mechanism for the production of methyl ketones from C terminal carboxyl groups in the presence of acetic anhydride and pyridine, our results do demonstrate that the side-chain carboxyls of glutamic acid yield reactive carbonyl groups, probably by the formation of mixed anhydrides. However, the C terminal carboxyl groups may yield methyl ketones, probably *via* azlactone formation, as acetic anhydride and pyridine-treated tetraglycine, subsequently treated with base, does not show a decreased dye-coupling reaction in the method. It is possible, therefore, to have a truly specific method for C terminal carboxyl groups (provided they are available and in sufficient number) by hydrolyzing the mixed anhydrides yielded by the side-chain carboxyls with suitable base or other treatment.

One of the puzzling features of the Barrnett-Seligman method was the origin of the numerous C terminal carboxyl groups purported to be shown in tissue sections. These authors explained intense staining on the presence of a high concentration of low molecular weight protein, containing a high local concentration of C terminal carboxyl groups, or a specific protein, rich in such groups. If these postulates be true, in regard to the tissues we have examined, a considerable degree of staining should still be present, even after base treatment had eliminated the mixed anhydrides derived from the side-chain carboxyls. In point of fact, this is not the case, as the tissues yielded negative results after base treatment, indicating sparse C terminal carboxyl groups of proteins of average or high molecular weight as would be expected in tissue sections. In a subsequent paper we shall show that this is especially relevant to the intense reaction obtained in hyaline cartilage, which is apparently due to the presence of large, high molecular weight aggregates of chrondromucoprotein, rather than to a high concentration of low molecular weight protein or polypeptide. In regard to our survey of tissues in the rat, we have not been able thus far to demonstrate C terminal carboxyl groups. However, the possibility that proteins of low molecular weight or polypeptides may be lost from the tissues during fixation, must be considered. It is possible that in some species or in certain pathological conditions, strongly positive staining in tissue sections could be attributed solely to the presence of these end groups. However, in such instances, care will have to be taken to ensure that the contribution of carbonyl groups derived from the side-chain carboxyls has been eliminated.

The question may well be asked if the method is even specific for protein carboxyl groups. From our initial results one would expect all carboxyl groups, including those of carbohydrates, to react. It will be shown (14) that the carboxyl groups of carbohydrates do not react, for reasons not at present clearly understood. It is possible that the many adjacent hydroxyl groups may react with the mixed anhydride to yield esters, thus preventing reaction with the hydrazide.

Due to the lack of reactivity of poly-α-L-aspartic acid one may postulate that the method may be specific for only γ-carboxyls of glutamic acid. However, further work is necessary to verify this statement.

A final word of caution might be added in regard to the interpretation of histochemical observations from a structural point of view. Barrnett and Seligman (1) have stated, "The first prerequisite for the histochemical demonstration of carboxyl groups of proteins 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." The necessity for immobilization of the protein is unquestioned but whether this denaturation has no histochemical significance can be questioned. The above authors go on to state: "When the protein-bound acylamido groups are few or widely spaced, monocoupling (of diazo blue B) resulted in a pink to red color, indicating sparse to moderate amount of carboxyl groups. When the carboxyls were numerous and close together, dicoupling occurred, resulting in a blue color."

It is felt that denaturation, necessary for fixation, may lead to serious difficulties in interpretation of histochemical observations from the structural viewpoint. In denaturation the stereorelations between the carboxyls in the native protein can be greatly altered and the conclusions drawn from the denatured protein must be extrapolated with great caution to structural interpretation of the native material.

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