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**MECHANISM OF DANSYLATION OF THE POLYAMINE
PENTAAZAPENTACOSANE 5 HCl**

by

Susan Klara Heimbecher

**A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHARMACY PRACTICE AND SCIENCE**

**In Partial Fulfillment of the Requirements
For the Degree of**

**DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PHARMACEUTICAL SCIENCES**

In the Graduate College

THE UNIVERSITY OF ARIZONA

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Pentaazapentacosane 5 HCl

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TO EUGENE

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ABSTRACT

Pentaazapentacosane pentahydrochloride (PAPC-HCl) is a synthetically produced aliphatic pentaamine that is being investigated for use as an anticancer agent. As part of this research project a rapid high-performance liquid chromatographic method for determination of the dansyl derivative of PAPC was developed. The chromatographic system uses a reverse phase C-8 column, a mobile phase of acetic acid buffer and acetonitrile and UV detection. The dansylation conditions were optimized with a pH of 11.0 and a 20 fold dansyl chloride excess. The yield of dansyl PAPC increased 10 fold as the reaction pH was changed from 9.5 to 10.5.

An investigation of the products formed in the dansylation reaction revealed that, even under conditions of pH and dansyl chloride concentration most likely to produce partially dansylated products, only perdansyl PAPC is present. This unexpected finding is explained by a mechanism whereby 1) only completely unionized amine molecules will dansylate and 2) the ratio of unionized molecules to ionized molecules increases as dansylation proceeds.

The proposed mechanism is verified by comparing the dansylation vs. pH profile of PAPC to that of a reference monoamine (piperidine ·HCl). After 4 hours at room temperature and pH 9.5, 100 % of piperidine is dansylated while under the same conditions only 10 % of PAPC is derivatized. A pH

greater than 10.5 is required to completely dansylate PAPC. This difference is significantly greater than would be predicted from the pK_a values but it is consistent with the proposed mechanism.

CHAPTER I. INTRODUCTION

Endogenous Polyamines

Endogenous polyamines are found in virtually all living cells (Campbell, et. al. 1978; Tabor and Tabor, 1984). As might be expected from their ubiquitous presence, these compounds play a critical cellular role. They are essential for cell growth and proliferation and, in higher plants and animals, for cellular differentiation (Tabor and Tabor, 1984; Pegg, 1986). Of significant interest to oncologists is the fact that the highest polyamine concentrations are found in rapidly dividing cells such as cancer cells or those of embryonic tissue (Takami, H. et. al., 1979; Caldarera, C. M., et. al., 1965) and, within both normal and malignant cells, polyamines are found primarily in the nucleus (Sarhan and Seiler, 1989).

Chemically, the polyamines are straight chain aliphatic compounds containing two or more basic nitrogen groups. The principle members of this class, putrescine, spermidine and spermine, contain two, three and four ionizable groups, respectively. The structures and pK_a values of these compounds are given in Table 1-1. On the basis of these values, the predominant form of these compounds, at physiological pH, will be where all the amine groups carry a positive charge. Although the mechanism of

polyamine cellular regulation is not known, the ability of these polycationic species to interact with negatively charged DNA and RNA probably plays a significant role (Symons, 1995; Marton and Morris, 1987). Continued interest in the potential use of polyamines to understand and control cell regulation has lead to extensive research on their metabolism and biomedical applications.

Metabolism

As described in Fig. 1-1, the synthesis of polyamines in mammalian cells begins with the decarboxylation of ornithine to form the diamine putrescine. This reaction is catalyzed by the enzyme ornithine decarboxylase (ODC) which is tightly regulated by both inhibitory substances such as ODC antizyme (Brosnan and Hu, 1988) and inducers such as hormones, drugs, and growth factors (McCann et. al. 1987). Spermidine is then formed from putrescine by the addition of an aminopropyl group. This reaction is catalyzed by the enzyme spermidine synthase and the aminopropyl moiety is donated by decarboxy-S-adenosylmethionine (dc-SAM). The aminopropyl donor is, in turn, formed from S-adenosylmethionine (SAM) in a decarboxylation reaction catalyzed by S-adenosylmethionine decarboxylase (SAM-dc). Finally, spermidine is converted to spermine by the addition of

another aminopropyl group from dc-SAM in a reaction catalyzed by spermine synthase.

Both nutrients and intestinal flora also provide polyamines which are absorbed by the gastrointestinal tract (Osborne, and Seidel, 1990). Typically, the absorption of these polyamines increases when either endogenous polyamine synthesis is inhibited i.e., as with the use of eflornithine i.e., 2-difluoromethylornithine (DFMO) in cancer treatment (Redgate et. al., 1995) or when the demand for polyamines increases, as in various malignant states (Quemener et. al., 1994).

The concentrations of spermine and spermidine are decreased metabolically through a two step process. In the first step the N1 amine groups of either spermine or spermidine react with acetyltransferase to form N1-acetyl-spermine or N1-acetyl-spermidine. These compounds are then oxidatively degraded by polyamine oxidase to form spermidine and putrescine as shown in Fig. 1-1. Putrescine can also be acetylated in this way (Redgate et. al., 1995) or it can be degraded by diamine and monoamine oxidase (McCann^a et. al. 1987). Both the acetylated polyamines and the free compounds are excreted in the urine (Redgate et. al., 1995).

Biomedical Applications

Due to the association between polyamines and cell proliferation, numerous studies have considered the possibility of retarding malignancies by decreasing polyamine concentrations (Redgate et. al, 1995; McCann et. al., 1987; Pegg, 1986). Typically, polyamine levels are decreased by targeting the enzyme required for the first step in polyamine synthesis, ornithine decarboxylase (ODC). The agent most widely used for this purpose is 2-difluoromethylornithine (DFMO) which, following enzymatic activation, irreversibly binds to ODC. DFMO has been shown to significantly decrease malignant growth in both *in vitro* and *in vivo* models (Pegg and McCann, 1982). However, its effectiveness against human cancers has been marginal. It has been suggested that this discrepancy is due to the fact that, in a clinical setting, cancer treatment generally begins after the rapid growth phase of most tumors; whereas, in an experimental setting this is not the case (Schechter et. al., 1987). (A more promising use of DFMO has been in the treatment of human protozoal diseases such as African trypanosomiasis, sleeping sickness, and pneumocystis carinii pneumonia, a life threatening infection in AIDS patients (Schechter et. al., 1987)).

Another approach has been to use polyamine analogs to interfere with the normal polyamine metabolic cycle and/or retard intracellular polyamine transport (Redgate et. al, 1995). Several dialkylspermines such as N¹, N¹²-

diethylspermine have been shown to inhibit cell proliferation (Edwards, et. al., 1990; Bergeron, et. al., 1988). Polyamine moieties have also been used as a template for attaching cytotoxic agents. In this way the polyamine transport system can be used to increase the intracellular concentrations of antineoplastic agents (Symons, 1995; Stark, et. al. 1992).

Pentaazapentacosane 5 HCl

Pentaazapentacosane 5 HCl (PAPC) is a synthetically produced aliphatic pentaamine that is being investigated by the National Cancer Institute as an anticancer agent. As shown below, PAPC is an analog of the naturally occurring polyamines putrescine, spermidine and spermine.

Pentaazapentacosane

It is important to note that this compound contains five secondary amine groups and, at physiological pH, it will be primarily in the 5⁺ ionization state. Consequently, PAPC will be more positively charged than any of the naturally occurring polyamines and would be expected to interact more strongly with negatively charged moieties such as DNA (Symons, 1995).

Physical Characteristics

Pentaazapentacosane 5 HCl is a white powder with a molecular weight 539.9. As shown in Fig. 1-2, its melting point, as determined by differential scanning calorimetry, is 338°C. This polyamine contains five ionizable amines and, as such, it is freely soluble in water. The molecular formula is $C_{20}H_{47}N_5 \cdot 5 HCl$.

Stability Studies

Aqueous solutions of 1 mg/ml PAPC in 0.08 M phosphate buffer were stable over a pH range of 4-8 for at least 3 months at room temperature. Similarly, autoclaved solutions of 1 mg/ml PAPC in normal saline were stable for at least 6 months at 4, 25 and 37°C.

Analytical Studies

Like the endogenous polyamines, PAPC does not contain a chromophore and therefore does not absorb in the UV region. However, several different agents, including dansyl chloride (Bontemps, et. al., 1984; Seiler, 1971; Seiler and Knödgen, 1979; Smith, et. al., 1991), benzoyl chloride, (Wongyai et. al., 1989), and 1,2-Naphthoquinone-4-sulfonate (Smith, et. al., 1989) can be used to add chromophoric groups to primary or secondary amines and thereby convert them to a UV absorbing derivatives.

In this study, an analytical method was developed whereby PAPC was derivatized with dansyl chloride and then assayed by HPLC.

Aims

The overall aim of this study is to examine the dansylation vs. pH profile of the polyamine PAPC. In order to achieve this goal the following specific objectives will be met:

- 1) Determine of the degree of dansylation of PAPC under unfavorable conditions.
 - 2) Propose a mechanism of dansylation.
 - 3) Verify the proposed mechanism by comparing the dansylation of PAPC with the dansylation of a monoamine.
-

Table 1-1. Structures and pK_a values of Spermine, Spermidine and Putrescine

Polyamine	pK _a values
$\text{H}_2\text{N}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}_2$ Spermine	7.9, 8.7, 10.2, 10.6 ¹
$\text{H}_2\text{N}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}_2$ Spermidine	8.2, 9.8, 10.2 ¹ and 8.3, 9.7, 10.9 ²
$\text{H}_2\text{N}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}_2$ Putrescine	9.6, 10.8 ³

¹Labadi et. al., 1991; ²Kimberly and Goldstein, 1981; ³Williams and Hardy, 1953

Figure 1-1. Polyamine Metabolism

Figure 1-2. DSC Scan of Pentaazapentacosane 5 HCl

CHAPTER II. pK_a DETERMINATION

Ionization Constants

Ionization constants (K_a values) are a measurement of the ability of a compound to gain or lose a proton. Due to the fact that ionization constants are small numbers i.e., negative powers of ten, it is common to use their negative logarithms or pK_a values.

pK_a values are typically determined experimentally by titration of the compound of interest with an acid or base. The pK_a values of a compound can also be determined by several different predictive methods which assign values to different functional groups. Frequently, these predictive methods are used when the solubility or stability of a compound make it difficult to titrate. In addition, consideration of the predicted pK_a values can serve as a check on the experimentally derived values as well as lead to a better understanding of a compound's chemical reactivity.

Predicted pK_a Values

Statistical Effects

In order to predict pK_a values for polyionic compounds such as PAPC, it is necessary to consider a statistical effect. This effect is due to an unequal

number of sites for proton removal versus proton addition. For instance, for a simple monoamine there is one site from which a proton can leave and, after dissociation, there is one site for proton addition. On the other hand, for the first dissociation of PAPC there are 5 sites from which a proton can leave but, after dissociation, there is only one site for proton addition. Consequently, the first pK_a will be decreased, from the value of a comparable monoamine, by the log of this ratio i.e. $\log (5/1)$. Similarly, for the second dissociation, the pK_a will be decreased by $\log (4/2)$. In this way the statistical effect is calculated for each ionization step and used to adjust the predicted pK_a values.

ΔpK_a Method

The pK_a values for PAPC can be predicted by using a ΔpK_a method (Perrin, et. al., 1981a). This method requires that, for polyionic compounds, the pK_a values be calculated in the order that the individual groups will dissociate. However, the assignment of these groups for PAPC is somewhat arbitrary since, for instance, each of the three central nitrogens has two positively charged neighbors four methylene groups away. In addition, pK_a values for polyionic compounds are macroscopic constants which describe the loss of protons from the entire molecule - not individual functional groups (although it is assumed that a proton would preferentially be donated from

one of the three central nitrogens). With these qualifications in mind, the pK_a values were calculated from a model of PAPC whereby the nitrogens dissociate in the order 3, 2, 4, 1, 5 as indicated below.

The computation starts with the pK_a value of the parent compound which, for a secondary amine is $\cong 11.1$. The effect of substituents added to the β carbon of each ionizable nitrogen is then calculated using equation 2.1

$$-\Delta pK_a = 0.28 + 0.87 \sigma^* \quad \text{Equation 2.1}$$

where σ^* is taken from a list of Taft values (Perrin, et. al., 1981b). It should be noted that, if the substituent of interest is separated from the β carbon by a methylene group, the calculated ΔpK_a value is multiplied by 0.4. For instance, a σ^* value of 2.24 is listed for the group; $-\text{CH}_2\text{NH}_3^+$, which is separated from β carbon of the central nitrogen by a methylene group. Therefore, using equation 2.1, the calculated ΔpK_a value is 2.23 and, adjusting this by a factor of 0.4, the final value is 0.89. Similarly, for every additional intervening methylene group the calculated ΔpK_a value will be multiplied by 0.4. Finally, it should be noted that only the affect of the nearest

neighbors is calculated i.e., amine groups further down the chain are not considered.

The σ^* and calculated $-\Delta pK_a$ values used to predict the five pK_a values of PAPC are shown in Table 2-1 and, sample calculations for the first two pK_a values are shown in Figures 2-1 and 2-2. Using this method, the predicted pK_a values are 8.6, 9.8, 10.1, 11.3 and 11.7.

Experimental pK_a Values

Titration Curves

Titration curves for PAPC were determined potentiometrically. The experimental set up consisted of the following. Solutions of 0.016 and 0.022 M PAPC (0.08 and 0.11 M as potassium hydroxide (KOH) equivalents) were titrated with 0.73 M KOH (which had previously been standardized with dried potassium acid phthalate). The water for all solutions was deionized and sparged with nitrogen gas before use. The titration set up included a Corning pH meter, Orion semimicro combination electrode and a Hamilton repeating dispenser. During the titration the tip of the dispenser was immersed in the solution and nitrogen gas was sparged over the surface.

Due to the fact that PAPC has five close pK_a values, the experimentally derived titration curves were evaluated according to a spreadsheet method

(Freizer, 1992). The first step involves calculation of S ; the average number of protons released, by substitution of the titration data into equation 2.2.

$$S = \frac{V_s C_s}{V_t C_s} - \frac{V_t + V_s}{V_t C_s} ([OH^-] - [H^+]) \quad \text{Equation 2.2}$$

where V_A is the volume of PAPC, C_A is PAPC concentration, V_B is the volume of NaOH and C_B is NaOH concentration. The values of OH^- and H^+ are calculated from the pH of the titration solution. Theoretically, the five pK_a values of PAPC should equal the pH at which S is 1/2, 3/2, 5/2, 7/2 and 9/2. As shown in Figure 2-3, the pK_a values determined on this basis were 8.0, 8.9, 9.8, 10.7 and 11.3. However, in order to make more precise pK_a determinations the titration data was further evaluated as described below.

As titration proceeds, the number of protons released, S , will equal the sum of the concentrations of each ionic species multiplied by the number of hydrogens that have been lost for that species, i.e., $5 [PAPC] + 4 [PAPC]^+ + \dots [PAPC]^{+5}$. Each of these concentrations can be written in terms of PAPC dissociation constants and the hydrogen ion concentration to give:

$$\text{Equation 2.3}$$

$$S = \frac{[H^+]^4 K_1 + 2 [H^+]^3 K_1 K_2 + 3 [H^+]^2 K_1 K_2 K_3 + 4 [H^+] K_1 K_2 K_3 K_4 + 5 K_1 K_2 K_3 K_4 K_5}{[H^+]^5 + [H^+]^4 K_1 + [H^+]^3 K_1 K_2 + [H^+]^2 K_1 K_2 K_3 + [H^+] K_1 K_2 K_3 K_4 + K_1 K_2 K_3 K_4 K_5}$$

Equation 2.3 can be rearranged to

$$S[H]^5 = (1-S) [H^+]^4 K_1 + (2-S) [H^+]^3 K_1 K_2 + (3-S) [H^+]^2 K_1 K_2 K_3 + (4-S) [H^+] K_1 K_2 K_3 K_4 + (5-S) K_1 K_2 K_3 K_4 K_5 \quad \text{Equation 2.4}$$

and both sides divided by $[H^+]^4(S-1)$ to give

$$\frac{S[H^+]}{1-S} = \frac{K_1}{(1-S)[H^+]} + \frac{(2-S) K_1 K_2}{(1-S)[H^+]^2} + \frac{(3-S) K_1 K_2 K_3}{(1-S)[H^+]^3} + \frac{(4-S) K_1 K_2 K_3 K_4}{(1-S)[H^+]^4} + \frac{(5-S) K_1 K_2 K_3 K_4 K_5}{(1-S)[H^+]^5} \quad \text{Equation 2.5}$$

which is of the form:

$$Y = a_0 + a_1 X_1 + a_2 X_2 + a_3 X_3 + a_4 X_4. \quad \text{Equation 2.6}$$

The fractions: $S[H]/1-S$, $(2-S)/(1-S)[H^+]$, $(3-S)/(1-S)[H^+]^2$, $(4-S)/(1-S)[H^+]^3$, and $(5-S)/(1-S)[H^+]^4$, from equation 2.5, are then solved over a range of pH values and substituted into equation 2.6 as Y , X_1 , X_2 , X_3 and X_4 , respectively (note that values of S are determined according to equation 2.2). The coefficients of equation 2.6, where $a_0 = K_1$, $b_1 = K_1 K_2$, $c_2 = K_1 K_2 K_3$, etc., are then determined with SAS (statistical analysis system).

The pK_a values for PAPC calculated from this procedure are 8.6, 9.4, 10.3, 11.0 and 11.8.

Conclusions

Although a number of assumptions are made in using the $-\Delta pK_a$ method of pK_a prediction, the predicted values (8.6, 9.8, 10.1, 11.3 and 11.7) and the experimentally determined values (8.6, 9.4, 10.3, 11.0 and 11.8) are in good agreement.

Table 2-1. Taft values for $-\Delta \text{pK}_a$ calculations

Substituent on β carbon	σ^*	$-\Delta \text{pK}_a^*$
$-\text{CH}_2\text{NH}_3^+$	2.24	2.23
$-\text{NH}_2$	0.62	0.82

*For every $-\text{CH}_2-$ group separating the specified substituent from the β carbon the $-\Delta \text{pK}_a$ value is multiplied by the factor 0.4.

pK_a of the central nitrogen (3) of PAPC

pK _a of a secondary nitrogen	11.1
- Δ pK _a of a -CH ₂ NH ₃ ⁺ group 1 carbon from the β carbon = 2.23 x 0.4 = 0.89	-0.89
- Δ pK _a for the other β carbon	-0.89
statistical factor	<u>-0.7</u>
predicted pK _a	8.6

Figure 2-1. Predicted value of the first pK_a of PAPC

pK_a of nitrogen (2) of PAPC

pK _a of a secondary nitrogen	11.1
- Δ pK _a of a -NH ₂ group 2 carbons	
from β carbon = 0.82 x 0.4 x .4 = 0.13	-0.13
- Δ pK _a for the other β carbon	-0.89
statistical factor	<u>-0.3</u>
predicted pK _a	9.8

Figure 2-2. Predicted value of the second pK_a of PAPC

Figure. 2-3. Plot of S vs. pH for PAPC. The pH at which $S = 0.5, 1.5, 2.5, 3.5$ and 4.5 should equal pK_a 1, 2, 3, 4 and 5 of PAPC.

CHAPTER III. ASSAY DEVELOPMENT

Introduction

Pentaazapentacosane (PAPC) is a long chain aliphatic compound which does not contain a chromophoric group. However, it does contain five secondary amines which can be derivatized to increase its UV absorbance. Because dansyl chloride (Dns-Cl) is frequently used for aliphatic polyamines (Bontemps, et. al., 1984; Seiler and Knödgen; 1979; Seiler, 1971) it was chosen as the derivatizing agent. Following derivatization, dansyl PAPC (dns-PAPC) is analyzed, without extraction, by HPLC under isocratic conditions and UV detection.

Preliminary work with PAPC showed that the typical conditions for amine derivatization i.e., excess Dns-Cl and pH 9-10 (Wilkinson and Hancock, 1984; Imai, et. al.; 1984; Seiler, 1975), were inadequate for analytical purposes. Therefore the effects of pH, Dns-Cl concentration and reaction time on the dansylation of PAPC were investigated. The PAPC concentrations analyzed (72 nmole/ml) were similar to those used for the assay of spermine from biological tissues (Kabra, et. al., 1986; Saeki, et. al., 1978; Peng, et. al. 1977).

It has been suggested that partially derivatized polyamines can be formed, especially at low pH or low dansyl chloride concentration (Seiler, 1975; Peng, et. al., 1977). However the presence or absence of partially dansylated products has not been specifically demonstrated because most procedures require sample extraction following dansylation (Henriks-Eckerman and Laijoki, 1985; Seiler, 1971) and partially dansylated products may be lost in this step. In this study, PAPC is analyzed without an extraction step to allow detection of all partially dansylated products that may be formed.

Materials and Methods

Chemicals

PAPC-HCl was supplied by the National Cancer Institute (Bethesda, MD). Piperazine HCl, 98% and Piperidine HCl, 99% were purchased from Aldrich (Milwaukee, WI). Dansyl chloride (Dns-Cl), 95%, glycine, 99% and horse skeletal muscle myoglobin, 95-100%, were purchased from Sigma (St. Louis, MO). Triethylamine (TEA), HPLC grade, and glacial acetic acid, ACS reagent grade, were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide GR, was purchased from EM Science (Gibbstown, NJ). Spectrometric grade acetonitrile was purchased from Baxter (Muskegon, MI).

Derivatization Procedure

Duplicate samples of PAPC-HCl (molecular weight 539.9) were derivatized at 5 different pH values and 6 different Dns-Cl concentrations. The following solutions were prepared: stock solution of 38.8 μg PAPC-HCl/ml water (equal to 72 nmole/ml); 1.2 mg/ml of dansyl chloride in acetonitrile (equal to 4.4 $\mu\text{mole/ml}$) and a buffer of 3 ml triethylamine (TEA) to 250 ml water (equal to 85.5 $\mu\text{mole TEA/ml}$). The TEA solution was adjusted to pH 8.5, 9.5, 10.0, 10.5 or 11.0 with glacial acetic acid (TEA buffer was used to minimize precipitation in the mobile phase).

Dansylation was carried out by adding 2.0 ml TEA buffer (at pH 8.5-11.0), 2.0 ml PAPC solution and 0.2, 0.5, 1.0, 2.0, 3.0 or 5.0 mL dansyl chloride solution to a 10 ml volumetric. The samples were then diluted to volume with acetonitrile and water to give a final ratio of 60:40 organic to aqueous. The final concentrations in the derivatization solutions were 14 nmoles/ml PAPC, 17 $\mu\text{moles/ml}$ TEA, and 0.09, 0.22, 0.44, 0.88, 1.3 or 2.2 $\mu\text{moles/ml}$ dansyl chloride. The samples were protected from light and allowed to react at room temperature for 4, 8, and 24 hours. The reaction was stopped by adding a 2.0 ml aliquot of the reaction solution to 2 ml of glycine (2 mg/ml) in a 10 ml volumetric. After 10 min. the neutralized solutions were diluted to volume with 0.04 M pH 5 acetic acid : acetonitrile (30:70).

HPLC Analysis

Samples were analyzed with a Beckman System Gold M406 liquid chromatograph. The system includes a 100 μ l injection loop, a Beckman 110B solvent delivery pump and a Beckman 168 diode array detector (Beckman Instruments, Fullerton, CA). Data analysis was performed using System Gold Chromatographic Acquisition Software (Beckman Instruments, Fullerton, CA). The column was an adsorbosphere RP-C₈ column, 5 μ m, 150 x 4.6 mm I.D. (Alltech Associates, Deerfield, IL).

The mobile phase was 0.04 M pH 5 acetic acid : acetonitrile (10:90). The acetic acid buffer pH had been adjusted with ammonium hydroxide. The HPLC conditions included a flow rate of 1.5 ml per minute, an injection volume of 100 μ l and the UV detection was at 254 nm. As shown in Figure 3-1, the Dns-PAPC peak eluted at 6 min. under these conditions. The spectrum of this peak was also determined with the HPLC diode array detector and, as shown in Figure 3-2, an absorbance maximum is seen at 254 nm.

Quantitation of Dansylated PAPC

The amount of dansylated PAPC detected is reported as percent relative recovery. This is calculated by assigning a value of 100% to the Dns-PAPC peak with the greatest peak area. The amount detected for all other peaks is then calculated relative to this value.

Results and Discussion

Dansylation Time

No significant difference was seen between samples derivatized for 4, 8 or 24 hours and therefore the average values for these time points are used. This result indicates that at room temperature dansylation is completed within 4 hours.

Dansyl Chloride Concentration

An excess of Dns-Cl is typically added in order to compensate for side reactions (Seiler, 1971; Seiler and Glick, 1970). In aqueous solutions significant amounts of Dns-OH are formed as the pH is increased above pH 9.5 (Gros and Labouesse, 1969). On the other hand, reagent peaks may interfere with the assay if too large an amount of Dns-Cl is used (Kabra, et. al., 1986). In this study the concentrations of Dns-Cl in the reaction solution (0.09 - 2.2 μ moles/ml) correspond to stoichiometric ratios of approximately 1-30 based upon 5 amines per PAPC molecule. Figure 3-3 shows the effect of Dns-Cl concentration on Dns-PAPC recovery at several different pH values. Each data point in the figure is the average of 6 measurements. As expected, when Dns-Cl concentrations are low an increase in Dns-Cl results in an increase in Dns-PAPC. However, for every pH tested, the effect of increasing

Dns-Cl concentration levels off at about 0.9 $\mu\text{moles/ml}$ (a 13 fold Dns-Cl excess).

pH

The pH of the solution, as well as the amine pK_a , are critical factors for dansylation. Because Dns-Cl will only react with uncharged amines, dansylation increases as the pH is increased. However, as mentioned above, the formation of Dns-OH also increases with pH. Therefore, the solution pH should ideally be well above the analyte pK_a but not so high that significant Dns-OH is formed (Gros and Labouesse, 1969). Figure 3-4 shows that the amount of Dns-PAPC formed consistently increases with pH. In all cases, very little Dns-PAPC is formed below pH 9.5. However, as the pH is increased from 9.5 to 10.5 the amount of Dns-PAPC increases and, at high Dns-Cl concentrations this is quite dramatic (a 10 fold increase). For these samples a slight change in pH could result in significant analytical variability. Finally, for the high Dns-Cl concentrations no significant change in the amount of Dns-PAPC is seen from pH 10.5 to 11.0. Therefore, using a pH within this latter range, along with sufficient Dns-Cl, should give both a good yield and consistent results.

PAPC Dansylation Products

PAPC-HCl has 5 secondary amines with a range of pK_a values of 8.6 to 11.8. Because dansyl chloride only reacts with uncharged amines, a partially dansylated product might be expected at a low pH. Also, incomplete derivatization may be expected at low dansyl chloride concentrations (Seiler, 1975). However, for all the conditions tested only one Dns-PAPC chromatographic peak is seen. Since all other peaks on the chromatogram are also found in the blank there is no evidence of partially dansylated PAPC. In order to ensure that these species were not missed due to interference, the early eluting peaks were further separated by increasing the aqueous portion of the mobile phase to 40%. Again, under these conditions only one Dns-PAPC peak was obtained.

Method Validation

Detection limit - The method validation was performed under the optimized dansylation conditions of 4 hour reaction time, pH 11.0 and 1.3 $\mu\text{mole/ml}$ Dns-Cl concentration. The minimal detection limits for Dns-PAPC were found by injecting 0.7 pmoles on to the column. The signal to noise ratio at this level was 2:1.

Reproducibility- Samples containing 5 different concentrations of PAPC in water (5 replicates each) were assayed as described in the experimental

section. This procedure was repeated a second day. As shown in Table 3-1, the intraday variation ranged from 1.3 to 7.6 percent and the interday variation was between 1.7 to 6.7 percent.

Linearity - The linearity between the polyamine concentration and the measured peak area was determined by analyzing PAPC concentrations of 6.1-122 $\mu\text{g/ml}$ (equal to 11.3-226 nmoles/ml). Sample concentrations of 2.4 $\mu\text{g/ml}$ were not within the linear range. At each concentration 5 replicates were dansylated and assayed as described in the experimental section. This procedure was repeated a second day. The regression equation for the concentration of PAPC standard solutions (y) vs. the peak areas (x) is $y = 3.6143x + 0.7128$ ($r = 0.99995$) for day 1 and $y = 3.5847x + 0.03599$ ($r = 0.99972$) for day 2.

Conclusions

The optimized dansylation conditions for PAPC-HCL include a pH of 11.0, a Dns-Cl concentration of 1.3 $\mu\text{moles/ml}$ and a dansylation time of 4.0 hours. Under these robust conditions samples containing 11-226 nmoles of PAPC can be quantitated. Dansylation below pH 10.5 will give smaller and, likely more variable chromatographic peaks. Under a variety of derivatization conditions only one dansylated PAPC product was found.

Figure 3-1. Chromatogram of dansylated PAPC (Dns-PAPC). The amount injected was 0.3 nmole. The sample had been dansylated in a solution containing TEA buffer (pH 10.5) and 2.2 μ moles/ml dansyl chloride for 24 hours. For separation conditions see text.

Figure 3-2. UV spectrum of the dansylated PAPC (Dns-PAPC) which eluted at 6 min. The spectrum was obtained from the HPLC diode array detector.

Figure 3-3. Dns-PAPC vs. Dns-Cl Concentration for 5 different pH values. pH 8.5 (◆), pH 9.5 (■), pH 10.0 (▲), pH 10.5 (□), pH 11.0 (●). The data point for pH 10.5 at 0.88 μmole/ml Dns-Cl was an outlier and is not shown.

Figure 3-4. Dns-PAPC vs. pH for 6 different Dns-Cl Concentrations. Dns-Cl concentration = 0.09 (■), 0.22 (◆), 0.44 (▲), 0.88 (●), 1.3 (□) and 2.2 (*) $\mu\text{mole/ml}$. The individual data points for the three highest Dns-Cl concentrations are not clearly visible due to overlap. The data point for pH 10.5 at 0.88 $\mu\text{mole/ml}$ Dns-Cl (o) was interpolated from the line in Fig. 3-3.

TABLE 3-1. Assay Reproducibility

PAPC	Day 1	Day 2	Combined Results
Concentration $\mu\text{g / mL}$	Intraday (n = 5) C. V. (%)	Intraday (n = 5) C. V. (%)	Interday (n = 10) C. V. (%)
6.10	3.7	2.9	5.6
12.20	1.6	6.5	4.6
24.38	7.6	1.9	6.7
48.76	2.5	1.3	2.5
121.9	1.9	1.4	1.7

CHAPTER IV. DETERMINATION OF COMPLETE DANSYLATION

Dansylation

Dansylation is a type of derivatization whereby a chromophore is added to compounds which would otherwise not absorb in the UV region. Although other types of derivatization are available, dansylation is most commonly used for primary and secondary amines as stable derivatives can be formed under relatively mild conditions. In addition, for HPLC analysis, the dansyl derivatives typically have satisfactory chromatography.

The reagent used for dansylation is dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride, Dns-Cl). As shown in figure 4-1, dansylation is a sulfonyl transferase reaction whereby one lewis base, in this case the chloride ion, is exchanged for another, such as a nucleophilic nitrogen or oxygen (Gordon and Maskill, 1989). This type of reaction has been described by both SN1 and SN2 mechanisms (Gordon and Maskill, 1989; Yang, et. al., 1995). The SN1 reaction involves two steps; 1) cleavage of the chloride - sulfonyl bond to form two separate ions and 2) reaction of the positively charged sulfonyl ion with a nucleophilic acceptor. On the other hand, an SN2 reaction is a one step reaction whereby new bond is formed while,

simultaneously, the original bond is broken. In this case there is a partial separation of charge but actual ions are not formed.

The fact that the sulfonyl group of dansyl chloride reacts with nucleophiles has several practical consequences. The first of these is that, in order for an amine group to act as a nucleophile, it must be in the uncharged state so that its two non-bonded electrons are available. Therefore, a low amine pK_a value and a high solution pH will, typically, increase the dansylation rate.

However, an increase in the solution pH also increases hydroxyl ion concentrations and, this nucleophile, also reacts with Dns-Cl. The product of this reaction, dansyl sulfonic acid (dansyl hydroxyl, Dns-OH), absorbs strongly in the UV region and can interfere with normal chromatography. Because of this well known side reaction (Gros and Labouesse, 1969) most dansylation procedures use an excess of dansyl chloride and a pH of 10 or below (Seiler, 1971; Seiler, 1970).

Dansyl chloride is a relatively non-polar compound which is insoluble in water. Therefore, dansyl chloride stock solutions are generally made with either 100 % acetone or acetonitrile. For this same reason, dansylation reactions are carried out in solutions which are partially non-aqueous; pure solvents are not used because the reaction rate is inversely related to the percent cosolvent (Gray, 1972). Amine groups which are derivatized with

dansyl chloride also become much more non-polar. This is due to both the addition of the non-polar naphthalene-sulfonyl group and, even more importantly, to the loss of a positive charge. The loss of charge results from converting a positively charged primary or secondary amine to a neutral sulfonamide.

As expected, the derivatized analytes will also have considerably different chromatography i.e., the retention times seen on reversed phase HPLC systems will increase. Some compounds have more than one functional group available for derivatization and, in this case, the retention times will increase with the number of dansyl groups attached (Seiler, et. al., 1978). For example, on a reverse phase system with a C₁₈ column and a mobile phase containing 80% methanol, the relative retention times of dansylated putrescine, spermidine and spermine were 1, 4 and 12, respectively (Gennaro, 1988).

Perdansylation of PAPC

PAPC contains five secondary amines with pK_a values of 8.6 to 11.8. Therefore, only some of the amine groups will be uncharged under the typical reaction conditions of pH 9-10. Consequently, because only neutral amines can react with dansyl chloride, partially dansylated products might be

expected. However, as described in chapter III, only one dansylated derivative of PAPC is formed. All other peaks on the chromatogram also appear in the blank. In this study, the identity of this derivative is investigated by mass spectrometry and by comparing its molar absorptivity to a dansylated monoamine and a dansylated diamine.

Experimental

Chemicals

PAPC-HCl was supplied by the National Cancer Institute (Bethesda, MD). Piperazine HCl, 98% and Piperidine HCl, 99% were purchased from Aldrich (Milwaukee, WI) and horse skeletal muscle myoglobin, 95-100%, was purchased from Sigma (St. Louis, MO).

Mass Spectrometry

As discussed in chapter III, the only dansyl PAPC product that is produced elutes at 6 min. on the HPLC chromatogram. Therefore, the fraction of HPLC mobile phase corresponding to this peak was collected and submitted for mass analysis. This sample solution was analyzed with a Finnigan TSQ 7000 (Finnigan, San Jose, CA) by electrospray mass

spectrometry. Calibration was against the multicharge envelop of equine skeletal muscle myoglobin.

Molar Absorptivity

Samples were derivatized as described in chapter III. The solutions contained either 7.22 nmoles/ml PAPC, 19.4 nmoles/ml piperazine or 37.3 nmoles/ml piperidine in addition to 1.3 μ moles/ml dansyl chloride (different concentrations were used to compensate for the fact that PAPC has five derivatizable nitrogens, piperazine has two and piperidine has one). The pH of the buffer in these solutions was 11.0. Following dansylation, these samples were diluted and analyzed by HPLC as described in chapter III except that the mobile phase was 45% acetonitrile for the piperidine and piperazine assay and 90% acetonitrile for the PAPC assay.

Results and Discussion

Mass spectrometry

Mass spectrometry of the peak collected at 6 min gave a mass number of 1522, which is equivalent to perdansyl PAPC. These results, together with the fact that the 6 min chromatographic peak was the only PAPC derivative

found, strongly indicates that only pentadansyl PAPC is formed in significant quantities.

Molar Absorptivity

The chromatographic peak area of Dns-PAPC was compared with that of the dansylated monoamine (piperidine) and a diamine (piperazine). Assuming that UV absorbance increases linearly with the number of dansyl groups, the molar absorptivity (calculated as area/pmoles) of pentadansylated PAPC should be five times that of a monodansylated compound and 2.5 times greater than a didansyl compound. As shown in Table 4-1, the results are consistent with complete dansylation of PAPC.

Figure 4-1. Reaction of dansyl chloride with water or with a secondary amine.

TABLE 4-1. Relative peak area per pmole on column for a mono, di and penta dansylated compound.

Relative AUC / pmole at 254 nm			
	Calculated	Found	Mobile Phase
monodansyl-	1.0	1.0	55:45 ACN:buffer
didansyl-	2.0	1.8	55:45 ACN:buffer
pentadansyl-	5.0	4.4	90:10 ACN:buffer

CHAPTER V. MECHANISM OF DANSYLATION

Introduction

Dansylation is a commonly used derivatization procedure for compounds which do not absorb in the UV region. For amines, the typical reaction conditions are pH 9-10, (Seiler, 1975; Imai et al., 1984; Wilkinson, 1984) and excess Dns-Cl (Seiler, 1970). However, only the neutral form of primary or secondary amines will react with dansyl chloride (Seiler, 1975) and, therefore, the optimal pH must be high enough that the amine is primarily unionized.

However, compounds such as spermidine, spermine and PAPC, which have multiple ionizable amines, will have a range of pK_a values over several pH units. For instance, the pK_a values for the four amine groups of spermine range from 7.9 to 10.6 (Labadi et. al., 1991) while those of PAPC range from 8.6 to 11.8. Therefore, for these types of compounds, only some of the amine groups will be uncharged under the typical reaction conditions of pH 9-10 and partially dansylated products might be expected to be produced (Seiler, 1975; Peng, et. al., 1977). In fact, a total of 18 different partially dansylated compounds could conceivably be produced from this pentaamine PAPC. However, as described in chapter IV, only perdansyl PAPC, in which all potential reaction sites have been dansylated, is obtained under standard

reactions conditions (although the yield is decreased under unfavorable conditions of low pH and low dansyl chloride concentration only the pentadansylated derivative is produced.) In addition, the presence of partially dansylated products has not been demonstrated for any diamines or polyamines despite the many reports of their dansylation.

In this study, a mechanism is proposed which explains the perdansylation of PAPC based on the assumption that 1) only completely unionized amine molecules will dansylate and 2) the ratio of unionized molecules to ionized molecules increases as dansylation proceeds. According to this mechanism, a significantly greater pH should be required to dansylate a polyamine vs. a monoamine and, in a mixture of partially reacted compounds, the one with the highest degree of dansylation will react preferentially. This aspect of the mechanism is tested by comparing the dansylation vs. pH curves for PAPC and the monoamine piperidine.

Materials and Methods

Materials

PAPC was supplied by the National Cancer Institute (Bethesda, MD). Piperidine HCl, 99%, was purchased from Aldrich (Milwaukee, WI). All other chemicals were at least reagent grade as described previously (Chapter III).

Methods

PAPC was derivatized with Dns-Cl as described previously (Chapter. III). The same conditions were used for the derivatization of piperidine except that only the 2.2 mmoles Dns-Cl /mL concentration is used and the molar concentration of piperidine is 5 times higher than that used for PAPC. The higher piperidine concentration is to account for the fact that there are five derivatizable nitrogens per molecule of PAPC. The HPLC conditions were as described previously (Chapter. III) except that the mobile phase was 45% acetonitrile for the piperidine assay and 90% acetonitrile for the PAPC assay.

Results

Dansylation vs. pH Profile of PAPC

PAPC was dansylated under conditions of pH 8.5, 9.5, 10, 10.5 and 11 and dansyl chloride concentrations of 0.88, 1.3 and 2.2 $\mu\text{mole/mL}$. As shown in Figure 5-1, the recovery of PAPC generally increases with increasing pH. However, no further increase in dansyl PAPC was seen at pH values above 10.5. It should also be noted that, as discussed in chapter III, for every condition tested, only one dansyl PAPC product is found.

Dansylation of PAPC vs. Piperidine

The amount of dansylated product obtained for piperidine, which has a pK_a of 11.1 (Weast, 1972), is compared to that obtained for PAPC as a function of pH as shown in Figure 5-2. Both the piperidine and the comparison PAPC samples contained 2.2 Dns-Cl mmoles/mL and 70 nmoles of titratable nitrogen. As shown in the figure, a much higher pH is required to achieve the same degree of PAPC dansylation as piperidine. For example, to get 80% dansylated product a pH of 10.0 is needed for PAPC while a pH of only 6.0 is sufficient for piperidine.

Discussion

The following mechanism of polyamine dansylation accounts for both the perdansylation of PAPC and the differences in the dansylation vs. pH profiles for the two amines. The mechanism is based on the premise that the polyamine molecule must be completely unionized before any one of the amine groups will react with dansyl chloride and secondly, that the fraction of uncharged molecules increases as dansylation progresses.

Requirement for Unionized Molecules

A possible reason for the first requirement is if dansyl chloride forms a positively charged intermediate i.e., to the extent that derivatization takes place by an SN1 mechanism the sulfonyl group will carry a positive charge (Gordon et. al., 1989). The formation of this positively charged intermediate would then be repulsed by any positive charges on the PAPC molecule. The repulsion could be due to direct affects by individual charged amine groups or, as suggested previously (King, et. al., 1965) any positive charges on the molecule could be distributed among all of the amine groups.

The requirement that the entire molecule be uncharged before dansylation will occur is crucial as this explains why no partial dansyl compounds are seen. Without this requirement each of the amine groups could dansylate 'independently', regardless of the ionization state of the other groups. For instance, PAPC has pK_a values of 8.6, 9.4, 10.3, 11.0 and 11.8. Therefore, at pH 8.5 about 50% of the PAPC molecules will have an unionized amine group and dansyl groups would be expected to react with these neutral sites as shown in Figures 5-3 and 5-4. However, no partially dansylated compounds were found under a wide range of conditions.

On the other hand, if dansylation depends on the concentration of completely uncharged molecules little or no dansylated products would be expected at pH 8.5 where, as described below, the fraction of uncharged

molecules is only 1×10^{-7} . This is in agreement with the results. As shown in Figure 5-1, no dansyl PAPC was detected at this pH. The requirement that all the amine groups be completely unionized could also explain the lack of partially dansylated compounds seen for other polyamines such as gentamycin (Peng, et. al., 1977).

Changes in the Fraction of Unionized Molecules

The second assumption of the proposed model is that the fraction of uncharged molecules increases as dansylation progresses. If only the uncharged PAPC molecules will react, then the higher the fraction of uncharged molecules (f_u) the more the reaction will be driven towards dansylation. The f_u values, can in turn, be readily calculated for any compound from the dissociation constants (K_a) and the hydrogen ion concentration (equation 5.1). In the case of a monoamine, such as piperidine,

$$f_u = K_a / ([H^+] + K_a) \quad \text{Equation 5.1}$$

In the case of a pentaamine, such as PAPC, five protons must be lost to form the unionized compound and the fraction unionized (f_{u1}) is calculated by equation 5.2 (Freizer, 1992).

Equation 5.2

$$f_{u1} = \frac{K_1 K_2 K_3 K_4 K_5}{[H^+]^5 + [H^+]^4 K_1 + [H^+]^3 K_1 K_2 + [H^+]^2 K_1 K_2 K_3 + [H^+] K_1 K_2 K_3 K_4 + K_1 K_2 K_3 K_4 K_5}$$

The constants K_1 , K_2 , K_3 , K_4 and K_5 are the five acid dissociation constants (K_1 is the effective K for dissociation of the penta-cationic compound, K_2 is the effective K for dissociation of the three possible tetra-cationic compounds, K_3 is the effective K for dissociation of the six possible tri-cationic compounds, etc.). The concentration of PAPC in the unionized form is the product of the total PAPC concentration and the fraction which is unionized i.e., $f_{u1} \cdot [\text{PAPC}]$.

Similar equations can be used to calculate the fraction uncharged for monodansyl-PAPC (dns-PAPC) compounds (f_{u1}). These compounds will have only four acid dissociation constants and, the value of these constants will differ from those of unsubstituted PAPC. Therefore, the K values used in equation 5.2 are replaced with K' values and the fraction uncharged is calculated for a tetracationic compound as shown in equation 5.3.

Equation 5.3

$$f_{u2} = \frac{K_1' K_2' K_3' K_4'}{[H^+]^4 + [H^+]^3 K_1' + [H^+]^2 K_1' K_2' + [H^+] K_1' K_2' K_3' + K_1' K_2' K_3' K_4'}$$

where K_1' , K_2' , K_3' , and K_4' are the four acid dissociation constants for monodansyl PAPC (Note that K_1' is the effective K for dissociation of the tetra-cationic compound, etc.). Comparable equations can also be derived for didansyl-PAPC ($\text{dns}_2\text{-PAPC}$), tridansyl-PAPC ($\text{dns}_3\text{-PAPC}$) etc.

It is significant that the primary difference between f_u values for a pentaamine (equation 5.2) vs. a tetraamine (equation 5.3) is due to the number of K_a values. For example, keeping any four, of PAPC's five K values, in equation 5.3 gives $f_{u2} > f_{u1}$ at any pH. This demonstrates that under all conditions the fraction of completely unionized molecules is inversely related to the number of similar ionizable groups on the molecule (assuming that the individual $\text{p}K_a$ values do not increase with dansylation). The latter provision is discussed below.

Electrostatic Effects

The dissociation constants for PAPC and its derivatives are affected by a combination of electrostatic and statistical factors (Perrin, et. al., 1981a). The $\text{p}K_a$ values of PAPC dansyl derivatives can be estimated by evaluating the effect that dansylation has on these factors. For PAPC, the electrostatic effect is mainly due to repulsion between positive charges on the molecule. Generally, the electrostatic lowering of the $\text{p}K_a$ of an amine group is proportional to the number of positive charges on the molecule. Although the

position of the charged amine groups will also affect dissociation, the primary effect results from the number of positive charges.

The first pK_a value for PAPC is quite low because, for the first dissociation, there are four positive charges that repel the proton of the fifth group. The second pK_a will be higher than the first because, following the first dissociation, there will be one less positive charge on the molecule. This trend continues until the highest pK_a , at which point no positive charges remain on the molecule and the electrostatic effect vanishes. (If the statistical effects are disregarded, the highest pK_a of a polyamine would be approximately the same as the pK_a of a structurally similar monoamine).

The electrostatic effect is also related to the number of positive charges on the dansyl derivatives of PAPC. For instance, dns-PAPC has four ionizable amine groups and, consequently, three positive charges are available to push the proton off of the first dissociating group. This is the same number of charges as are present for the second dissociation of PAPC and therefore, the electrostatic effects for these two species should be similar. Accordingly, if the statistical factors are ignored, and it is assumed that there are no specific interactions of the dissociating group with the dansyl moieties, the pK_a values for the first dissociating group of dns-PAPC and the second dissociating group of unsubstituted PAPC should be approximately equal. In

a like manner, the pK_a values for the second dissociating group of dns-PAPC and the third dissociating group of PAPC should also be equal, and so forth.

Statistical Effects

The statistical effect is based on the number of sites available for proton removal vs. the number available for proton addition (Perrin, et. al., 1981a). For example, for the fifth dissociation step of PAPC there is only one site for removing a hydrogen atom but, after this hydrogen is removed, there are five sites for adding a hydrogen, i.e., a 1:5 ratio. The effect this has on the pK_a will be $\log 5$ or 0.7 pK_a units. This is in agreement with the experimentally found pK_a value of 11.8 which is 0.7 pK_a units higher than the pK_a value of a typical secondary amine which is 11.1 (Perrin, et. al., 1981a).

Because the magnitude of the statistical effect depends on the number of ionizable amines, the statistical effect will decrease as dansylation proceeds and ionizable amines are converted to unionizable sulfonamides. For instance, after the first dansyl group is added there are only four ionizable amines and the difference between the first dissociation constant and the last, due to just this effect, would be calculated from $(4:1)/(1:4)$ i.e., 16. Likewise, the ratio between the first and last dissociation constants of di, tri, and tetra dansyl PAPC are 9, 4 and 1. Note that the statistical effect, like the

electrostatic effect, vanishes for the pK_a of tetradansyl PAPC which is effectively a monoamine.

pK_a Values of Dansyl Derivatives

Since the addition of each dansyl group decreases the number of potential positive charges by one, the first step in estimating pK_a values for dns-PAPC is to delete the lowest pK_a value of PAPC. Then, the changes in the remaining 4 pK_a values, (9.4, 10.3, 11.0 and 11.8), due to the statistical effect i.e., going from 5 to 4 ionizable groups, is calculated. This involves first subtracting the statistical component from the pentaamine pK_a values and then adding in the statistical component for the four monodansyl pK_a values.

For example, the statistical component of the second pK_a of PAPC is - $(\log 4/2)$ or -0.3 while the statistical component of the first pK_a of monodansyl PAPC is - $(\log 4/1)$ or - 0.6 (Perrin, et. al., 1981).

The statistically corrected pK_a of the first dissociating group for monodansyl PAPC is equal to 9.1 i.e., $9.4 + 0.3 - 0.6$. This procedure is repeated to generate pK_a values for all the dansylated PAPC compounds. The pK_a values generated in this way, for each of the partially dansylated compounds, are shown in Table 5-1.

Kinetic Requirement for Exclusive Perdansylation

The reaction for formation of dns-PAPC is shown in Figure 5-5. If the Dns-Cl reagent is in excess and the reaction solution is buffered, the concentrations of Dns-Cl and HCl are relatively constant and dansylation is pseudo first order. Dansylation of PAPC can now be represented by

Scheme 1

where $A = f_{u1} \cdot [\text{PAPC}]$, $B = f_{u2} \cdot [\text{dns}_1\text{-PAPC}]$, and $C = f_{u3} \cdot [\text{dns}_2\text{-PAPC}]$, etc. and k_1 thru k_5 are the rate constants for the uncharged species.

Scheme 1 can be further simplified to $A \xrightarrow{k_n} F$ since, as described in chapter III, none of the partially dansylated compounds are produced in measurable quantities. Therefore, the changes in concentration of B, C, D, and E must be negligible relative to those of A and F, and the rate of formation of F is equal to the rate of loss of A. This is consistent with a reaction scheme whereby $k_5 \gg k_4 \gg k_3 \gg k_2 \gg k_1$ (Connors, 1981) and dns₅-PAPC is the most energetically favorable derivative.

Rationale for Perdansylation of PAPC

The basis for this stepwise increase in rate constants can be seen by examining the changes in the fraction of the various uncharged PAPC

derivatives. As shown in Table 2-2 at any specific pH the f_u value increases as dansylation proceeds. Furthermore, because each f_u value of each species is a constant at a constant pH, it can be combined with the corresponding rate constant shown in scheme 1 to give overall rate constants, k_1' , k_2' , ..., which represent the rate constants in terms of the total concentration (charged and uncharged) for each species. For example the overall rate constant for formation of dns₁-PAPC is $k_1' = k_1 \cdot f_{u1}$. Similarly, for dns₂-PAPC the overall rate constant is $k_2' = k_2 \cdot f_{u2}$, and so forth (Connors, 1981).

If the k values are equivalent the k' values must increase progressively with dansylation, and $k_5' \gg k_4' \gg k_3' \gg k_2' \gg k_1'$. Therefore, the formation of dns-PAPC will facilitate the formation of dns₂-PAPC which in turn will facilitate the formation of dns₃-PAPC, etc.

Dansylation Profiles of PAPC and Piperidine

Figure 5-2 shows the relative amounts of dansylated product obtained for PAPC and the monoamine piperidine as a function of pH. It is apparent that to achieve the same degree of dansylation PAPC requires a much higher pH than piperidine. However, this difference cannot be explained solely by the individual pK_a values for these two compounds. The dansylation of PAPC

will be most limited by its highest pK_a value (11.8), and the difference between this value and the pK_a of piperidine is only log 5 or 0.7 pK_a units. Based on these values PAPC would require a pH only 0.7 units higher than piperidine to obtain equivalent amine dissociation and dansylation. It is therefore evident that the pH dependency of PAPC dansylation is not simply related to the concentration of uncharged amine groups.

These results are, however, consistent with a mechanism whereby dansylation, and its pH dependency, are related to the concentration of uncharged molecules (f_u). Figure 5-5 shows the change in f_u vs. pH for PAPC and piperidine. As with the dansylation data, higher pH values are required for PAPC to obtain values equivalent to piperidines's and the slope of the curve for PAPC is much sharper. Note that the ratio of f_u piperidine to f_u PAPC approaches infinity as pH is decreased and unity as pH is increased. These changes in f_u values are consistent with the facts that dansylation of piperidine is much greater than dansylation of PAPC at low pH and that this difference diminishes as the pH is increased.

Conclusions

It is well known that for dansylation to occur, the amine group must be in the unionized form (Gros and Labouesse, 1969). Consequently, the effect of pH on dansylation is usually thought of in terms of individual amine groups.

However, in this study it is shown that for a polyamine, such as PAPC, the ionization state of the entire molecule, and not just the amine group, is the key factor. The mechanism proposed requires an uncharged molecule for dansylation and predicts that formation of perdansyl PAPC is energetically favorable. This mechanism is consistent with both the complete lack of partially dansylated products and the pH / dansylation profile of PAPC and piperidine.

Figure 5-1. Dns-PAPC vs. pH. Mean $\pm$ SD for samples derivatized with 0.88, 1.3 or 2.2 μ mole dansyl chloride /mL for 4, 8 or 24 hours ($\blacklozenge$, $n=9$).

Figure 5-2. Dansylation of Piperidine (●) and PAPC (▲). Sample concentrations of 70 nmoles piperidine /mL and 14 nmoles PAPC /mL were derivatized (as described in the text).

Figure 5-3. Predominant Species of PAPC at pH 8.5

Figure 5-4. Expected dansyl derivatives at pH 8.5 if a completely neutral molecule is not required.

Figure 5-5. Formation of a monodansyl PAPC derivative.

Figure 5-6. Fraction of uncharged molecules vs. pH for Piperidine (●) and PAPC (▲). The fu values were calculated according equation 5-1 and 5-2 described in the text.

Table 5-1. pK_a values for PAPC and its dansyl derivatives.

Number of ionizable amine groups					
5	4	3	2	1	0
PAPC	dns-PAPC	dns₂-PAPC	dns₃-PAPC	dns₄-PAPC	dns₅-PAPC
8.6					
9.4	9.1				
10.3	10.1	9.8			
11.0	10.9	10.7	10.4		
11.8	11.7	11.6	11.4	11.1	none

Table 5-2. The fraction of unionized molecules (f_u)^a

pH	PAPC (f_{u1})	dns-PAPC (f_{u2})	dns ₂ -PAPC (f_{u3})	dns ₃ -PAPC (f_{u4})	dns ₄ -PAPC (f_{u5})	piperidine (f_u)
9	1.7 E-7	8.5 E-7	6.3 E-6	1.5 E-4	7.9 E-3	7.9 E-3
10	4.4 E-4	9.9 E-4	2.7 E-3	1.2 E-2	7.3 E-2	7.3 E-2
11	6.7 E-2	9.5 E-2	1.4 E-1	2.4 E-1	4.4 E-1	4.4 E-1

^aThe f_u values are calculated by using equations of the form shown for equations 5-1 - 5-3. The K_a values are calculated from pK_a 11.1 for piperidine and from the pK_a values shown in Table 5-1 for PAPC compounds.

SUMMARY

Pentaazapentacosane pentahydrochloride (PAPC-HCl) is a synthetic analog of the endogenous polyamines i.e., putrescine, spermidine and spermine, which is being investigated as an anticancer agent. However, this compound does not contain a chromophore and, therefore it was derivatized with dansyl chloride to increase its UV absorbance. Because only the neutral form of an amine will react with dansyl chloride the effect of pH on the dansylation of PAPC was examined. It was found that the yield of dansyl PAPC increased 10 fold as the reaction pH was changed from 9.5 to 10.5 and that only pentadansyl PAPC was produced. Both these findings were unexpected but could be explained by a mechanism whereby 1) only completely unionized amine molecules will dansylate and 2) the ratio of unionized molecules to ionized molecules increases as dansylation proceeds. This proposed mechanism has been verified by comparing the dansylation vs. pH profile of PAPC to that of a reference monoamine (piperidine HCl).

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