curves were interpreted in terms of two sets of binding sites, and the derived n and k values are listed in Table II. The points on Fig. 4 are experimental; the lines are drawn from the equation:

$$\frac{\bar{v}}{A} = \frac{n_1 k_1}{1 + k_1 A} + \frac{n_2 k_2}{1 + k_2 A} \qquad \text{(Eq. 8)}$$

using the derived n and k values. It is clear that the addition of salicylate at a free concentration of 0.5 mM affects the first set of binding sites; at the higher concentrations of free salicylate (2.5 mM), methyl orange is displaced from both the first and second sets of binding sites. The urate albumin interaction was analyzed previously (4), and the effect of 0.5 mM sodium salicylate on the derived n and k values is shown in Table II.

After determining n and k values, it is possible to predict fractional binding at any concentration of protein and ligand. The fraction bound (β) is given by:

$$\beta = \frac{1}{1 + \frac{1}{(P)nk} + \frac{(A)}{n(P)}} \qquad \text{(Eq. 9)}$$

(P) and (A) being protein and free ligand concentrations, respectively.

Preequilibration of the protein with the competing ligand (ligand B) (Procedure 4, Table I) is necessary, because useful data on displacement may only be obtained when the binding of the primary ligand is studied in the presence of a constant concentration of the competing ligand.

A full discussion of the analyses that allow the association constant of the displacing ligand (ligand B), in this case of salicylate–albumin interaction, to be determined was made by Edsall and Wyman (5).

This method is ideally suited to the *in vitro* determination of drug–protein interaction and drug displacement. It is fast, and analysis of a few experiments yields as much information on the molecular interactions involved as many conventional ultrafiltration, equilibrium dialysis, or gel filtration experiments. Adequate analysis of the data allows a precise prediction of free drug concentration to be made in the presence of varying concentrations of displacing compounds. The results, if indicating displacement, may also alert one clinically to potentiation of action and/or to toxicity when the therapeutic regimen requires the patient to take more than one drug.

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2-Nitrophenylhydrazine: A Selective Reagent for Colorimetric Determination of Carboxylic Acid Anhydrides and Chlorides

JAMES W. MUNSON

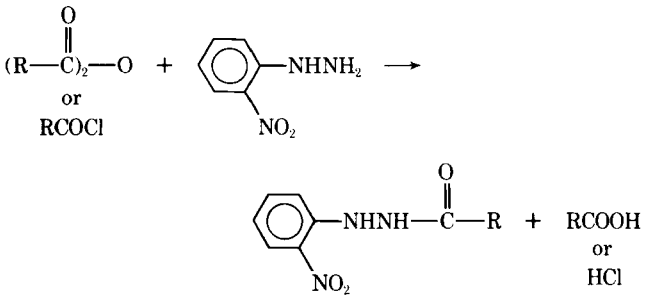
Abstract □ Carboxylic acid anhydrides and chlorides react with 2-nitrophenylhydrazine to form the corresponding hydrazides. These hydrazides in aqueous basic solution give a blue color which can be utilized for colorimetric analysis. Both acid anhydrides and acid chlorides undergo this reaction in water, while only the acid chlorides react readily in acetonitrile. The use of acetonitrile as the solvent allows the analysis of acid chlorides in the presence of acid anhydrides. A variety of other carboxylic acid derivatives and carbonyl compounds gave no interference. Suggested analytical procedures for acid anhydrides and acid chlorides are given along with analytical data.

Keyphrases □ 2-Nitrophenylhydrazine—colorimetric reagent for determination of carboxylic acid anhydrides and chlorides □ Carboxylic acid anhydrides and chlorides—colorimetric determination using 2-nitrophenylhydrazine □ Colorimetry—determination, carboxylic acid anhydrides and chlorides using 2-nitrophenylhydrazine

The analysis of carboxylic acid anhydrides and carboxylic acid chlorides has generally been accomplished by one of three methods: direct titration, condensation with a nucleophile and subsequent back-titration of excess nucleophile, or condensation with a nucleophile and subsequent spectrophotometric determination of the product.

Carboxylic acid anhydrides have been titrated directly with sodium methoxide in a nonaqueous medium (1). Carboxylic acid chlorides are readily hydrolyzed to the parent carboxylic acid with the addition of water. The parent acid can be then titrated directly with a standard base. However, any mineral acid present, such as hydrochloric acid, will also be titrated and lead to higher values (2). Acid anhydrides are also hydrolyzed by water, but the reaction is slower than that of acid chlorides.

Anhydrides have been determined by condensation with excess aniline to form the anilide. The unreacted aniline is determined *via* titration with a standard acid (3). An interference encountered in this procedure is the formation of anilides from carboxylic acids which may be present. Other nucleophiles such



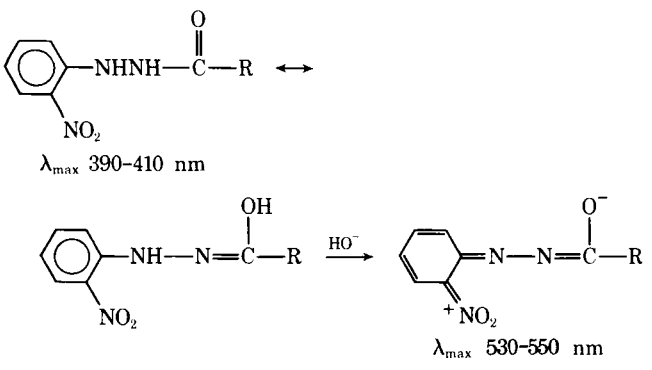
Scheme I

as *p*-nitroaniline (4) and 2,4-dichloroaniline (5) have been used to minimize this interference. Morpholine has also been used successfully as a nucleophile, with the excess morpholine being titrated with a standard methanolic hydrochloric acid solution (6). Moreover, anhydrides have been determined by reaction with excess *m*-chloroaniline and determination of the unreacted amine by titration with sodium nitrite (7).

Acid anhydrides and acid chlorides have also been determined by the conventional ferric hydroxamate procedure (8). A neutral hydroxylamine reagent is preferred because esters and amides do not react readily under these conditions. However, higher yields were obtained when an alkaline hydroxylamine reagent was used. Acid anhydrides, acid chlorides, and phosgene have been determined by reaction with a known excess of piperidine (9). The excess piperidine was determined by reaction with 3,5-dinitrobenzoyl chloride and subsequent colorimetric determination of the 3,5-dinitrobenzamide.

A fluorescence analysis for acid chlorides and acid anhydrides involves condensation of the substrate with 2-carboxyisonitrosoacetanilide in an alkaline aqueous acetone medium (10). The resulting products fluoresce when exposed to UV light. This method is suitable for measuring small quantities (1×10^{-12} mole) of substrate. However, the fluorescence produced is not stable.

Legradi (11) recently reported that acid anhydrides and acid chlorides react with 2-nitrophenylhydrazine to form a blue color in basic media. It was also reported that esters, imides, amides, and carboxylic acids do not react under the same conditions. The reactions involved are shown in Scheme I. The resulting hydrazide ionizes in basic solution to give a blue color. A resonance form of the chromophore is given in Scheme II (11). In acidic solutions, these hydrazides exhibit an absorption maximum between 390 and 410 nm; in basic solutions, the maxima are shifted to 530–550 nm.



Scheme II

This paper describes the development of an analytical method for acid anhydrides and acid chlorides utilizing this reaction. A method for the determination of acid chlorides in the presence of acid anhydrides is also described.

EXPERIMENTAL[1]

Materials—Unless otherwise stated, chemicals were analytical reagent grade and were used directly. All water was redistilled from alkaline permanganate. Standard pH buffer solutions were prepared according to Bates (12). Acetonitrile[2] was redistilled from phosphorus pentoxide; the fraction boiling at 81.5° was collected. Acetone[3] was refluxed with potassium permanganate, dried with anhydrous sodium sulfate, and redistilled, bp 56° [lit. (13) bp 56–60°].

2-Nitrophenylhydrazine hydrochloride[2] was recrystallized from methanol, mp 195–199° dec.

Anal.—Calc. for $C_6H_7N_3O_2 \cdot HCl$: C, 38.00; H, 4.26; N, 22.16. Found: C, 38.26; H, 4.37; N, 21.97.

2-Nitrophenylhydrazine[2], mp 90° [lit. (11) mp 90°] was used without further purification. Acetamide[4] was recrystallized from benzene, mp 81° [lit. (14) mp 81°]. Benzoic anhydride[5] was recrystallized from benzene–methanol, mp 41–42° [lit. (15) mp 42°]. Phthalic anhydride[2] was recrystallized from an ether–methanol solution, mp 128° [lit. (16) mp 132°]. Succinimide[4] was recrystallized from methanol, mp 124° [lit. (17) mp 123–125°]. *p*-Nitrophenyl acetate was synthesized by the method of Chattaway (18), mp 81–82° [lit. (18) mp 83°]. Succinic anhydride[5] was recrystallized from an ether–methanol solution, mp 116–117° [lit. (19) mp 119°]. Maleic anhydride[6], mp 52–53° [lit. (20) mp 52°], was used without further purification.

The following liquids were redistilled at atmospheric pressure: acetyl chloride[4], bp 51–52° [lit. (21) bp 50–55°]; acetic anhydride[4], bp 137–138° [lit. (22) bp 139°]; acetophenone[5], bp 198–200° [lit. (23) bp 201°]; benzaldehyde[3], bp 176–177° [lit. (24) bp 175–180°]; benzoyl chloride[7], bp 194° [lit. (21) bp 194–196°]; butyrolactone[5], bp 203–204° [lit (25) bp 206°]; ethyl acetate[4], bp 76° [lit. (26) bp 77°]; phthaloyl dichloride[2], bp 271° [lit. (27) bp 281°]; pivaloyl chloride[5], bp 104° [lit. (28) bp 103–104°]; propionic anhydride[2], bp 162° [lit. (29) bp 168°]; and propionyl chloride[2], bp 78–79° [lit. (21) bp 75–80°]. Thionyl chloride[6], *p*-toluoyl chloride[2], *p*-toluenesulfonyl chloride[2], and sodium perchlorate[8] were used directly.

Synthesis of Acetyl- and Phthalyl-2′-nitrophenylhydrazides—Acetyl-2′-nitrophenylhydrazide and phthalyl-2′-nitrophenylhydrazide were synthesized by the following method. The appropriate anhydride (0.01 mole) was dissolved in 40 ml of benzene, and 0.015 mole of 2-nitrophenylhydrazine was suspended in 25 ml of benzene. The anhydride solution was added dropwise to the stirred suspension of 2-nitrophenylhydrazine. The resultant mixture was allowed to stand at room temperature for 30 min. The precipitated hydrazide was collected on a filter, washed with benzene and 0.1 *N* hydrochloric acid, and recrystallized from a methanol–water mixture.

1. Acetyl-2′-nitrophenylhydrazide, mp 140–141° [lit. (30) mp 140°].

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[1] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are corrected. Boiling points are uncorrected. Spectral measurements were made with either a Cary model 15 or a Beckman model DU spectrophotometer. The Cary model 15 was fitted with a thermostated cell compartment that maintained the temperature constant to ±0.1° with a circulating water bath (Precision Scientific Co.). Thermometers for use at 25.0° were calibrated against a thermometer carrying an ASTM certificate. The pH measurements were made with a Radiometer pH meter model 52 equipped with a Radiometer combination electrode (GK2321C). All elemental analyses were performed by Baron Consulting Co., Orange, Conn.

[2] Aldrich Chemical Co.
[3] J. T. Baker Chemical Co.
[4] Allied Chemical Co.
[5] Eastman Organic Chemicals.
[6] Fisher Chemical Co.
[7] Matheson, Coleman and Bell.
[8] G. F. Smith Chemical Co.

Table I—Effect of Solvent Composition on Reaction of Phthalic Anhydride and 2-Nitrophenylhydrazine

Solvent[a]	t_∞[b], sec
Water	300
Methanol	1500
Ethanol	1500
2-Propanol	2500
tert-Butanol	3250
Acetonitrile	—[c]

[a] Solvent composition is: methanol 5 ml, acetonitrile 2 ml, solvent listed—add a sufficient quantity to make 25 ml. [b] Time required to reach maximum absorbance. Conditions were: 2-nitrophenylhydrazine, 0.005 *M*; and phthalic anhydride, 0.0001 *M*. [c] No reaction within 2 hr.

Anal.—Calc. for $C_8H_9N_3O_3$: C, 49.22; H, 4.66; N, 21.53. Found: C, 49.44; H, 4.93; N, 21.80.

2. Phthalyl-2′-nitrophenylhydrazide, mp 291° [lit. (31) mp 293–294°].

Anal.—Calc. for $C_{14}H_{11}N_3O_5$: C, 55.81; H, 3.69; N, 13.95. Found: C, 56.05; H, 3.76; N, 13.92.

Kinetics of 2-Nitrophenylhydrazine Decomposition—The decomposition of 2-nitrophenylhydrazine in aqueous solutions was followed by measuring the decrease in absorbance at 435 nm as a function of time. Pseudo-first-order rate constants were calculated from plots of log $(A_\infty - A_t)$ *versus* time, where A_∞ = the absorbance at infinity, and A_t = the absorbance at the time interval taken. Small contributions of the buffer to the decomposition of the substrate were eliminated by measuring the rate at different buffer concentrations and extrapolating to the rate constant at zero buffer concentration. All solutions were adjusted to an ionic strength of 0.15 *M* with sodium perchlorate.

Effect of Solvent on Extent of Reaction—The effect of solvent on the extent of reaction was measured by reacting phthalic anhydride with 2-nitrophenylhydrazine in various solvent mixtures. One-milliliter aliquots of the reaction mixture were removed as a function of time and made basic with sodium hydroxide. The final concentration of hydroxide was approximately 0.1 *M*. The absorbances of the final solutions were measured at 550 nm. Solvent compositions are listed in Table I.

Determination of Reaction Time and Reaction Yields for Anhydrides—Reaction mixtures were prepared by mixing appropriate volumes of stock solutions of 2-nitrophenylhydrazine hydrochloride, phosphate buffer, anhydride, and water. Stock solutions of anhydrides were freshly prepared in acetonitrile. One-milliliter aliquots were removed and diluted to 10 ml with an aqueous sodium hydroxide solution, the final concentration of hydroxide being 0.1 *M*. The visible spectra of these solutions were measured to determine the wavelength of maximum absorbance for each anhydride. All reactions were carried out using the following conditions: 2-nitrophenylhydrazine, 0.0023 *M*; phosphate buffer, 0.005 *M*; anhydride, approximately 0.0001 *M*; pH 6.10 (±0.05); and 25°. Reaction yields were determined by reacting different concentrations of the anhydride for a sufficient time, removing an aliquot, making it basic, and measuring the absorbance at an appropriate wavelength. The absorbances were plotted against the apparent concentration of hydrazide in the spectral solution (complete conversion of anhydride to hydrazide was assumed). At least four different substrate concentrations were used for each anhydride.

Determination of Reaction Time and Reaction Yields for Acid Chlorides—Reaction mixtures were prepared by mixing stock solutions of 2-nitrophenylhydrazine (in acetonitrile), acid chloride (freshly prepared in acetonitrile), and acetonitrile. One-milliliter aliquots were removed as a function of time and diluted to 10 ml with an aqueous sodium hydroxide solution, giving a final hydroxide concentration of 0.1 *M*. The visible absorption spectra of these solutions were measured to determine the wavelength of maximum absorption. Reaction yields were obtained by plotting absorbance against apparent concentration of hydrazide in the spectral solution (complete conversion of the acid chloride to hydrazide was assumed). At least four different substrate concentrations were used for each acid chloride. All reactions were carried out under the following conditions: 2-nitrophenylhydrazine, 0.004 *M*; acid chloride, approximately 0.0001 *M*; and 25°.

Table II—Reaction of Acid Anhydrides with 2-Nitrophenylhydrazine in Aqueous Solution

Substrate	t_∞[a], min	λ_{max}, nm	Apparent Molar Absorptivity[b], liters mole^{-1} cm^{-1}
Acetic anhydride	<2	530	5710
Phthalic anhydride	<2	550	7330
Maleic anhydride	4	550	3500
Propionic anhydride	10	530	4800
Succinic anhydride	10	530	1330
Benzoic anhydride	60	540	1190

[a] Time required for maximum color yield. [b] In 0.1 *N* sodium hydroxide. Conditions were: 2-nitrophenylhydrazine, 0.0023 *M*; phosphate buffer, 0.005 *M*; pH 6.10 (±0.05); and 25°.

Determination of Interferences—The possibility of interference by other carboxylic acid derivatives and carbonyl compounds was investigated by reacting various substrates in the aqueous and acetonitrile systems. Aliquots were removed at 30 and 60 min and made basic. The appearance of a blue color (or absorbance between 520 and 570 nm) was used to indicate interference. Concentrations of substrates used were 10–100 times greater than those used in determining reaction yields for the anhydrides and acid chlorides.

Investigation of Decomposition Products of 2-Nitrophenylhydrazine—One hundred milligrams of 2-nitrophenylhydrazine was dissolved in 50 ml distilled water. A saturated sodium hydroxide solution was added dropwise until the solution was decolorized. The solution was adjusted to pH 7 and extracted with three 25-ml portions of ether. The ether fractions were combined, dried with anhydrous sodium sulfate, and evaporated to dryness. The aqueous fraction was made basic (pH 10–11) and the extraction procedure was repeated. The aqueous fraction was then made acidic (pH 1–2) and extracted with ether as before. Only the acidic fraction yielded a significant residue. This residue was recrystallized from hot water, yielding white needles melting at 155° and having the following elemental composition: C, 53.55%; H, 3.69%; and N, 30.08%.

Suggested Analytical Procedures—*Anhydrides*—Prepare the reaction mixture by mixing 5.0 ml of a stock solution of 2-nitrophenylhydrazine hydrochloride (0.110 g in 50.0 ml methanol), 5.0 ml of phosphate buffer solution (1.69 g of dried reagent grade potassium dihydrogen phosphate, KH_2PO_4, and 1.76 g of dried reagent grade disodium hydrogen phosphate, Na_2HPO_4, dissolved in 1000 ml distilled water), and sufficient distilled water to make 25.0 ml. Pipet 5.0 ml of the reaction mixture into a 10.0-ml volumetric flask. Add 1.0 ml of a suitably diluted solution of the anhydride, mix thoroughly, and allow to stand at 25° for the appropriate time (Table II). Add 1.0 ml of 1.0 *N* sodium hydroxide and dilute to 10.0 ml with distilled water. Measure absorbance at the appropriate wavelength.

Table III—Reaction of Acid Chlorides with 2-Nitrophenylhydrazine in Acetonitrile

Substrate	t_∞[a], min	λ_{max}, nm	Apparent Molar Absorptivity[b], liters mole^{-1} cm^{-1}
Acetyl chloride	<2	530	7908
Propionyl chloride	<2	530	6560
Pivaloyl chloride	3	530	5990
Phthaloyl chloride	4	550	7420
Benzoyl chloride	6	540	9090
p-Toluoyl chloride	9	540	8910

[a] Time required for maximum reaction. [b] In 0.1 *N* sodium hydroxide (aqueous). Conditions were: 2-nitrophenylhydrazine, 0.004 *M*; and 25°.

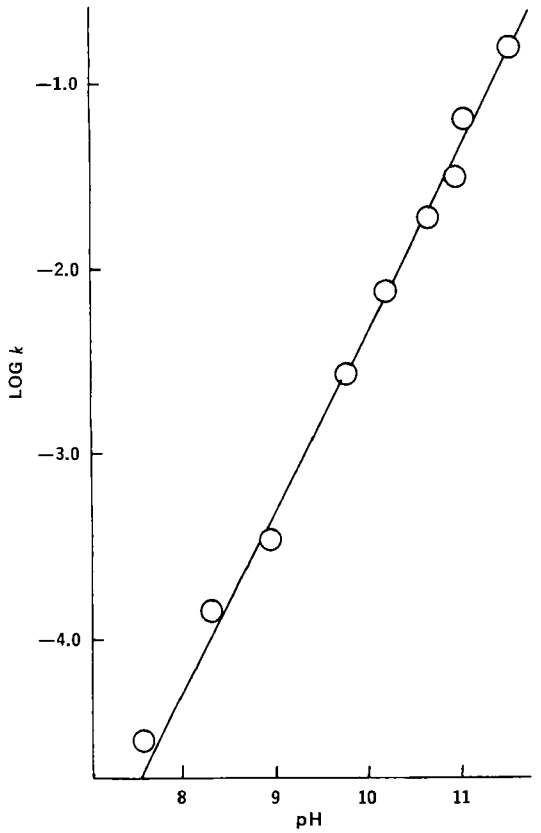

Figure 1—*The pH–rate profile for the decomposition of 2-nitrophenylhydrazine in aqueous solutions at 25.0° and ionic strength of 0.15* M.

Acid Chlorides—Pipet 2.0 ml of a 0.04 *M* solution of 2-nitrophenylhydrazine in acetonitrile into a 10.0-ml volumetric flask. Pipet 2.0 ml of a suitably diluted solution of the acid chloride in acetonitrile into the flask. Mix and allow to react for the appropriate time at 25° (Table III). Add 1.0 ml of 1.0 *N* sodium hydroxide and dilute to volume with distilled water. Measure the absorbance at the appropriate wavelength.

RESULTS AND DISCUSSION

Effect of Solvent on Extent of Reaction—Table I gives the effect of different solvents on the reaction of phthalic anhydride and 2-nitrophenylhydrazine (I). Since the solvolysis of the substrate was a possible competing reaction in the analysis, this study was performed to find a solvent that would give a minimum amount of solvolysis while not severely lowering the reaction rate. The reaction rate decreased with a decrease in the dielectric constant of the solvent within the hydroxylic solvent series. However, yields of the 2-nitrophenylhydrazide were not significantly different. Therefore, water was used as the solvent for the rest of the anhydride studies. Acetonitrile (an aprotic solvent) gave no reaction within 2 hr; this finding was utilized in later experiments on the analysis of the acid chlorides. Further work on the solvent requirement for the reaction is currently underway.

Table IV—Reaction of Anhydrides with 2-Nitrophenylhydrazine in Acetonitrile

Substrate	Half-Life[a], hr
Acetic anhydride	10
Propionic anhydride	10
Maleic anhydride	44
Phthalic anhydride	90
Succinic anhydride	>150
Benzoic anhydride	>150

[a] Taken from plots of absorbance *versus* time. Conditions were: 2-nitrophenylhydrazine, 0.004 *M*; and 25°.

Table V—Compounds Not Reacting within 1 hr at 25° in Either the Aqueous or Acetonitrile System

Acetone[a]	*p*-Nitrophenyl acetate
Acetophenone[a]	Butyrolactone
Benzaldehyde[a]	Acetamide
Succinimide	Thionyl chloride
Ethyl acetate	*p*-Toluenesulfonyl chloride

[a] Reacted to form precipitates but did not produce a blue color in aqueous sodium hydroxide. For other possible interferences, consult Ref. 37.

Kinetics of 2-Nitrophenylhydrazine Decomposition—In early investigations, it was found that I in solution underwent a decoloration from orange to colorless as the solution is made basic. Addition of an acid failed to reverse this color change, suggesting that a decomposition is involved rather than an acid–base equilibrium. A partial pH–rate profile for the decomposition of I in aqueous solutions is given in Fig. 1. The slope of the line indicates that the reaction is first order with respect to hydroxide. The only product isolated from the basic degradation mixture is consistent with the compound 1-hydroxy-1,2,3-benzotriazole, mp 155° [lit. (32) mp 159–160°].

Anal.—Calc. for $C_6H_5N_3O$: C, 53.33; H, 3.74; N, 31.09. Found: C, 53.55; H, 3.69; N, 30.88.

The presence of this compound is consistent with the findings of Nietzki and Braunschweig (33). A similar product, 6-nitro-1-hydroxy-1,2,3-benzotriazole, was isolated as a basic decomposition product of 2,4-dinitrophenylhydrazine (34). However, in the case of 2,4-dinitrophenylhydrazine, dinitrobenzene and *m,m'*-dinitroazoxybenzene were also isolated. For a review of the condensation reactions of I and related compounds, the reader is referred to Ref. 35.

The rapid decomposition of I at basic pH presents a potential problem because it would be difficult to maintain a sufficient concentration of nucleophile under these conditions. The pKa for I was found to be 3.46 at 25° by potentiometric titration. This value agrees well with a literature value of 3.50 (36). Because of this low pKa, I will be essentially in the free base form at pH values as low as 6 or 7. From Fig. 1, it can be seen that this compound is reasonably stable at these pH values. Therefore, all further investigations of I reactions in water were carried out between pH 6 and 7. An additional benefit of working at this pH is that the hydroxide-catalyzed hydrolysis of the anhydrides is minimized.

Reaction Time and Yields for Anhydrides—The results of the study of the reaction of anhydrides and I are given in Table II. Multiple determination of the apparent molar absorptivity of the acetic anhydride reaction gave excellent agreement (5710 ± 100 liters $mole^{-1}$ cm^{-1}). The molar absorptivities of acetyl-2′-nitrophenylhydrazide and phthalyl-2′-nitrophenylhydrazide in 0.1 *N* sodium hydroxide were found to be 6850 and 8570 liters $mole^{-1}$ cm^{-1}, respectively. These values indicate that yields are less than quantitative, probably due to a competing hydrolysis reaction, and that standards should be run with each unknown. An attempt to analyze cinnamic anhydride under these conditions was not successful due to the insolubility of the substrate. The color of the basic solutions was stable for at least 3 hr.

Table VI—Calibration Data for Determination of Acetic Anhydride by Reaction with 2-Nitrophenylhydrazine in Aqueous Solution

10^4C, M[a]	A_{530}[b]	$\bar{A}_{530}$
0.336	0.175, 0.175, 0.175	0.173
0.672	0.370, 0.378, 0.378	0.375
0.840	0.480, 0.480, 0.480	0.480
1.008	0.570, 0.581, 0.583	0.578
1.681	0.946, 0.953, 0.958	0.952
2.689	1.501, 1.501, 1.575	1.526

[a] Concentration in the spectral solution. This represents a 10-fold dilution of the original anhydride solution. [b] One-centimeter cells.

Table VII—Calibration Data for Determination of Acetyl Chloride by Reaction with 2-Nitrophenylhydrazine in Acetonitrile

10^4C, M[a]	A_{530}[b]	$\bar{A}_{530}$
0.298	0.246, 0.240, 0.270	0.252
0.349	0.255, 0.257, 0.260	0.257
0.596	0.485, 0.500, 0.495	0.493
0.948	0.760, 0.758, 0.756	0.758
1.190	0.928, 0.925, 0.925	0.926
1.49	1.203, 1.208, 1.195	1.202
1.75	1.367, 1.390	1.379

[a] Concentration in spectral solution. This represents a fivefold dilution of the original acetyl chloride solution. [b] One-centimeter cells.

Acid chlorides also react under these conditions, but the yields are very low, probably due to rapid hydrolysis of the substrate. No further work was carried out on the reaction of acid chlorides in water.

Reaction of Acid Chlorides with 2-Nitrophenylhydrazine in Acetonitrile—Table III presents the results of the reaction of various acid chlorides and I in acetonitrile. The apparent molar absorptivity of the product of the acetyl chloride reaction is slightly higher than that of acetyl-2′-nitrophenylhydrazide. While the yields are very reproducible, this slightly higher value (7908 liters $mole^{-1}$ cm^{-1}) is unexplained at this time. The reaction products of the acetonitrile reaction are currently under investigation. As a product is formed in this reaction, an equivalent amount of 2-nitrophenylhydrazine hydrochloride is formed which precipitates from solution. This effect is more noticeable at higher substrate concentrations but does not interfere in the analysis.

Reaction of Acid Anhydrides with 2-Nitrophenylhydrazine in Acetonitrile—Based on the early observation that phthalic anhydride did not react with I in acetonitrile, a study was carried out to measure the extent of the reaction of anhydrides in this system over a longer period. The large difference in the reaction times between acid chlorides and anhydrides in acetonitrile (Table IV) suggests that acid chlorides could be measured in the presence of anhydrides in an acetonitrile system with as little as 1% error. This analysis is feasible if the anhydride and acid chloride are of comparable concentrations. In the event that the anhydride is in a large excess of the acid chloride, the interference from the anhydride may become too great for accurate analysis.

Reaction of Other Compounds with 2-Nitrophenylhydrazine—A variety of carboxylic acid derivatives, carbonyl compounds, and related substrates were examined for reaction in both the aqueous and acetonitrile systems. Absorbance at 520–570 nm or the production of a blue color was used to indicate reaction. Table V lists those compounds that gave no reaction within 1 hr at 25°. The concentrations of substrates used were 10–100 times those used in the anhydride and acid chloride studies. Acetone, acetophenone, and benzaldehyde reacted with I in the aqueous system, as evidenced by the formation of a precipitate. However, no blue color was formed on the addition of sodium hydroxide. This reaction, probably hydrazone formation, represents a possible interference since it consumes nucleophile. Legradi (37) reported that acetyl and benzoyl acetone, substituted benzaldehydes, and formaldehyde reacted with I to give colors ranging from orange to violet.

Results of Suggested Analytical Procedures—*Anhydrides*—A variety of solutions of acetic anhydride (freshly prepared in acetonitrile) were subjected to the suggested procedure (Table VI). Over the concentration range used, the calibration curve is linear; the linear regression line for all data is $A_{530} = 5715.5C - 0.008$, with a correlation coefficient of 0.999 and a standard error of 0.016.

Acid Chlorides—The results of several determinations of acetyl chloride are presented in Table VII. The calibration curve is linear over this concentration range; the linear regression line for all data is $A_{530} = 7908C + 0.005$, with a correlation coefficient of 0.999 and a standard error of 0.018.

The reaction of I with acid chlorides and anhydrides is potentially useful for the analysis of these substrates. The low nucleophilicity and consequent high selectivity of the nucleophile make it an excellent choice for analysis of these substrates in the presence of other carboxylic acid derivatives. Since the nucleophile decomposes to colorless products under the final analysis conditions, large concentrations of it can be used without causing an interference in the final measurement.

Throughout this study, very low concentrations of nucleophile were used. Obviously, faster reactions can be achieved at higher nucleophile concentrations, but the possibility of interferences should be investigated under these conditions. However, solubility of I becomes a limiting factor in aqueous reaction mixtures.

Other functional groups that can be converted easily to acid chlorides and anhydrides may be analyzed using this reaction. Legradi (38) utilized this reaction as a spot test for acids after first converting the acid to acid chloride by the action of thionyl chloride. Higher molecular weight acids such as fatty acids were analyzed by this method in this laboratory. However, yields are not entirely reproducible and further work is in progress.

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Anticancer Activity of Zoanthids and the Associated Toxin, Palytoxin, against Ehrlich Ascites Tumor and P-388 Lymphocytic Leukemia in Mice

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Abstract □ Crude alcoholic extracts of zoanthids, indigenous to Hawaii, have been shown to have general antitumor activity against Ehrlich ascites tumor in mice. The toxic component, palytoxin, was effective in the control and cure of Ehrlich ascites at extremely low dosages using several different methods of administration and displayed marginal activity against P-388 lymphocytic leukemia.

Keyphrases □ Zoanthids—antitumor activity of crude extracts, palytoxin as potent antitumor component □ Anticancer agents, potential—pharmacological testing of zoanthids and palytoxin against Ehrlich ascites tumor and P-388 leukemia □ Palytoxin—pharmacological studies as antitumor agent

Observations of antitumor activity in extracts of marine invertebrates (1–3), particularly the phylum Coelenterata (4, 5), have now been extended to extracts of a number of species belonging to the family Zoanthidae, order Zoantharia. The water-soluble materials, from ethanolic extracts of eight Hawaiian species representing three genera of zoanthids, have been found to inhibit growth of Ehrlich ascites carcinoma in Swiss Webster mice.

The specific Ehrlich ascites activity increased with increasing toxicity of the extracts, suggesting that the toxic component itself may be responsible for the antitumor activity. Palytoxin, the toxic principle of "limu-make-o-Hana," a zoanthid from Maui, Hawaii (6), which has been identified as *Palythoa toxica* Walsh and Bowers (7), was found to be a potent antitumor agent that completely controlled Ehrlich ascites carcinoma in mice at doses as low as 84 ng/kg and exhibited an inhibitory effect on the tumor in doses as low as 5.25 ng/kg. Several different modes of administration and injection schedules were used to evaluate its activity against Ehrlich ascites tumor. Palytoxin was tested also against P-388 lymphocytic leukemia in mice but showed only marginal activity.

RESULTS AND DISCUSSION

The activities of the crude extracts against Ehrlich ascites tumor are given in Table I. In this laboratory, during the past 5 years, more than 2500 mice were used as infected controls and none survived for as long as 30 days. Consequently, the presence of any survivors in the treated group at 30 days indicates significant activity. The absence of observable abdominal distension from ascitic fluid was the basis for describing survivors as nonascitic (8).

Since the specific Ehrlich ascites activity generally increased with increasing toxicity of the extracts, the antitumor activity may be primarily due to palytoxin. In this study, the most toxic and Ehrlich ascites-active sample was obtained from an unidentified zoanthid from Haleiwa, Oahu; nontoxic extracts, at least at the levels tested, with low Ehrlich ascites activity were obtained from *Zoanthus pacificus*, *Palythoa psammophilia*, and an unidentified zoanthid from a tidepool adjacent to Makapuu Beach, Oahu. The palytoxins from *Palythoa tuberculosa*, *Palythoa vestitus*, and the zoanthid from Haleiwa were found to have UV spectra identical with that of palytoxin from *P. toxica*. Whether the palytoxins from these various sources are all structurally identical or subtly different remains to be demonstrated. Recently, it was found that the toxicity of *P. tuberculosa* is seasonally dependent and, more importantly, is linked with the reproductive cycle of the zoanthid because the toxin appears to be associated with the bisexual and female polyps and is located in the eggs of the female polyps (9). This finding might explain the low toxicity of the specimen of *P. toxica* that was collected in September at the Halona Blowhole, Oahu, since all previous specimens of this organism had shown toxicities comparable to that of *P. toxica* from Muolea, Maui.

Only a few Hawaiian zoanthids have been adequately described in the literature (7), and all have been classified in the three genera *Palythoa*, *Zoanthus*, and *Isaurus*. In this work, six known and two new species of zoanthids[1] were collected and evaluated for anticancer activity. For a preliminary chemotaxonomic identification of each organism, the sterol complement of the zoanthid was isolated, analyzed by GLC and mass spectrometry, and compared with those from known zoanthids. The two unidentified zoanthids from Haleiwa and Makapuu, as well as *P. toxica*, contained essentially a single sterol (24-methylenecholesterol), the

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