

Interaction of Di- and Tricarboxylic Acids with Glutaric Anhydride in Aqueous Solution

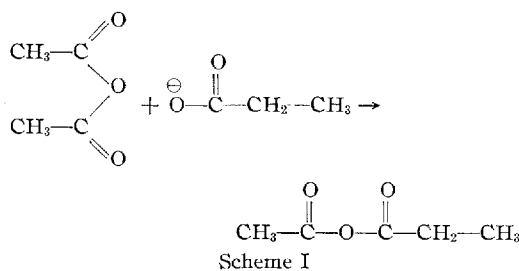
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Anionic forms of polycarboxylic acids such as succinic, citric, etc., interact reversibly and rapidly with glutaric anhydride in aqueous solution at room temperature to produce species which undergo subsequent hydrolysis. The species formed *in situ* from citrate was reacted with aniline and the resulting products isolated by column chromatography. Results of the chromatographic study suggested a mechanism based on the initial formation of a mixed anhydride which cleaved to produce an anhydride of the attacking anionic species. Spectrophotometric investigations have shown that the rate of formation and subsequent hydrolysis of the anhydride are dependent upon the pH and the buffer concentration. These reactive species are presumed to be formed to a varying extent in any formulation containing citrate, particularly under autoclaving conditions.

MANY BIOCHEMICAL systems as well as pharmaceutical formulations contain polycarboxylic acids. Citric acid, tartaric acid, malic acid, aconitic acid, and succinic acid are examples of food acids which occur naturally and are also often used in pharmaceutical preparations. In an earlier report (1) it was shown that these acids exist in solution in equilibrium with their corresponding cyclic acid anhydride forms which are capable of reacting with any nucleophilic species present. The present communication is concerned with results of studies designed to determine the rate and mechanism of transference of this anhydride character in a mixture containing two of these polycarboxylic acid species. Specifically, the interaction of citrate species with glutaric anhydride was investigated.

In aqueous solution, since water is a weak but an effective nucleophile, acid anhydrides undergo relatively rapid hydrolysis with half lives of the order of minutes. In the presence of other more potent electron donors, which are often constituents of frequently used pharmaceutical buffers, other reactions may take place preferentially. Thus, it has been suggested that acetic anhydride reacts with propionate ions with subsequent formation of a mixed anhydride (2).

Scheme I as written would be expected to be largely irreversible in the presence of a large excess of the attacking carboxylate species. If an analogous reaction takes place between a cyclic anhydride and a polycarboxylic acid species, on the other hand, an equilibrium system such as



that shown in Scheme II may be expected to take place (3).¹

Formation of citric anhydride probably results from intramolecular attack from the neighboring carboxyl group of the citrate moiety on a carbonyl carbon in the mixed anhydride. The mixed anhydride may also revert back to glutaric anhydride by the same mechanism and is probably present only in relatively low concentration.

The present investigation was designed to determine whether the postulated reactions would occur and to establish the individual rates and products of the system. This was done by observing spectrometric changes in the reacting systems and by chromatographic analysis and identification of the end products.

RESULTS AND OBSERVATIONS

Spectrophotometric Studies.—Glutaric anhydride has a characteristic ultraviolet absorbance spectrum that can be utilized to follow its hydrolysis in aqueous solution. When citric acid is employed as a buffer at a pH where substantial quantities of the di-ionized species are present, a change in ultraviolet absorbance is observed. This interaction can be conveniently followed since the reactants, intermediate species, and the final products apparently

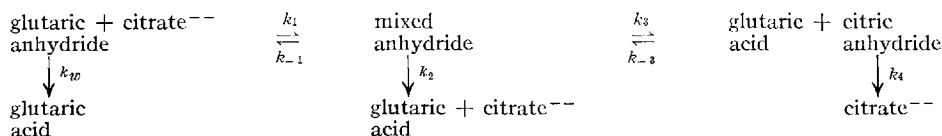
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¹ Higuchi *et al.* (3) have also shown that acyl exchange of this type takes place with great facility. Their paper should be consulted for specific details.



possess sufficiently different ultraviolet molar absorptivities to permit observation of the various reactions. A typical absorbance change at 248 $m\mu$ with time for a system containing initially 7.05×10^{-3} moles of glutaric anhydride in 0.3 M citrate buffer at $\text{pH} = 5.0$ is shown in Fig. 1. There is a small increase in absorbance initially, followed by a logarithmic approach to an equilibrium value. This corresponds presumably to a $G + B \rightleftharpoons C \rightarrow D$ type relationship where species C possesses a slightly higher molar absorptivity than the reactants or the final product. The experimental observation noted above is in agreement, at least in form, to the reactions implicit in Scheme II.

It is apparent from the proposed reaction scheme that there are two possible species which could correspond to species C, the mixed anhydride and citric anhydride. Similarly, the deterioration of the species could be attributable to decomposition of the mixed anhydride or hydrolysis of citric anhydride.

If one assumes $k_3 \gg k_1, k_{-1}, k_2, k_2,$ and k_w , and that k_{-3} is negligible,² the concentration of species such as citric anhydride can be expressed as:

$$[\text{CA}] = \frac{k_1[\text{B}][\text{GA}]_0}{k_1[\text{B}] + k_w - k_4} \times [e^{-k_4 t} - e^{-(k_1[\text{B}] + k_w)t}] \quad (\text{Eq. 1})$$

where $[\text{CA}] =$ citric anhydride, $[\text{GA}] =$ glutaric anhydride, and $[\text{B}] =$ citrate buffer. If the absorbance of a reacting system initially containing glutaric anhydride and citrate is followed at 248 $m\mu$, a plot of $\log [A_T - A_\infty]$ against time (where A_T is the absorbance at any time, t , and A_∞ is the limiting absorbance) will yield a straight line in the terminal phase, provided $(k_1[\text{B}] + k_w) \gg k_4$. This is because under such a circumstance $(A_T - A_\infty)$ will be expected to be essentially proportional to the concentration of citric anhydride when the total anhydride concentration becomes very small. The slope of the logarithmic plot would then be equal to $-k_4$. Extrapolation of this line will give an imaginary $(A_0' - A_\infty)$ value (which is proportional to $[\text{CA}]$) at time zero. A semilogarithmic plot of $[(A_T' - A_\infty) - (A_T - A_\infty)]$ against time will yield a linear relationship with a slope equal to $-(k_1[\text{B}] + k_w)$.

The rates of appearance {corresponding to $(k_1[\text{B}] + k_w)$ } of the strongly absorbing intermediate species (citric anhydride?) were determined from plots of $(A_T' - A_T)$ for systems at several pH values and different buffer concentrations. Results are shown in Fig. 2. The data suggest that the initial reaction between the anhydride and citrate probably involves the di- and/or tri-ionized form of citric acid.

The apparent rates of loss of the active species (k_4) determined as outlined above are shown in

² Pseudo first-order conditions were maintained and there was always at least a sixtyfold excess of citrate buffer.

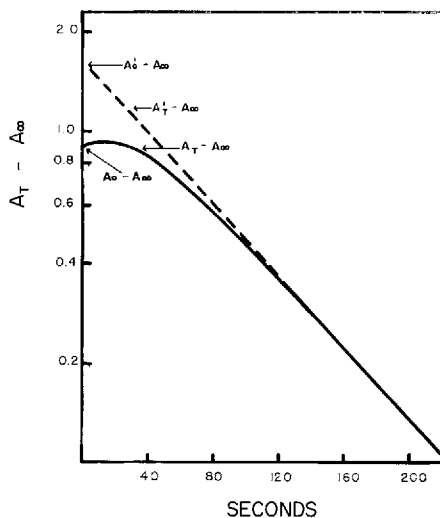


Fig. 1.—Semilogarithmic plot of absorbance change at 248 $m\mu$ for the system glutaric anhydride in citrate buffer. $\text{pH} = 5.0$, 0.3 M citrate; $T = 25^\circ$.

Fig. 3 for several values of pH and various buffer concentrations. The pH range studied was limited to that where citrate behaved effectively as a buffer. Based on the proposed mechanism of relatively rapid formation of citric anhydride followed by a slower hydrolysis, the apparent pH dependency could be ascribed to a slower rate of formation of citric anhydride at lower pH values and buffer concentrations. The relationship presented above permitting separation of the individual rate constants requires that $(k_1[\text{B}] + k_w)$ be at least 4 to 5 times larger than k_4 . Since in the lower pH range this minimum ratio in the reaction velocities is not apparently obtained, the limiting slopes do not reflect the true magnitude of k_4 in this range. The terminal slopes above $\text{pH} = 4.5$ are considered to reflect the true values of k_4 in that at these pH values, the rate of formation is considerably faster than the subsequent hydrolysis. In Fig. 3, above $\text{pH} = 4.5$, the lines connecting the experimental points extrapolate back to a common value of $11.5 \times 10^{-3} \text{ sec}^{-1}$. This is assumed to be the rate constant for hydrolysis of citric anhydride in water.

The observed decrease in absorbance may also be attributed to slow disappearance of the mixed anhydride (to form citric anhydride) followed by rapid hydrolysis of citric anhydride. A mechanism such as this would require that the glutaric-citric mixed anhydride would be a relatively stable species which would not be expected based on the structure. Direct hydrolysis of the mixed anhydride is also a possibility, but again a stable mixed anhydride could be required. Further work is

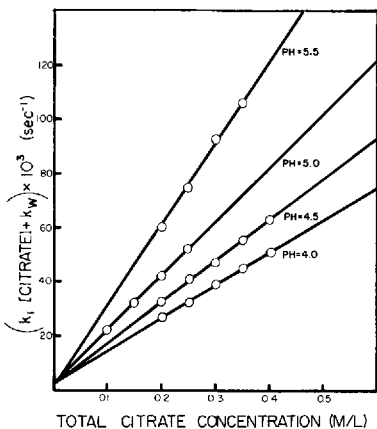


Fig. 2.—Rate of formation of the species formed from the glutaric-citric system at various pH values and different buffer concentrations.

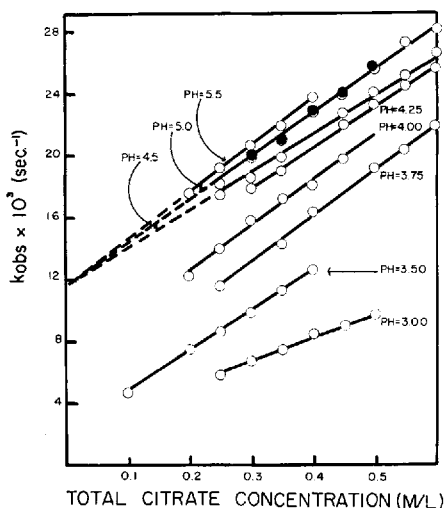


Fig. 3.—Observed rate of hydrolysis of the species formed from the interaction of glutaric anhydride in citrate buffer at various pH values and different buffer concentration. Key: ●, 1 M KCl; ○, H₂O.

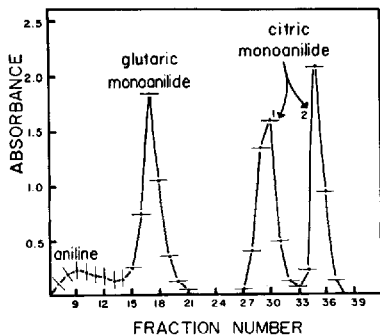


Fig. 4.—Typical chromatogram for the anilides formed by the addition of aniline to the reaction mixture. The reaction was quenched with aniline after 30 sec. The eluate was extracted with 0.1 N NaOH and the alkaline solution analyzed spectrophotometrically at 241 m μ .

required to definitely establish which mechanism is correct, but intuitively the scheme involving rapid formation of citric anhydride followed by a relatively slow hydrolysis would seem the more likely.

Chromatographic Studies.—Although results of rate studies were strongly indicative of formation of citric anhydride species from mixtures of glutaric anhydride and citrate buffers, the data were not considered definite by themselves. Attempts were made to substantiate these findings by direct chemical examination of the reacting systems. The procedure adopted was isolation and quantification of the reaction products resulting from addition of aniline to the anhydride mixture, all anhydride species present presumably reacting extremely rapidly with the strong nucleophile aniline.

Separation of reaction products following addition of aniline to the reacting systems are evident in the chromatogram shown in Fig. 4. The particular run contained initially 0.3 M pH = 5 citrate buffer and 3.95×10^{-5} moles of glutaric anhydride with a trace of dioxane. The reaction was quenched with aniline after 30 sec. and separated by column partition chromatography and analyzed by ultraviolet spectrophotometry as described earlier (4). The double peak ascribed to citric monoanilide corresponds to the isomeric forms as previously suggested (4). Elemental analysis, equivalent weight, ultraviolet spectrum, and infrared spectrum of the compounds corresponding to peaks 1 and 2 were determined and found to be compatible with that of citric monoanilide.

Supportive evidence to corroborate the postulate that the two peaks correspond to the isomeric forms of citric monoanilide was obtained by hydrolyzing citranilic acid³ and chromatographing the resultant products. Random hydrolysis of this citric acid imide will produce the two isomeric monoanilides of citric acid. Chromatographic separation of this mixture in the same manner as that employed for the anilides formed in the glutaric anhydride-citric acid mixture gave two peaks. The retention volume required for these two peaks was the same as that required for the two peaks in the glutaric-citric case.

It is conceivable that citric monoanilide could have formed through a lactone intermediate. However, previous work with the lactones of tartaric acid (5) has shown them to be unreactive toward aromatic amines under the conditions employed here, and therefore, this was ruled out as a possible pathway.

Citric monoanilide theoretically could have been formed through a mixed anhydride as well as citric anhydride. Previous work has shown (6) that addition of aniline to a mixed anhydride usually gave anilides of both compounds. Depending upon the nature of the mixed anhydride, the yield of one of the anilides may be favored over the other. When aniline was added to the reaction mixture where the pII of citrate buffer was 2.0, citric monoanilide could not be isolated.⁴

³ Citranilic acid was prepared according to Higuchi *et al.* (4), m.p. 187–188° (literature values vary from 185–189°). Molecular weight determined by direct titration with sodium hydroxide was found to be 253 (calculated 249).

⁴ At pII = 2.0, the fraction of aniline in the protonated form is quite high and therefore a large excess was employed. Isolation of glutaric monoanilide from the reaction mixture indicated that sufficient unprotonated aniline was present to react with anhydrides in the system.

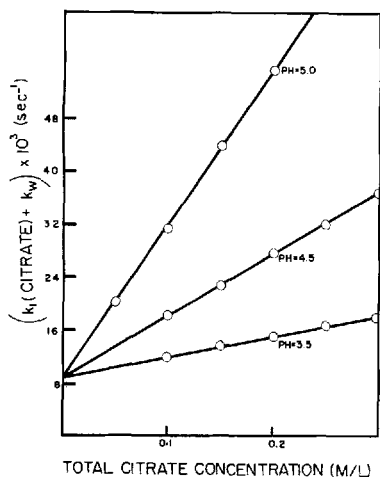


Fig. 5.—Disappearance of phthalic anhydride at 302 $m\mu$ as a function of pH and buffer concentration.

At a pH = 2.0, using 3.08 and 4.74 as the respective first and second ionization constants of citric acid (7), the monoionized and free acid would be present almost exclusively with very little of the di-ionized species present. If the monoionized citrate species can attack glutaric anhydride, and since citric monoanilide was not isolated, it is reasonable to assume that either the mixed anhydride is present in a very low concentration or that glutaric monoanilide is exclusively favored in this system.

An additional system that was tried was glutaric anhydride in a pH = 5.5 acetate buffer. Addition of aniline to this reaction mixture did not produce any acetanilide. This would indicate that this system, like the previous one, hydrolyzes *via* an unstable mixed anhydride or formation of glutaric monoanilide is exclusively favored. An alternative possibility is that acetate ion serves as a general base in this system.

To demonstrate the generality of the reaction, phthalic anhydride was used in place of glutaric anhydride. Phthalic anhydride hydrolysis was followed at 315 $m\mu$ in citrate buffer. Unlike the glutaric case, there was no apparent anomaly observed in the ultraviolet spectrum, indicating that if a new species was being formed in solution, it had less absorbance at this wavelength than the reactants. The rate of hydrolysis of phthalic anhydride as a function of pH and buffer concentration is shown in Fig. 5. Apparently this system, like glutaric anhydride, is sensitive to the di- and/or tri-ionized species of citric acid. Addition of aniline to the reaction mixture in a manner the same as before gave substantial quantities of citric monoanilide. This indicates that phthalic anhydride like glutaric anhydride is capable of reacting with citrate to form a species in solution which has anhydride properties.

DISCUSSION

Polycarboxylic acids are often used in combination as buffer components; in light of the findings of this study an exchange of anhydride character also

presents a possible pathway for drug loss from solution. Equally important is the possibility that a myriad of products could result.

EXPERIMENTAL

Equipment and Reagents.—A Cary model 11 M.S. recording spectrophotometer was utilized for the spectrophotometric determinations. All pH measurements were made with a Beckman Zeromatic pH meter with an expanded scale.

Aniline was purified by repeated distillation and was stored under nitrogen prior to use. Dioxane was purified according to Vogel (8). Glutaric anhydride was recrystallized from ether until a m.p. of 56–57° was obtained. All other chemicals were of analytical or reagent grade.

Procedure for Kinetic Runs on Citric Acid–Glutaric Anhydride Reactions.—Citrate buffers of appropriate concentration and pH were prepared. One-hundred microliters of a 0.450 *M* glutaric anhydride in dioxane solution was mixed with 6 ml. of citrate buffer in a 2-cm. photometer cell. The reaction was followed spectrophotometrically at 248 $m\mu$. All reaction solutions were equilibrated at 25 ± 0.1° prior to use. The concentration of dioxane used in all cases was determined to have a negligible effect on the rate constants.

Procedure for Kinetic Runs on Citric Acid–Phthalic Anhydride Reactions.—Citrate buffers of appropriate concentration and pH were prepared. Fifty microliters of a 0.0338 *M* phthalic anhydride in dioxane solution was mixed with 6 ml. of citrate buffer in a 2-cm. photometer cell. The reaction was followed spectrophotometrically at 302 $m\mu$. All reaction solutions were equilibrated at 25 ± 0.1° prior to use.

Chromatographic Separation of the Reaction Mixture.—The chromatographic columns were prepared as outlined in a previous communication (4). The reaction mixture was prepared by adding 15 ml. of citrate buffer to 100 μ l. of a 1.58 *M* glutaric anhydride in dioxane solution. The reaction was allowed to continue the requisite period of time and was quenched with 4 ml. of a 0.33 *M* aqueous aniline solution. The pH was adjusted to 3.13 with HCl and a 5-ml. sample was placed on the column with 5 Gm. of silicic acid. The eluting solutions consisted of 100 ml. each of chloroform, 1.5% butanol in chloroform, 10% butanol in chloroform, and 30% butanol in chloroform; each solution was saturated with the internal phase prior to use. The eluate was collected in 10-ml. fractions; the collecting vessel was rinsed with 10 ml. of chloroform and added to the 10-ml. fraction. Each sample was extracted with 20 ml. of 0.1 *N* NaOH.

Preparative Chromatography of the Reaction Mixture.—A chromatographic column with the following dimensions was employed: length = 60 cm., i.d. = 3.40 cm. Three-hundred grams of silicic acid was utilized as the support and 300 ml. of pH 3.13 phosphate buffer was employed as the internal phase. The eluting solutions consisted of 500 ml. each of chloroform, 1.5% butanol in chloroform, 5% butanol in chloroform, 15% butanol in chloroform, and 35% butanol in chloroform; each solution was saturated with the internal phase prior to use. The reaction mixture was prepared by mixing 100 ml. of 0.3 pH = 5 citrate buffer

with 3.2 ml. of 1.58 *M* glutaric anhydride in dioxane solution. The reaction mixture was quenched at the end of 30 sec. with 16 ml. of a 0.33 *M* aqueous aniline solution. The pH of the resulting solution was adjusted to pH 3.13 with HCl and 30 ml. of this solution was placed on the column with 30 Gm. of silicic acid. The eluate was collected in 50-ml. fractions. The solvent was removed from each sample under reduced pressure and a mixture of chloroform-petroleum ether added to precipitate the compounds.

Characterization of Reaction Compounds.—*Glutaric Monoanilide*.—This was recrystallized from methanol-water, m.p. 129–130°. Molecular weight found by direct titration against standard NaOH was 205 (calculated 207). Known glutaric monoanilide was prepared by adding glutaric anhydride dissolved in dioxane to an aqueous solution of aniline. The sparingly soluble anilide precipitated and when recrystallized gave a m.p. 129–130°. Both known and unknown compounds gave identical infrared and ultraviolet absorption spectrums.

Citric Monoanilide.—1.—This was recrystallized from chloroform, m.p. 136–137°.

Anal.—Calcd. for C, 53.94; H, 4.87; N, 5.23. Found: C, 53.90; H, 4.94; N, 5.3.

Molecular weight determined by direct titration against NaOH was 269 (calculated 268). Infrared

and ultraviolet spectra were characteristic of an anilide. NMR spectrum strongly suggested this to be the symmetrical isomer, 2-hydroxy-2-*N*-phenylcarbamide-1,3-propanedicarboxylic acid.

Citric Monoanilide.—2.—This was recrystallized from chloroform, m.p. 127–128°. Molecular weight determined by direct titration against sodium hydroxide solution was 270 (calculated 268).

Anal.—Calcd. for C, 53.94; H, 4.87; N, 5.23. Found: C, 53.87; H, 4.85; N, 5.20.

Infrared and ultraviolet absorption spectra were characteristic of an anilide. NMR spectrum suggested this to be the unsymmetrical isomer, 2-hydroxy-1-*N*-phenylcarbamide-2,3-propanedicarboxylic acid.

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Interaction of Acetic Anhydride with Di- and Tri-carboxylic Acids in Aqueous Solution

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Earlier studies have shown that cyclic anhydrides, such as succinic, glutaric, etc., interact with citrate ions in aqueous solution to form what appears to be a citric anhydride species. These interactions have been assumed to be highly reversible through intermediate formation of a mixed anhydride. The present studies were concerned with a comparable system which was expected to be essentially irreversible. Spectrophotometric investigations have shown that acetic anhydride apparently reacts with citrate in aqueous solution to form a new species which undergoes rapid hydrolysis. The rate of the initial reaction and the rate of the subsequent step appear to depend on the citrate concentration and pH. Results of chromatographic studies on products obtained by reaction with aniline at different phases of the reaction are presented.

IN AN EARLIER report (1), experimental evidence was presented which suggested that glutaric and presumably other cyclic anhydrides formed an equilibrium system in the presence

of a large excess of citrate buffer in which the total anhydride concentration was distributed among glutaric anhydride, citric anhydride, and perhaps glutaric-citric anhydride. An attempt has been made in the present study to obtain a clearer picture of the reacting system by employing acetic anhydride to furnish the initial anhydride concentration; the noncyclic anhydride not being expected to participate effectively in any reversible process.

If the forward reaction pathway remains essen-

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