A Nuclear Magnetic Resonance Study of the Anhydrides of Argininosuccinic Acid and Related Guanidino Compounds*

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ABSTRACT: Argininosuccinic acid forms two cyclic anhydrides. Inconsistencies between the chemical and physical properties of anhydrides I and II and the structures previously considered for these compounds have been clarified by a study of the proton magnetic resonance spectra of argininosuccinic acid, the two anhydrides, and other model compounds (guanidinoacetic acid, guanidinosuccinic acid, their respective anhydrides and creatinine). The resonances of the carbon-bound protons observed in D_2O and also in trifluoroacetic acid have been assigned. Changes in the resonance positions of certain protons of the guanidino acids as compared with their respective anhydrides served to indicate the positions involved in cyclization. The results show that anhydride I contains a five-membered ring structure in which the α-carboxyl group of the succinyl moiety is in anhydride linkage with the terminal nitrogen of the ornithine moiety. Anhydride II also contains a five-membered ring structure, the same carbonyl group being linked to the imino nitrogen of the amidine group. The resonances of the nitrogen-bound protons (in trifluoroacetic acid solution) suggest that in several of the guanidino acids, protonation may occur on the CNC nitrogen as well as on the imino nitrogen. The spectra for the guanidino anhydrides strongly support a five-membered ring structure for guanidinosuccinic anhydride similar to that of anhydride II. In these two structures and in the structures assigned to guanidinoacetic anhydride and to creatinine, the double bond is located in the cyclic configuration, conjugate with the carbonyl oxygen. Anhydride I is exceptional in that the double bond is nonconjugate. In accord with this, anhydride II and the model anhydrides are apparently protonated on the carbonyl oxygen in trifluoroacetic acid while anhydride I is protonated on nitrogen.

The physical and chemical properties of these compounds are discussed and shown to be in harmony with these conclusions, particularly with the location of the anhydride linkages, with the protonation in acid solution and with the conjugate or nonconjugate assignments of the double bonds.

Argininosuccinic acid undergoes reversible ring closure by anhydride formation under relatively mild conditions. Two such anhydrides have been described, anhydrides I and II, respectively, and these differ from each other in many physical and chemical properties (Ratner *et al.*, 1953b; Westall, 1960; Ratner and Kunkemueller, 1966). Neither anhydride is active as substrate for the enzymes argininosuccinate synthetase (Petrack and Ratner, 1958) and argininosuccinase (Ratner *et al.*, 1953a) which utilize argininosuccinate itself. However, mutual interconversion among the three compounds occurs with great ease and it is quite likely that the anhydride forms may be encountered in biological materials in the presence of, or even in the absence of, the parent compound, depending upon the conditions of exposure.

In a recent study of these compounds, large differences were found in the behavior of the two anhydrides toward mild oxidizing conditions and toward heat (Ratner and Kunkemueller, 1966). The unusual lability shown by anhydride I, in contrast to anhydride II, suggested that the chemical configuration (Figure 1B) originally suggested (Ratner *et al.*, 1953b) for anhydride I was not in harmony with such lability. It also became evident that the structure for anhydride II should be reconsidered since the new findings were inconsistent with the configuration originally suggested (Figure 1C) for this compound (Westall, 1960).

The structure of the parent acid and the various structures for the two anhydrides are shown in Figure 1. In these structures, the carbon atoms are distinguished with numbers and the nitrogen atoms with letters. For ease of comparison and to avoid confusion, the numbers of the carbons in argininosuccinate and its anhydrides and the letter designation of the nitrogens are retained in the constituent acids shown in later figures. Thus, C-9 and N-d of guandinosuccinate are related to C-9 and N-d of argininosuccinate. The structure given, in each case (except as noted), is the one supported experimentally. Other tautomeric forms are discussed later.

A study of the nuclear magnetic resonance spectra was undertaken in an effort to gain further insight into these problems. The high-resolving power thus afforded under very mild conditions of sample exposure appeared to us to have many advantages for elucidation of the structure of the highly polar compounds con-

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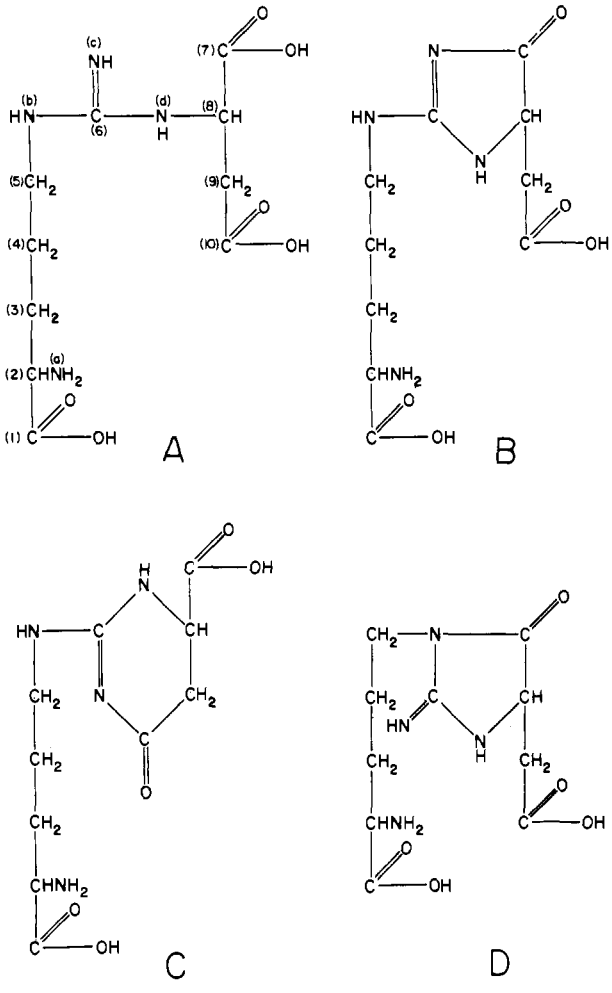

FIGURE 1: Structures of argininosuccinic acid and its anhydrides. (A) Argininosuccinic acid, (B) anhydride II, (C) structure previously considered for anhydride II, and (D) anhydride I.

sidered here. The application of mass spectroscopy or infrared spectroscopy proved to be far less satisfactory.

The spectra in D_2O and trifluoroacetic acid presented here support the view that anhydride I has the five-membered ring structure given in Figure 1D. On the other hand, the spectra for anhydride II are consistent with the five-membered ring structure given in Figure 1B, the configuration previously assigned to anhydride I. The six-membered structure, C, has been excluded from consideration as far as either anhydride is concerned. It has also been possible to assign the five-membered rather than the six-membered ring structure to the anhydride of guanidinosuccinic acid. A definitive conclusion had not been possible from previously available evidence (Ratner *et al.*, 1953a; Ratner and Kunkemueller, 1966).

Since little was known of the nmr spectra of compounds possessing the guanidino acid and guanidino acid anhydride groupings, several model compounds of biological interest were also examined. These include guanidinoacetic acid (glycocyamine), guanidinosuccinic acid, their respective anhydrides, and creatinine. Two resonances have been observed for the protons bonded to nitrogens in the guanidino and guanidino anhydride groups.

The assignments for the protons bonded to the nitrogens in the guanidino anhydride grouping has led to the formulation of a structure for creatinine in which the double bond is located in the cyclic configuration, conjugate with the carbonyl group, rather than in the exocyclic position usually depicted. On the basis of similar results, the conjugate configuration must also be formulated for all of the guanidino anhydrides examined except for anhydride I of argininosuccinate. In the case of anhydride I, the spectra support the nonconjugate structure, as shown in structure D. The proton acquired in trifluoroacetic acid has been assigned to nitrogen N-c (see Figure 1). The fact that protonation occurs at the nitrogen in this anhydride is consistent with the relatively high basicity of this grouping and adds further proof for the unusual location of the anhydride linkage.

Experimental Section

Materials. Deuterium oxide was purchased from the Stewart Oxygen Co. and was distilled before use; anhydrous trifluoroacetic acid was obtained from the Fluorochemical Division of Minnesota Mining and Manufacturing Co. and was redistilled through an all-glass fractionating column. Tetramethylsilane was obtained from the Anderson Laboratories, Inc., Weston, Mich. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate was the gift of Dr. G. V. D. Tiers of the Minnesota Mining and Manufacturing Co. Creatinine, purchased from Mann Research Laboratories, was recrystallized from aqueous acetone. Guanidinoacetic acid anhydride, guanidinosuccinic acid anhydride, argininosuccinic acid, and its two anhydrides were prepared as previously described (Ratner *et al.*, 1953b; Ratner and Kunkemueller, 1966).

Preparation of Solutions. For the runs carried out in D_2O, the amino acid was dissolved in D_2O, lyophilized, and then redissolved in D_2O for examination. The compounds were examined at a concentration of approximately 0.1 M, the pH of the solution being that of the isoionic point of the compound.

Anhydride I had to be used as a supersaturated solution in order to reach this concentration range. Aspartic acid and guanidinosuccinic acid were brought into solution at pH 6–7 by adding 1 equiv of solid K_2CO_3. Argininosuccinate was used as the barium salt which gave a pH of about 8.5 in aqueous solution. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate was present in a concentration of 1%.

The trifluoroacetic acid solutions of the compounds were prepared in the same concentration as in D_2O and tetramethylsilane was added to the extent of 1% by volume. In the case of argininosuccinate, which was used as the barium salt, the observations were taken within 10–15 min after the solution was prepared. Ring closure was negligible within this time; the spectra showed no change. Aspartic acid and guanidinosuccinic acid were used as the free acids allowing 15–30 min for solution in the trifluoroacetic acid.

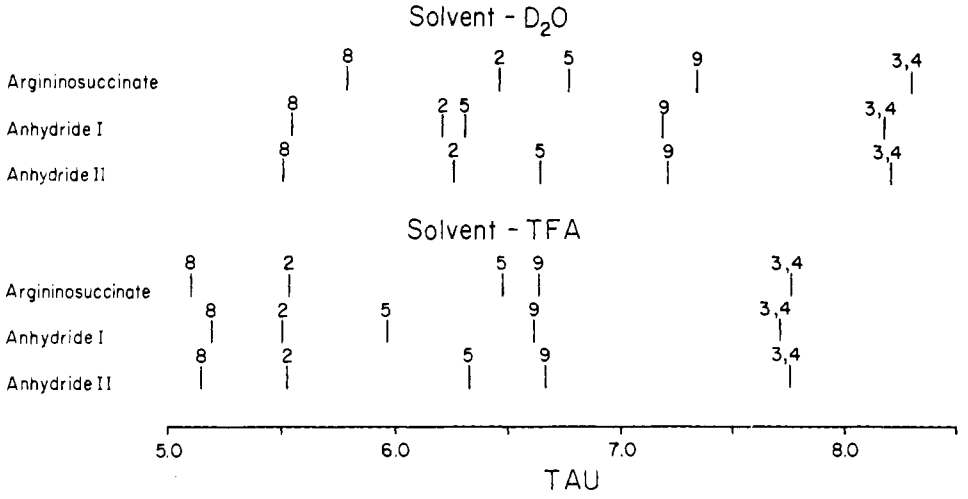

FIGURE 2: Positions of the resonances of carbon-bound protons of argininosuccinic acid, anhydride I, and anhydride II in D_2O and in trifluoroacetic acid. The numbers above each position designate the carbon.

Spectra. Samples in D_2O were observed at room temperature in capped nuclear magnetic resonance tubes; samples in trifluoroacetic acid were sealed. No attempt was made to remove oxygen. Spectra were observed at 60 Mcycles on a Varian HA-60A spectrometer. The positions of the resonances are expressed in terms of τ units as defined previously with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal reference for the aqueous solutions (Kowalsky and Tiers, 1960; Kowalsky, 1962) and tetramethylsilane for the trifluoroacetic acid solutions. Reproducibility to within 0.01–0.02 ppm could readily be achieved. Areas were measured with the Varian V-3521A integrator and were good to 10%. The area of the C-3,4 proton resonances was used as a calibrating standard. The values given in the tables represent the average of three or four runs.

Results and Discussion

Protons Bonded to Carbon

It was anticipated that resonances of one or more of the carbon-bonded protons would be shifted upon ring closure. For the three anhydride structures under consideration (structures B, C, and D, Figure 1), the displacement of resonances associated with protons at C-5, C-8, and C-9 were expected to be the most useful in discriminating among these several structures.

Spectra of Argininosuccinate and Its Anhydrides in D_2O. The spectrum for argininosuccinate in D_2O showed five peaks and these five were also observed in approximately the same positions in the spectra of the two anhydrides. The positions and respective assignments are summarized in Table I and in Figure 2. The four methylene protons at C-3 and C-4 are assigned to the unresolved resonance at 8.30; the two protons at C-9 to the 7.34 resonance and the two protons at C-5 to the absorption at 6.77. For the least shielded α protons at C-2 and C-8, the resonance at lower field, 5.79, has been assigned to the C-8 proton in view of the proximity of the second carboxyl group to this carbon atom. The positions of the C-8 and C-9 proton resonances agree with the positions of the corresponding resonances of aspartic acid and of guanidinosuccinic acid. In the latter compound the CH_2 resonance at C-9 lies at 7.30 and the α-CH resonance at C-8 lies at 5.71.[1]

Although the relative positions of the C-2, C-3,4, and C-5 proton resonances of argininosuccinic acid correspond well with those of arginine, the actual positions differ somewhat, those for arginine being found at about 0.3–0.5 ppm lower field. The differences may arise from the fact that at the pH of the solutions at which the measurements were made, the arginine would have a net positive charge while the argininosuccinate would have a net negative charge.[2] The assignments of all resonances were unequivocally substantiated by the number of protons as determined by integration. In both solvents and for argininosuccinate and its two anhydrides, the values found were in the expected ratio of 4:2:2:1:1, respectively, for the five peaks. The close similarity in the τ values for analogous protons indicates that substitution at the amidine nitrogen of arginine (or the amino nitrogen of aspartate), as it occurs in argininosuccinate, affects the positions of the resonances of the carbon-bonded protons in the side chains very little. The assignments for arginine and aspartic

[1] In guanidinosuccinic acid the CH_2 resonance would be expected, in a first-order splitting, to be a doublet, and the α-CH resonance, a triplet. They appear in D_2O solvent as a complex septuplet and quintuplet, respectively. However, area measurements support the assignments. In trifluoroacetic acid the CH_2 resonance of this compound does appear as a doublet but the α-CH resonance is still complex. However, the anhydride in trifluoroacetic acid clearly shows the expected doublet and triplet in the predicted positions.

[2] The pH of the solutions at which the measurements were made were 6.5, 9.0, 5.7, and 4.2 for arginine, argininosuccinic acid, and anhydrides I and II, respectively.

TABLE I: Positions of Resonances of Carbon-Bound Protons of Guanidino Amino Acids and Related Compounds.[a,b]

Compound	Solvent	CH_2CH_2 (C_3—C_4)	CH_2COOH (C_9)	CH_2NH (C_5)	α-CH (C_2)	α-CH (C_8)
Argininosuccinic acid	D_2O	8.30 (m, 4.0)	7.34 (t, 1.6)	6.77 (t, 2.0)	6.46 (m, 0.9)	5.79 (m, 0.8)
	Trifluoroacetic acid	7.77	6.64	6.48	5.54	5.11
Argininosuccinic anhydride I	D_2O	8.18 (m, 4.0)	7.19 (d, 2.2)	6.31 (m, 1.7)	6.21 (m, 0.7)	5.55 (m, 0.9)
	Trifluoroacetic acid	7.72	6.62	5.97	5.51	5.20
Argininosuccinic anhydride II	D_2O	8.21 (m, 4.0)	7.21 (d, 2.2)	6.64 (t, 1.7)	6.26 (t, 0.7)	5.51 (t, 0.9)
	Trifluoroacetic acid	7.76	6.67	6.33	5.53	5.15
Arginine	D_2O	7.83 (m, 4.0)		6.41 (t, 2.0)	5.88 (t, 1.0)	
	Trifluoroacetic acid	7.78		6.55	5.48	
Aspartic acid	D_2O		7.24 (t, 2.0)			6.07 (m, 1.0)
Guanidinosuccinic acid	D_2O		7.30 (d, 2.0)			5.71 (m, 1.0)
	Trifluoroacetic acid		6.72			5.19
Guanidinosuccinic anhydride	Trifluoroacetic acid		6.62 (d, 2.0)			5.08 (t, 1.0)
Guanidinoacetic acid	Trifluoroacetic acid					5.72[c] (s, 2.0)
Guanidinoacetic anhydride	Trifluoroacetic acid					5.47[c] (s, 2.0)
Creatinine	Trifluoroacetic acid			6.65[d] (3.0)		5.48[c] (s, 1.9)

[a] Positions are given in τ values (4,4-dimethyl-4-silapentanesulfonic acid, sodium salt, or tetramethylsilane as reference). [b] s = singlet; d = doublet; t = triplet; m = multiplet. The positions are given as the center of gravity of the signal. The numbers are the number of protons calculated using the C_3—C_4 proton resonance as a standard (4.0 protons). [c] α-CH_2. [d] CH_3—N.

acid agree with those of Jardetzky and Jardetzky (1958) and Bovey and Tiers (1959).

Spectra of Argininosuccinate and Its Anhydrides in Trifluoroacetic Acid. The assignments of the various resonances of argininosuccinic acid in trifluoroacetic acid solution follow those in D_2O. Here however the agreement with the position of the arginine resonances is closer than in D_2O (Table I). In addition, the resonances of guanidinosuccinic anhydride in trifluoroacetic acid correlate very well with those of anhydrides I and II.

The spectra for argininosuccinate and its anhydrides taken in trifluoroacetic acid as solven showed a shift to lower fields as compared with those in D_2O. As may be seen in Figure 2, the effect varied from about 0.3 τ for the C-5 proton to 0.7 or more for the C-2 proton, depending upon the distance from the protonation sites, the C-1 carboxyl and the α-amino group. The C-3,4, C-2, and C-5 protons which lie in the arginine chain show the same order in trifluoroacetic acid as in D_2O for all three compounds. The protons at carbons C-8 and C-9, which lie in the succinic acid moiety, are also adjacent to carboxyl or amide protonation sites, and these shifts to lower field are also large (see below).

Position of Anhydride Linkage in Anhydrides I and II. Although the positions of the resonances for the two anhydrides were in general similar to those for the parent compound, some changes were found as a result of ring closure. On comparing the spectra observed in trifluoroacetic acid (Figure 2), it may be seen that a significantly large downward displacement (0.51) in the position of the C-5 proton resonance was observed for anhydride I while very little change (0.15) was found at C-5 for anhydride II. A displacement of this magnitude implicates structure D for anhydride I. In D_2O solution (Table I and Figure 2) the displacements were essentially the same, 0.46 for anhydride I and 0.13 for anhydride II.

A small shift was observed in D_2O solution for the C-2 proton resonance for anhydrides I and II. The dis-

TABLE II: Positions of Resonance of Nitrogen-Bound Protons of Guanidino Amino Acids and Related Compounds in Trifluoroacetic Acid.[a,b]

	Guanidino Group[c]			Guanidino Anhydride Group[c]	
	HC(=NH)—NH (—C or —H)	HC—NH—C	α-NH_2	HC(=N (—C or —H))—NH (—C or —H)	HC—NH—C
Compound	N-b; N-c	N-d	N-a	N-b; N-c	N-d
Argininosuccinic acid barium salt	3.37 (s, 2.3)	3.16 (d, 1.3)[d]	2.35 (s, 3.00)		
Anhydride I of above			2.42 (s, 3.0)	1.84 (s, 1.8)	1.17 (s, 1.1)
Anhydride II of above			2.37 (s, 3.4)	1.63 (s, 1.2)	1.40 (b, 0.8)
Arginine hydrochloride	3.77 (b, 1.7)	3.39 (b, 2.3)	2.27 (d, 3.0)		
Guanidinosuccinic acid	3.53 (s, 3.4)	2.91 (d, 1.3)[d]			
Guanidinosuccinic anhydride				2.06 (s, 2.0)	1.45 (b, 1.0)
Guanidinoacetic acid	3.58 (b, 2.5)	3.17 (t, 1.7)[d]			
Guanidinoacetic anhydride				2.18 (b, 1.9)	1.78 (b, 1.0)
Creatinine				2.20 (s, 2.0)	

[a] Positions are given in τ units, tetramethylsilane reference. [b] s = singlet; d = doublet; t = triplet; b = broad. These designations indicate the form only and do not necessarily imply spin–spin interactions. The positions given are the center of gravity of the signal. The numbers are the number of protons calculated using the carbon-bound protons as standard, *i.e.*, for the arginino derivatives the C_3—C_4 proton resonance (4.0 protons) was used. [c] Represented in the unprotonated form. [d] Splittings: argininosuccinic acid, 9 cps; guanidinosuccinic acid, 8 cps; guanidinoacetic acid, 6 cps (0.15, 0.13, and 0.10 ppm, respectively).

placement of this resonance in both anhydrides (about 0.2) is not attributable to ring closure but may be due in both cases to the change in the degree of ionization of the α-NH_2 group[2,3] since this change was not seen in trifluoroacetic acid as the solvent.

It is of interest to point out that in trifluoroacetic acid solution there is also a significant difference between the resonance positions of the C-5 proton in ornithine (6.60) and in proline (6.30) (Bovey and Tiers, 1959). However, the *N*-methyl proton resonances of *N*-methylpiperidine (7.62) and *N*-methylcyclohexylamine (7.58) are quite close. A possible explanation of such displacements toward lower field lies in the fact that in proline the carboxyl group is maintained in specific spatial relationship to the CH_2N group, whereas in ornithine the carboxyl group, being at the end of a flexible chain, may assume various orientations with respect to the CH_2N group. In *N*-methylpiperidine and *N*-methylcyclohexylamine, the lack of a carboxyl group renders such an interaction impossible. A situation somewhat similar to that in proline would obtain in structure D. Here the exocyclic double bond would have a fixed orientation with respect to the N—C-5 bond. This would place the C-5 protons in the deshielding region of the double bond, thus explaining the displacement of the resonance peak to lower field. In argininosuccinic acid, and in structures B and C, the orientation is not fixed since the methylene group at C-5 can rotate around the C—N bond at C-6; the ring itself in the latter two structures can rotate around this bond.

As pointed out above, the magnitude of the shift of the proton resonance peaks to lower field in trifluoroacetic acid solution increases with the proximity to a protonation site. The protons at C-9 in structures B and D are closer to a protonation site (the carboxyl group at C-10) than the proton at C-8. For the C-8 proton resonances the displacements are 0.68, 0.35, and 0.36 and for the C-9 proton resonances they are 0.70, 0.57, and 0.54 for argininosuccinate and anhydrides I and II, respectively. The smaller displacements for the proton resonance at C-8 thus provides evidence for the position of the free carboxyl as in structures B and D, and at the same time excludes structure C from consideration. The data also excludes another possible six-membered ring structure (not shown) involving anhydride formation between C-10 and N-b.

Thus evidence for the position of the anhydride linkage in anhydride I as shown in D is given by the large displacement of the proton resonance at C-5; evidence for the position of the free carboxyl at C-10 comes from the relative magnitude of the shifts of the C-8 and C-9 protons to lower field in trifluoroacetic acid. The observations also furnish evidence for the structure of anhydride II. The small shift of the C-5 proton resonance and the fact that the small shifts of the C-8 and C-9 proton resonances are similar to those found for anhydride I support the structure given in structure B.

[3] The pK_a values for the α-NH_2 group were close to 9.55 for the three compounds but the pH of the solutions at which the measurements were made varied. Jardetzky and Jardetzky (1958) have observed that the α-CH resonance is shifted to lower fields when the α-amino group is protonated.

Protons Bonded to Nitrogen

Protons bonded to nitrogen usually cannot be observed in a D_2O medium because of rapid exchange with the solvent. However, in anhydrous trifluoroacetic acid the exchange is sufficiently slow to allow the detection of characteristic resonances. The assignment of resonances for the nitrogen-bonded protons in argininosuccinate was puzzling. Since compounds of this kind have not previously been examined in trifluoroacetic acid, several additional guanidino compounds of biological interest were examined for comparison. The model compounds chosen for this purpose were guanidinosuccinic acid, guanidinoacetic acid, their respective anhydrides, and creatinine.

Nitrogen-Bonded Protons of Argininosuccinic, Guanidinosuccinic, and Guanidinoacetic Acids. The simplest model compound in this series, guanidinoacetic acid (Figure 3E), showed two broad but well-resolved resonances for the guanidino protons, located at 3.58 and 3.17 (Table II). The resonance at 3.17 was attributed by Bovey and Tiers (1959) to the proton at N-d since it clearly shows splitting to a triplet by the neighboring α-carbon protons. This was confirmed in the present study. With the increased resolution, integrations were carried out for each of the two resonances. Although the N-b,c and N-d proton resonances are clearly separated, they do merge somewhat and a clear and unequivocal assignment of integration values is not possible. However the ratio of protons, N-b,c:N-d is found to be 2.5 to 1.7. The sum of these numbers is less than the expected value of 5 in trifluoroacetic acid, a descrepancy which was also found with arginine (see below). The value of 1.7 suggests that it may be possible to protonate the guanidino group at N-d. Two similar, well-separated resonances were also observed for the nitrogen-bonded protons in guanidinosuccinic acid (Figure 3F); a broad single peak located at 3.53 and a sharp doublet at 2.91 showing a splitting of 8 cps by the proton at the α-carbon. The same assignments based on position and splitting were made for this compound also although the integration values did not in this case permit the respective assignments of the protons. However the total number of protons approximated a value of 5.

Argininosuccinic acid in trifluoroacetic acid showed partially resolved resonances at 3.16 and 3.37. The doublet at 3.16 showed a splitting of 9 cps which corresponds well to the 8 cps for the C-9 proton splitting of the N-d doublet in guanidinosuccinic acid (Figure 3F). Here also on the basis of position and splitting the resonance at 3.16 is assigned to the C—NH—C group (N-d) and that at 3.37 to the R—NH—C=NH group (N-b and N-c). The total integrated intensity of these resonances indicate a total of 3.6 protons, somewhat less than the expected value of 4 in trifluoroacetic acid. Although it is difficult, because of the closeness of the resonances, to break down the area assignments further, the doublet at 3.16 represents approximately 1.3 protons and the resonance at 3.37, approximately 2.3 protons. For all three compounds just discussed, the data suggest that there may be protonation on N-d as well as on N-c.

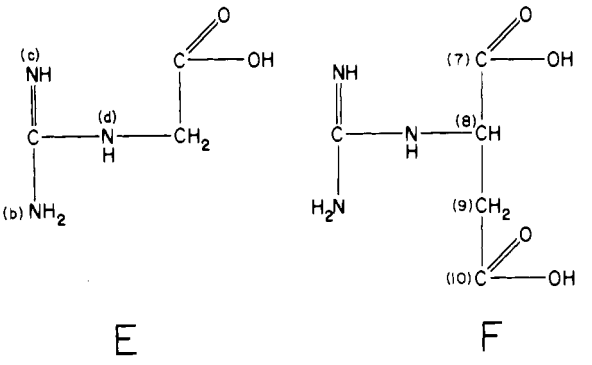

FIGURE 3: Structure of guanidinoacetic acid (E) and guanidinosuccinic acid (F).

Arginine in trifluoroacetic acid was found not to be a suitable model for these studies. Its resonances are too broad for detailed observation and the integrated intensity of its guanidine NH proton resonances is less than the theoretical value. The arginine resonances consist of a prominent broad peak at 3.39 fused to a broader resonance of lower intensity at 3.77. The integrated intensity of these resonances together totals approximately 4.0 protons. The reason for this discrepancy with the predicted value is now known. The α-amino protons of arginine and of argininosuccinic acid exhibit a resonance at 2.27 and 2.35, respectively, and each has an integrated intensity of three protons showing the presence of an acquired proton, in agreement with expectation.

Anhydrides of Guanidino Acids Model Compounds. The assignment of the resonances of the nitrogen-bonded protons in guanidinoacetic anhydride and guanidinosuccinic anhydride follows from that of creatinine. As may be seen from Table II, the two resonances of guanidinoacetic anhydride (Figure 4G) were located at 2.18 and 1.78. On the other hand, only one resonance located at 2.20 was observed for creatinine (Figure 4H). This latter resonance must be assigned to protons bonded at C—NH_2 (N-b) since no proton is present at the N-d position in creatinine. Thus, in guanidinoacetic anhydride, the assignment can be made: C—NH_2 protons, 2.18 and C—NH—C proton, 1.78. Guanidinosuccinic anhydride, structure K or L in Figure 4, showed resonances at 2.06 and 1.45, in positions similar to those of the C—NH_2 peak for guanidinoacetic anhydride and the C—NH—C peak for anhydride II (see below). For this reason, in either of the proposed structures, K or L, the resonance at 1.45 is again assigned to the C—NH—C proton. The resonance at lower fields in all cases was so broad that the expected splitting by the adjacent C-H could not be observed.

In making the assignments summarized above, consideration has also been given to various alternative tautomeric structures in which each of these compounds might exist. For creatinine, the resonances of the carbon-bound protons are in the ratio of 3:2 and only one type of nitrogen-bound proton, corresponding to the two protons, is observed. These nuclear magnetic

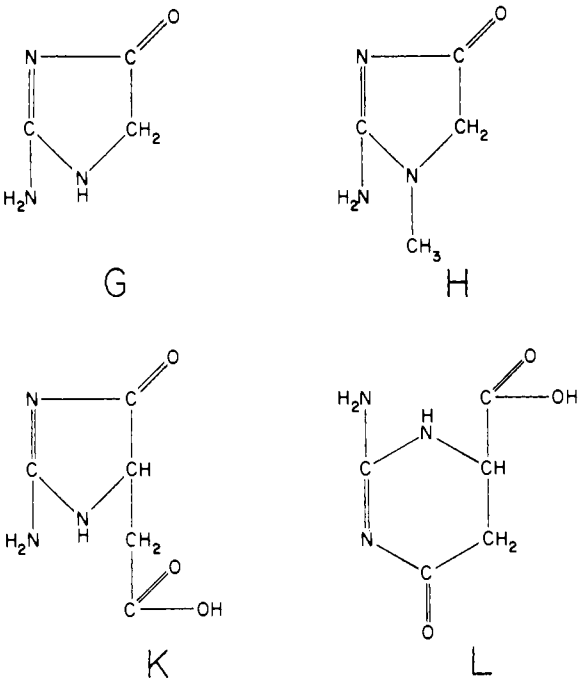

FIGURE 4: Structure of guanidino anhydrides. (G) Guanidinoacetic anhydride (glycocyamine), (H) creatinine, (K) guanidinosuccinic anhydride, and (L) a structure previously considered for guanidinosuccinic anhydride.

resonance data are consistent with only one of the four possible tautomeric forms, structure H.

Other possible tautomeric structures which can be written for guanidinoacetic and guanidinosuccinic anhydrides can be eliminated since they involve a change in the number of carbon-bound protons. The spectra in D_2O and in trifluoroacetic acid indicate a satisfactory accounting of protons on carbon in terms of the constituent acids.

Evidence for the position of the anhydride linkage in guanidinosuccinic anhydride comes from the nitrogen-bonded protons. The results given in Table I show that very small shifts (0.1) of the C-9 methylene proton resonance and of the C-8 α-proton resonance take place on ring closure. The changes are too low to be of significance in discriminating between the two structures under consideration and the spectra were observed only in trifluoroacetic acid. However, the close similarity in the resonance positions of the nitrogen-bonded protons (Table II) with (1) the guanidinoacetic anhydride resonance for the N-b protons, (2) with the anhydride II resonance for the N-d proton, and (3) the present evidence for the five-membered ring structure for anhydride II strongly suggest structure K for guanidinosuccinic anhydride.

The formulas H, G, and K represent the structures in aqueous solution. In trifluoroacetic acid, protonation would be expected to take place on the amide oxygen to form the ion shown in Figure 5. This proton would not be observed because of fast exchange with the solvent. Protonation on the oxygen would also explain the low basic strength of these guanidino

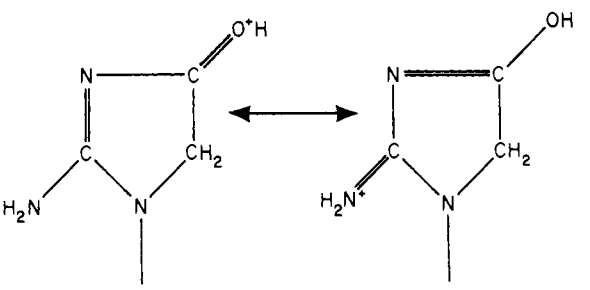

FIGURE 5: Structure of the ion formed by model guanidino anhydrides in trifluoroacetic acid.

anhydrides. The pK_a values for this grouping are given in Table III and the value is about 5 in all cases. Area integrations of the resonances are consistent with the location of the proton acquired in acid solution on the oxygen atom rather than the nitrogen. For both guanidinoacetic anhydride and guanidinosuccinic anhydride, integration of the resonance indicated a value of one for the N-d proton and a value of two for the N-b proton. The latter value was also found for creatinine.

Nitrogen-Bonded Protons of Anyhdrides I and II. For anhydride I and anhydride II, the α-amino resonances were located at 2.42 and 2.37, respectively, very close to the corresponding resonances for both argininosuccinate and arginine (Table II). The integrated intensity of each resonance confirmed the assignment since each gave a value of three. The two peaks characteristic of the guanidino protons were displaced to lower fields upon cyclization, shifting to 1.84 and 1.17 for anhydride I and 1.63 and 1.40 for anhydride II. Since the two nitrogen-bonded proton resonances observed were located in positions corresponding to those in the model compounds, they have been given analogous assignments as shown in Table II. The resonances were only moderately broad but resolution was insufficient to reveal any multiplicity.

The question of alternative tautomeric structures occurs also with anhydrides I and II. If we eliminate, as with the model compounds, those structures in which the labile proton is transferred to or from a carbon atom, then only one formulation of structure B (Figure 1) has a conjugated double-bond system, but structure D has no formulation with a conjugated double-bond system. For structure D, a comparison of the nuclear magnetic resonance spectra of trifluoroacetic acid solutions (number of nonequivalent nitrogen-bonded protons and their relative areas) with the various tautomeric forms indicates that structure D itself, protonated at N-c, is the most probable structure for anhydride I.

Integration of the areas of the N-d proton resonance gave, in agreement with the results on the model anhydrides, a value of one proton for each anhydride. Integration of the areas for the N-b (or N-c) proton resonances gave a value of two protons for anhydride I at N-c, and but one proton for anhydride II at N-b. Thus anhydride I is the only compound of the guanidino anhydrides in which the guanidino group is protonated

TABLE III: Some Physical Properties of Guanidino Acids and Their Anhydrides.

Compound	Ultraviolet Absorption, λ_{max} (mμ)	pK_a of Guanidino or Guanidino Anhydride	Isoionic Point pH
Argininosuccinic acid[a]		>12	3.5
Anhydride I[a]	End absorption	8.10	5.7
Anhydride II[b]	215	5.15	4.2
Creatine		13.6	
Creatinine[a]	234	5.0	5
Guanidinoacetate		>12	
Guanidinoacetic anhydride	224	5.0	5
Guanidinosuccinic acid[a]		>12	
Guanidinosuccinic anhydride[a]	226	5.60	4.4

[a] Ratner *et al.* (1953b). [b] Ratner and Kunkemueller (1966).

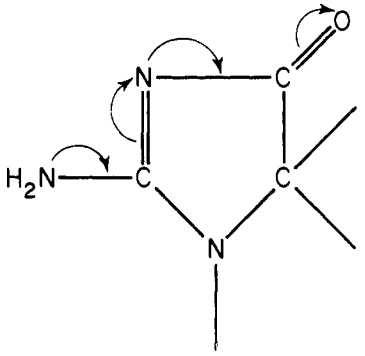

FIGURE 6: Possible electronic shifts in guanidino anhydrides.

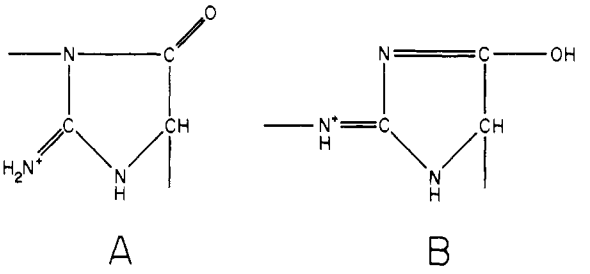

FIGURE 7: Structure of the ions formed by the anhydride moiety of anhydrides I and II in trifluoroacetic acid. (A) Anhydride I and (B) anhydride II.

on nitrogen in trifluoroacetic acid solution. These observations are consistent with the absence of a conjugated structure in anhydride I and the presence of a conjugated structure in anhydride II and in the three model compounds. In the latter group, the conjugated systems are capable of electronic shifts as shown in Figure 6. Such shifts would favor protonation on oxygen. A proton on oxygen would exchange at a very fast rate with the trifluoroacetic acid and not be observable in the nuclear magnetic resonance spectrum. This conjugated system is absent in the structure given for anhydride I, electron shifts favoring protonation on oxygen cannot take place, and consequently protonation on nitrogen occurs.

Structure in Relation to Physical and Chemical Properties. In strong acid, the structure for the guanidino portion of the argininosuccinic anhydrides I and II, respectively, would then be as shown in Figure 7. The pK_a values for the guanidino anhydride grouping are 8.1 for anhydride I and 5.15 for anhydride II (Ratner *et al.*, 1953b; Ratner and Kunkemuller, 1966). The latter value is close to that for the three model anhydrides (see Table III), and can be explained as before on the basis of protonation on the oxygen. In contrast, anhydride I is exceptional in the higher basicity of the anhydride grouping. It can be seen from the nuclear magnetic resonance spectra that this is due to the fact that this compound is protonated on the nitrogen at N-c rather than on oxygen.[4]

The conjugate position of the double bond in the guanidino anhydride grouping is consistent with ultraviolet absorption as well as with nuclear magnetic resonance spectra. As summarized in Table III, anhydride II, guanidinosuccinic anhydride, guanidinoacetic anhydride, and creatinine exhibit absorption maxima in the ultraviolet at 215, 226, 224, and 234 mμ, respectively, while anhydride I shows only end absorption. Differences in the chemical properties of anhydride I and II are also in harmony with the assigned structures. For example, anhydride I is extremely susceptible to oxidative cleavage under very mild conditions while anhydride II, stabilized by the conjugate structure,[5] is entirely resistant to oxidative cleavage under the same conditions (Ratner and Kunkemuller, 1966).

In the earlier studies, some evidence for the location of the anhydride linkage had been sought from a com-

[4] It is of interest to mention that although anhydride II is the more stable of the two anhydrides, argininosuccinate at pH 2 is converted much more rapidly into anhydride I than to anhydride II.

[5] Creatinine also undergoes oxidative cleavage in dilute alkali (Van Pilsum *et al.*, 1956). This instability is probably due to the fact that the products formed (oxalic acid and *N*-methylguanidine) are each highly stable.

parison of the carboxyl group dissociations in the expectation that location at C-7 as compared with C-10 would be reflected in some change. The pK_a values found for the carboxyl group at C-7 and at C-10 were 2.65 and 4.26, respectively, and these were the same for argininosuccinic acid as for guanidinosuccinic acid. After anhydride formation, the pK_a value found for the carboxyl remaining in the succinic moiety (C-7 or C-10) was 3.30, 3.26, and 3.23 for anhydride I, anhydride II, and guanidinosuccinic anhydride, respectively (Ratner *et al.*, 1953b; Ratner and Kunkemueller, 1966). Since there were no differences, such data could not aid in descriminating among the structures considered. It is now evident, from the new assignments, that these values all represent the dissociation of the carboxyl group at C-10 and therefore little variation can be expected.

Definitive Proof of Structure. The structure for anhydride I has also been studied by degradative means employing isotopic labeling. Exposure of this anhydride to mild oxidation at alkaline pH results in degradative cleavage so as to form arginine, pyruvic acid, and CO_2. With a preparation of anhydride I labeled exclusively in the C-10 position, degredation led to the location of the ^{14}C in the evolved CO_2. The results were consistent with structure D (Ratner and Kunkemueller, 1966). However, the fact that anhydride II is not susceptible to cleavage under these conditions and that the model guanidino anhydrides with selective ^{14}C labeling were not available limited the scope of this approach.

The structures proposed here are consistent with and offer an explanation for the various physical and chemical properties previously gathered. Thus the weight of the nuclear magnetic resonance studies has permitted a definitive assignment of structure to be made for the two anhydrides of argininosuccinate and for the guanidino anhydrides of the model compounds.

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