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THE ANHYDRIDES OF ARGININOSUCCINIC ACID

C. R. LEE and R. J. POLLITT

Medical Research Council Unit for Metabolic Studies in Psychiatry,
University Department of Psychiatry, Middlewood Hospital, P.O. Box 134, Sheffield S6 1TP

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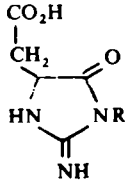
Abstract—Several model compounds, including N^2 – and 3-methylglycocyamidines and the α- and β-lactams of guanidinosuccinic acid are compared with argininosuccinic anhydrides I and II in terms of pK values, NMR spectra, stability to alkali and reactivity in the Jaffé reaction. The properties of the argininosuccinic anhydrides can be explained largely in terms of the differences between N^2- and 3-substituted glycocyamidines.

ARGININOSUCCINIC acid, an intermediate in the Krebs-Henseleit urea cycle, undergoes ready cyclization to form two anhydrides*.[1, 2] There are four possible acylguanidinium structures† involving the guanidinosuccinate moiety only. Two (A and B) are 5-membered anhydrides involving the α-carboxy-group and two (C and D) are 6-membered involving the β-carboxy-group. Westall[1] tentatively assigned structures B and D to anhydrides I and II respectively on the grounds that anhydride I has a higher isoelectric point and hence probably has the stronger α-carboxy-group involved in the cyclization. However, detailed examination of the titration curves showed that the values for pK_1, pK_2, and pK_4 are very similar, and that the difference lies in pK_3, assumed to be the pK of the acylguanidinium ring.[2, 3]



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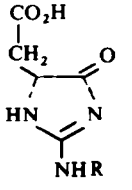

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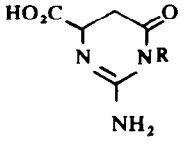

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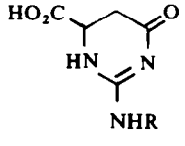

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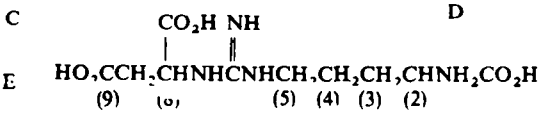

E

* In this paper the notation of Ratner and co-workers is used. Anhydrides C and B of Westall[1] correspond to anhydrides I and II respectively.

† The convention of Matsumoto and Rapoport[5] is followed in drawing the tautomeric structures. Support for the exocyclic double bond in (A) comes from the N-proton resonance spectra.[4]

The only convincing evidence as to the structures of these anhydrides is the recent NMR study by Kowalsky and Ratner.[4] These authors assigned structures A and B to anhydrides I and II respectively on the C-proton resonance spectra in D_2O and trifluoroacetic acid solutions and on the distribution of N-bonded protons in trifluoroacetic acid solution. This assignment was based, as far as ring-size is concerned, on relatively small differences (0·2 τ units) in changes of chemical shift between D_2O and trifluoroacetic acid solution, and a number of important deductions were not confirmed experimentally because of lack of model compounds. Thus alternative confirmation of these assignments is desirable.

In addition, a number of features of the anhydrides remain unexplained. For example, the similarities of the ultraviolet spectra and pK values of argininosuccinic anhydride II (pK_3), a lactam of guanidinosuccinic acid (pK_2) and creatinine were noted by Ratner and Kunkemueller.[3] Since the tautomerism of creatinine and the effect of substitution were not known and no data were available on analogous 6-membered rings no definite conclusions could be drawn from this. Recently, however, Matsumoto and Rapoport[5] have prepared a number of cyclic acylguanidines substituted in various positions and have studied their pK values, ultraviolet spectra and stabilities to alkali. We have extended this series by inclusion of the two simplest models, 3-methylglycocyamidine and N^2-methylglycocyamidine (glycocyamidine is the lactam of glycocyamine – guanidinoacetic acid). We have also isolated and characterized a 6-membered lactam of guanidinosuccinic acid. Consideration of the pK values and stabilities of these compounds, their NMR spectra, and their reactivity in the Jaffé reaction has enabled us to assign structures in agreement with those of Kowalsky and Ratner. These models also help in the understanding of other properties of the anhydrides.

Model compounds

Johnson and Nicolet[6] showed that the methylglycocyamidine obtained by Korndorfer[7] by the action of iodomethane on glycocyamidine was the 3-isomer. N^2-Methylglycocyamidine was prepared from 2-thiohydantoin and by other methods, but was converted directly to the 5-benzylidine derivative without isolation.[6]

ω-Methylglycocyamine, prepared by ring opening of crude N^2-methylglycocyamidine with alkali, gave on cyclization in acid a mixture of 15% N^2-methylglycocyamidine and 85% 3-methylglycocyamidine.[6] The production of the N^2-compound in this way was denied by Lempert.[8] We have repeated this work, using 3-methylglycocyamidine as starting material, and examining the products of ring closure by paper electrophoresis at pH 5·3. A compound having a slightly lower mobility than creatinine was found, in addition to the faster major product, 3-methylglycocyamidine. The N^2-isomer isolated later had similar electrophoretic properties, supporting Johnson and Nicolet's original observation.

We were unable to prepare N^2-methylglycocyamidine by the 2-thiohydantion method. Attempts to prepare ω-methylglycocyamine by the action of N,S-dimethylisothiourea on glycine in aqueous ammonia or sodium hydroxide also failed. Matsumoto and Rapoport reported[5] similar failures with N,N',S-trimethylisothiourea. The methylglycocyamidines were obtained in low yield from N,S-dimethylisothiourea and glycine ethyl ester in ethanol. Several unidentified Jaffé-positive products were produced, but the desired compounds were readily isolated by column

chromatography. The structure of the N^2-methylglycocyamidine follows by elimination, as it is the only N-methylated glycocyamidine not previously isolated. Its pK and NMR spectrum also confirm the assignment.

The lactam of guanidinosuccinic acid originally prepared by Ratner *et al.*[2] was tentatively given the 5-membered structure on the basis of the pK of the carboxy group.[3] High-voltage electrophoresis at pH 2 showed that the reaction mixture from this preparation contained another compound of lower mobility, with no net charge at pH 5·3. Chromatography on a sulphonic acid resin in the H^+ form, eluting with M hydrochloric acid, separated this compound from the α-lactam and unchanged guanidinosuccinic acid. It was found that the formation of this compound, the β-lactam, is favoured by heating at pH values above 3 and that the compound's low solubility allowed the separation of the isomers without chromatography. The structure of the compound follows from its preparation, analysis, and the physical properties discussed below.

pK Values of the acylguanidinium group

Table 1 shows the pK values and stabilities at pH 12 of a variety of 5- and 6-membered acylguanidinium compounds. The compounds are classified into four groups corresponding to the four possible anhydride structures for argininosuccinic

TABLE 1. pK VALUES AND STABILITIES AT pH 12 OF VARIOUS CYCLIC ACYLGUANIDINIUM COMPOUNDS

Compound	pK_a	Half life at pH 12 (25°)	Reference
Type A			
N^2,3-Dimethylglycocyamidine	7·96	70 min	5
3-Methylglycocyamidine	7·94	17 min	this paper
Type B			
Glycocyamidine	4·80	Stable	9
N^2-Methylglycocyamidine	4·80	Stable	this paper
1-Methylglycocyamidine (creatinine)	4·83	Stable	10
Guanidinosuccinic α-lactam	5·01 (pK_2)	Stable	this paper
	5·60 (pK_2)*		2
Type C			
2-Methylamino-3-methyl-4-oxo-3,4,5,6-tetrahydropyrimidine	9·48	8 min	5
Type D			
β-Guanidinopropionic lactam	7·05	4 hr	5, 11
Guanidinosuccinic β-lactam	6·76	1 day	this paper
Argininosuccinic anhydride I	8·10 (pK_3)	1 day	2
Argininosuccinic anhydride II	5·15 (pK_3)	Stable	3

* Perhaps this sample contained some of the β-lactam.

acid. For the 5-membered compounds creatinine, glycocyamidine and guanidinosuccinic α-lactam the preferred tautomer has the endocyclic double bond (type B). These compounds are probably protonated on oxygen in acidic solution.[4] Substitution

on the 3-position produces the exocyclic double bond form (A), which has a higher pK value and is less stable at high pH. An analogous situation is seen in the 6-membered series, where compounds with the double bond conjugated with the carbonyl group are more stable and have lower pK values. The differences between the B and D types of structure are sufficient to assign the two lactams of guanidinosuccinic acid with confidence. Similarly, argininosuccinic anhydride II (pK_3 5·15) must correspond to structure B. This is also the more stable of the two anhydrides.[1, 3] The pK_3 of anhydride I is 8·1, which corresponds most closely with the A type of structure. The 4-amino-4-carboxybutyl side chain is unlikely to have any great effect on the pK of the ring: nevertheless, the differences between the expected pKs for the structures A, C and D are not sufficiently large for certainty, and the structures of the D type are stable enough to be considered seriously. The stability of anhydride I in alkali is greater than that of any of the corresponding models. This is discussed below.

pK Values of the ring carboxy group

Argininosuccinic anhydrides I and II have very similar values for pK_2 (3·26 and 3·30 respectively[2, 3]), ascribed to the free succinic acid carboxy group. In structures A and B the carboxy group is β to the guanidino function, whereas in C and D it is in the α-position. By analogy with the amino acids there should be large differences in the pK values of the two types of carboxy group. The pK_1 of guanidinosuccinic acid α-lactam (β-carboxy free) is found to be 3·25 (lit[2] 3·23) and that of the β-lactam is 1·9. This indicates clearly that argininosuccinic anhydride II corresponds to structure B in agreement with the conclusions from the pK of the acylguanidino group. Argininosuccinic anhydride I is assigned to A rather than C or D on the basis of pK_2. It is unlikely that the change of ring charge distribution (O- versus N-protonation) between D and C would change the pK of the α-carboxy group on a type C structure sufficiently to upset this assignment.

NMR spectra

The NMR spectra of carbon-bonded protons were obtained in M-D_2SO_4 in D_2O. The results (Table 2) agree generally with those of Kowalsky and Ratner[4] who used D_2O and trifluoroacetic acid solutions. Both argininosuccinic anhydrides give similar τ-values for C_2, $C_{3,4}$, C_8 and C_9 protons. Those for C_5, C_8 and C_9 protons agree quite well with the analogous protons in the 5-membered ring models. In contrast, guanidinosuccinic β-lactam gave a complex spectrum with a triplet at τ 4·79 for the original α-proton, and a symmetrical septuplet centred at τ 6·35 for the two β-protons. The line intensities are consistent with this approximating to an ABX-type spectrum with the two β-protons being centred at τ 6·24 and 6·47, $J_{AX} = J_{BX} = 6{\cdot}5$ Hz, and $J_{AB} = 18$ Hz. The equality of J_{AX} and J_{BX} causes the α-proton to appear as a triplet and the τ values for the A and B protons are such that the two adjacent peaks of the quartets nearly coincide, appearing as the centre peak of a septuplet. The absence of any significant differences between the two C_9 protons in the argininosuccinic anhydrides seem to rule out the possibility of a 6-membered structure with C_9 in the ring.

Jaffé reaction

Argininosuccinic anhydride I gives a positive Jaffé reaction with picric acid,[2]

whereas anhydride II is reported to be negative.[1] When applied to electrophoretograms on filter paper[12] the Jaffé reaction is more general and shows up quite a range of compounds,[8] including both argininosuccinic anhydrides, 3-methyl and N^2-methylglycocyamidines, guanidinosuccinic α-lactam but not the guanidinosuccinic β-lactam or β-guanidinopropionic lactam. In aqueous solution, however, there were significant differences in the rates of reaction of the various compounds (Table 3). Anhydride II reacts more slowly than anhydride I. Both are slower than the corresponding methylated glycocyamidines, but this is probably due to the effect of the carboxymethyl group in the 5-position of the glycocyamidine ring. In both cases the

TABLE 2. NMR SPECTRA OF ARGININOSUCCINIC ANHYDRIDES AND SOME ANALOGUES

The numbering is based on argininosuccinic acid (E) and follows that of Kowalsky and Ratner. Spectra were taken in M D_2SO_4 in D_2O except for that of guanidinosuccinic β-lactam which required more concentrated acid (3 M) for solution.

Compound	C_8	C_2	C_5	C_9	$C_{3,4}$
Argininosuccinic anhydride I	5·45 (t) (J = 4·5 Hz)	5·95 (t) (J = 6 Hz)	6·32 (m)	7·00 (d) (J = 4·5 Hz)	8·30 (m)
Argininosuccinic anhydride II	5·43 (t) (J = 4·5 Hz)	5·96 (t) (J = 6 Hz)	6·67 (t) (J = 6 Hz)	7·02 (d) (J = 4·5 Hz)	8·26 (m)
3-Methylglycocyamidine	5·25 (s)		6·35 (s)		
N^2-Methylglycocyamidine	5·23 (s)		6·50 (s)		
Guanidinosuccinic α-lactam	5·50 (t) (J = 5)			7·10 (d) (J = 5)	
Guanidinosuccinic β-lactam	4·79 (t)			6·35 (septuplet)	

N^2-substituted derivative gives a slower reaction and a slower decay than the 3-substituted derivative. The 6-membered guanidinosuccinic β-lactam probably does not react at all, confirming the assignment of the 5-membered structures to the argininosuccinic anhydrides.

Other properties

In agreement with Matsumoto and Rapoport[5] we did not find systematic variations of infrared or ultraviolet spectra very helpful. N^2-methylglycocyamidine showed λ_{max} (pH 12) 214 nm, ε 14,000 which compares well with argininosuccinic anhydride II (λ_{max} 214 nm, ε 14,400). At pH 12, both argininosuccinic anhydride I and 3-methylglycocyamidine had λ_{max} 206 nm. Absorbance at these wavelengths is rather non-specific, however.

In an attempt to obtain direct chemical proof of the carboxy group involved in the anhydride link, argininosuccinic anhydrides I and II and guanidinosuccinic α- and β-lactams were treated with anhydrous hydrazine. Both at 100° and at room temperature, the guanidinosuccinic lactams gave moderate yields of the appropriate aspartyl hydrazides. At 100° both argininosuccinic anhydrides gave spots corresponding to aspartyl α-hydrazide and an unidentified product, yellow with ninhydrin which also is obtained from both the guanidinosuccinic lactams. In addition, however, there were other compounds present, including a minor product, yellow with ninhydrin, which was obtained only from the argininosuccinic anhydrides and guanidinosuccinic

α-lactam. At room temperature, the hydrazinolysis was much less complete and the reaction mixture from argininosuccinic anhydride I contained large quantities of anhydride II. This rather unexpected transformation seems to invalidate this line of approach and consequently no attempt was made to identify the products rigorously.

Reduction of the free carboxy groups with diborane was also attempted.[13] Argininosuccinic anhydrides I and II and guanidinosuccinic α-lactam gave poor yields of a product tentatively identified as homoserine. Guanidinosuccinic β-lactam gave no detectable product, however, so this approach too was abandoned.

Discussion

Most of the physical and chemical properties of the argininosuccinic anhydrides agree with those expected from consideration of the model compounds. In particular, the differences in pK_3 values, stability at high pH, and reactivity in the Jaffé reaction can be accounted for in terms of the differences between N^2-substituted and 3-substituted glycocyamidines. However, compared with previously studied compounds, argininosuccinic acid forms anhydrides very readily. The process is detectable on electrophoresis at pH 2, as shown by streaking between the argininosuccinic acid and the anhydride spots. It is also noticeable on ion-exchange columns at 35° and pH 3·2. This contrasts with the concentrated acid and prolonged heating used in most of the preparations of the glycocyamidines. Quantitative data are not available, but a rough guide to reactivity may be had from the conditions used preparatively. Thus, alkylation on nitrogen increases the ease of cyclization of glycocyamidine,[6, 14] as also does substitution on the α-carbon atom.[15] Similarly, β-guanidinobutyric acid cyclizes more readily than β-guanidinopropionic acid.[16, 17] These effects may be sufficient to account for the ease of cyclization of guanidinosuccinic and argininosuccinic acid, but it may be that some form of neighbouring group participation occurs. Argininosuccinic anhydride I is unexpectedly stable to alkali compared with the model compounds, and this is almost certainly due to the substitution of the acylguanidinium ring.

The relative proportions of argininosuccinic anhydrides I and II formed in acid solution (approx 9:1) are in agreement with Johnson and Nicolet's results on the cyclization of ω-methylglycocyamine.

Table 3. Jaffé reaction of argininosuccinic acid and some analogues

Compound	Maximum colour yield (relative to creatinine)	Rise time (min)	$t_{\frac{1}{2}}$ decay (min)
Argininosuccinic anhydride I	42	3	9·8
Argininosuccinic anhydride II	25	75	390
Glycocyamidine	82	17	138
Creatinine	100	20	(several days)
5-Methylglycocyamidine	45	55	122
3-Methylglycocyamidine	72	0·25	1·5
N^2-Methylglycocyamidine	72	35	37
Guanidinosuccinic α-lactam	25	30	190
Guanidinosuccinic β-lactam	0·22*	—	—

* Possibly due to a trace of the α-isomer.

In view of the appreciable amount of 6-membered β-lactam formed from guanidinosuccinic acid, particularly in the pH range 3–7, it is a bit surprising that 6-membered anhydrides of argininosuccinic acid have not been encountered. Argininosuccinic acid or anhydride I give complex mixtures on heating in solution in the pH range 3–7. One of the components has electrophoretic mobilities at pH 2 and 6·5 consistent with its being anhydride IV (D) and it is possible that anhydride III (C) may also be present in these mixtures.

EXPERIMENTAL

NMR spectra were taken on a Varian Associates A-60 or HA-100 spectrophotometer. Ultraviolet spectra were obtained on a Beckman DB spectrophotometer. For stability studies, 0·01 M NaOH was used in 1 mm cells.

Potentiometric titrations were carried out under N_2 at 25° observing the precautions detailed by Albert and Sergeant.[18] The compounds were present initially as the hydrochlorides. For guanidinosuccinic α-lactam, this was made *in situ* by the addition of the calculated quantity of hydrochloric acid to the zwitterionic form, and in the intermediate stages of the titration the solution was supersaturated. Guanidinosuccinic β-lactam was too insoluble for even this procedure. Hence, pK_2 was obtained by dissolving the compound in one equivalent of 0·01 M KOH and partial titration with HCl. An approximate value for pK_1 was calculated from the electrophoretic mobilities at two pH values (1·6 and 2·0) by the formula.

$$K_1 = [H_a^+]\,[H_b^+]\,(u_a - u_b)/([H_a^+]\,u_b - [H_b^+]\,u_a)$$

Where u_a is the relative mobility at $[H_a^+]$. The electro-osmotic flow was measured by the mobility of urea, and n-butylamine was taken as a reference for comparing the mobilities at the two pH values. The electrophoresis was carried out on paper in a cooled plate apparatus with the cooling water regulated at 25°.

Argininosuccinic acid was isolated by the barium salt method[1] from the urine of a patient with argininosuccinic aciduria. This patient is one of the original pair described by Allen *et al.*[19] The anhydrides were prepared from this by the methods of Ratner and Kunkemueller.[3] Glycocyamidine hydrochloride,[15] β-guanidinopropionic acid and its lactam,[17] 5-methylglycocyamidine[15] and guanidinosuccinic acid[20] were prepared by literature methods.

3-Methylglycocyamidine. This was prepared by the methylation of glycocyamidine by a modification of the method of Korndorfer.[7] Glycocyamidine hydrochloride (6·8 g), dissolved in water, was converted to the free base by treatment with De-Acidite FF resin (quaternary ammonium) in the bicarbonate form followed by evaporation to dryness. The resultant solid was then heated with CH_3I (10 ml) and MeOH (10 ml) at 100° for 12 hr in a sealed tube. The reaction mixture was applied to a column of Amberlite IRA 410 hydroxide form resin made up in MeOH. Elution with MeOH, addition of HCl to the eluate and evaporation gave 3-methylglycocyamidine hydrochloride which was recrystallized from ethanol-ether, m.p. 251–261° dec (change in crystalline form 200–210°) (Y. 3·3 g, 44%).

N^2-Methylglycocyamidine and 3-methylglycocyamidine from glycine ethyl ester. N,S-dimethylisothiouronium iodide[21] (11·6 g, 0·05 mole) and glycine ethyl ester hydrochloride (14 g) were heated under reflux in EtOH (300 ml). NaOEt (0·1 mole) in EtOH (200 ml) was added over 3 hr to the stirred mixture and stirring and heating were continued for a further 3 hr. The suspension was filtered and the filtrate taken to dryness. The residue was taken up in M HCl and heated under reflux for 5 hr to hydrolyse any excess of glycine ethyl ester—this step was omitted where only the N^2-methyl isomer was required. The solution was again taken to dryness. The solid was taken up in sodium formate—acetate buffer (0·5 M, pH 3·5) and applied to a 60 × 4 cm column of Dowex 50WX8 (sulphonic acid) resin 52–100 mesh equilibrated with the same buffer. The column was eluted successively with starting buffer (1 l), 0·5 M NaOAc pH 5·5 buffer (3·5 l), and for 3-methylglycocyamidine, 0·5. M NaOAc (500 ml) and M Na_2CO_3-$NaHCO_3$ pH 9·5 (1·5 l). The fractions containing the glycocyamidines were located by monitoring the effluent at 235 nm. The appropriated fractions were combined, acidified with conc HCl and evaporated to dryness. The products were extracted with warm EtOH and chromatographed in EtOH on a short column of silica gel. N^2-Methylglycocyamidine hydrochloride (1·0 g, 13%) had m.p. 190–193° dec (from ethanol-ether), IR spectrum different from creatinine and 3-methylglycocyamidine (Found: C, 32·0; H, 5·5; N, 27·8. $C_4H_8ClN_3O$ requires C, 32·1; H, 5·4; N, 28·1%). 3-Methylglycocyamidine hydrochloride (1·1 g, 15%) was identical by

electrophoresis and IR spectrum with the sample obtained by Korndorfer's method. (Found: C, 32·15; H, 5·8; N, 28·2%).

DL-Guanidinosuccinic α-lactam (2-amino-4-oxo-5-carboxymethyl-4,5-dihydroimidazole). DL-Guanidinosuccinic acid monohydrate 2·0 g) dissolved in 2 M HCl (50 ml) was heated under reflux for 10 hr. The mixture was evaporated to dryness, and the residue was dissolved in water (15 ml). After several hours, the soln was filtered to remove a small amount of β-lactam and then adjusted to pH 4 with NaOH. The ppt was collected, suspended in a small quantity of water and dissolved by the addition of the minimum amount of 2 M HCl. Addition of NaOH to pH 4 gave the α-lactam (1·48 g, 83%) m.p. 272–274° dec (lit[2] m.p. 268–270°).

DL-Guanidinosuccinic β-lactam (2-amino-4-carboxy-6-oxo-3,4,5,6-tetrahydropyrimidine). DL-Guanidinosuccinic acid monohydrate (2·0 g) in water (50 ml) was heated under reflux for 24 hr. The soln was filtered while hot, and the ppt washed with hot water. The product was recrystallised by dissolving in the minimum quantity of HCl and precipitating at pH 4. The β-lactam (0·82 g, 46%) decomposes at 280°. (Found: C, 38·0; H, 4·9; N, 26·9. $C_5H_7N_3O_3$ requires C, 38·2; H, 4·5; N, 26·7%). This compound was detected on electrophoretograms by chlorination followed by starch-KI spray or by the α-naphthol-diacetyl reagent.[12]

Hydrazinolysis

The compound (4–5 mg) was sealed in an evacuated tube with anhydrous hydrazine[22] (1 ml). After the reaction, the hydrazine was removed over conc H_2SO_4 *in vacuo*, and the products examined by high-voltage electrophoresis at pH 2 and pH 5·3. Aspartyl α- and β-hydrazides were prepared by the methods of Narita and Ohta.[23]

Diborane reduction[13]

The compound (3–5 mg) was dissolved in trifluoroacetic acid (0·05 ml). Excess of trifluoroacetic acid was removed *in vacuo* and M diborane in THF[24] (4 ml) was added gradually at 0°. After being left for 2 hr at 0°, the soln was evaporated to dryness in a stream of N_2. The residue was dissolved in 3 M $Ba(OH)_2$ (1 ml) and heated in a sealed tube at 105° for 12 hr. The mixture was then cooled and adjusted to pH 2–3 with dil H_2SO_4. The $BaSO_4$ was removed by centrifugation and a portion of the supernatant examined by electrophoresis at pH 2.

Jaffé reaction

The compound in solution (3 ml) was treated with 0·04 M picric acid (1 ml) and 0·75 M NaOH (1 ml). The colour development and decay were followed at 500 nm against an appropriate blank. The cell housing was maintained at 25°. The times for maximum colour development were noted and first order plots were obtained for the later portions of the decay curves.

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