foamy residue was recrystallized from ethyl acetate to yield benzyl reserpate, m.p. 202–204°.

Preparation of Derivatives of 18-O-Benzoylreserpic Acid.—The compounds listed in Table II were prepared by the following methods: (A) Benzyl 0-(3,4,5-trimethoxybenzoyl)-reserpate (1.0 g.) was suspended in 400 ml. of methanol and hydrogenated over 500 mg. of palladium black at atmospheric pressure for 4 hr. Most of the hydrogen was absorbed rapidly during the first half-hour. The mixture was then diluted with 100 ml. of methylene chloride to dissolve crystalline products, filtered and the filtrate evaporated *in vacuo*. The residue was dissolved in methylene chloride, a small amount of methanol added and the solution evaporated carefully under nitrogen on the steam-bath to crystallize the desired 0-(3,4,5-trimethoxybenzoyl)-reserpic acid. After recrystallization the yield was 870 mg.

Proof of constitution was afforded by reaction of 45 mg. of the substance in methylene chloride–methanol–ether with excess of diazomethane overnight to yield 40 mg. of a compound, m.p. 266–271°, identical by infrared spectra with an authentic sample of reserpine.

Anal. Calcd. for $C_{33}H_{40}N_2O_9$: C, 65.11; H, 6.62; N, 4.60. Found: C, 64.91; H, 6.61; N, 4.70.

(B) The general procedure followed using the corresponding acyl halides and reserpic acid esters is illustrated by the preparation of 2-dimethylaminoethyl 0-(3,4,5-trimethoxybenzoyl)-reserpate.

To 2.37 g. (0.005 mole) of 2-dimethylaminoethyl reserpate, dried *in vacuo* at 85–90° for 2 hr., was added 30 ml. of dry pyridine and 1.38 g. (0.006 mole, 20% excess) of 3,4,5-trimethoxybenzoyl chloride. The mixture was cooled under tap water with constant shaking for a period of 5 minutes and allowed to stand at room temperature overnight. The pyridine solution was poured into 300 ml. of ice-cold water containing 10 ml. of ammonium hydroxide. Precipitated solid, which became granular on standing, was filtered, washed with water and air-dried. The crude ester, dissolved in methylene chloride, was filtered through a short Florisil column and recrystallized from ethyl acetate–petroleum ether; yield 1.5 g.

(C) The procedure was the same as B except that the reaction mixture was poured into water and carefully adjusted with ammonia to approximately *p*H 8–9. An excess of ammonia was avoided in order to minimize possible ammonolysis of the ethoxyformyloxy group.

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[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

The Association of Nickel(II) Ion with Peptides[1]

BY R. BRUCE MARTIN, MICHAEL CHAMBERLIN AND JOHN T. EDSALL

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In solutions of peptides such as glycylglycine and nickel(II) ion with a ligand-to-metal ratio greater than two to one, two equivalents of acid, in addition to that found when the peptide is titrated in the absence of Ni(II), are titrated at about *p*H 9. Since titration of the corresponding solutions of glycylsarcosine and nickel(II) give no indication of additional equivalents in this region, the amide hydrogen is implicated as the source of the protons, as has previously been demonstrated for copper(II) complexes of peptides. Complexes of nickel(II) with glycinamide, triglycine and tetraglycine, which are blue in relatively acid solutions, yield yellow solutions on ionization of the amide hydrogens. Absorption maxima for the three compounds named above were at 438, 430 and 412 mμ, respectively, with molar extinction coefficients of 61, 240 and 215. For tetraglycine the titration curve of the three amide hydrogens showed extremely high buffer capacity; the three *pK* values were closer together than would be expected statistically for equivalent and independent groups, and an appreciable time was required for equilibration during titration. The resulting complex contains one mole of tetraglycine per mole of Ni(II). The color change presumably indicates a transition from an octahedral to a planar configuration.

Heretofore, the association of divalent copper with peptides has been regarded as unique, in that Cu(II) was the only metal ion which was known to induce ionization of an amide hydrogen after combination with the amino group of a peptide such as glycylglycine. Convincing evidence has been presented that it is the amide hydrogen and not a proton from the hydration sphere of the cupric(II) ion that ionizes at about *p*H 4.[2–5] The ionization of the amide hydrogen apparently does not occur for simple peptide complexes of divalent cobalt,[3] manganese,[3,4] magnesium[4] and zinc.[6] In this paper evidence is presented that divalent nickel complexes are also effective in promoting the ionization of amide hydrogens.

Experimental

Standardized sodium hydroxide solution was added from a Gilmont ultramicroburet of 1.00-ml. capacity to solutions of nickel nitrate, ligand and sufficient KNO_3 to yield an ionic strength of about 0.16. The temperature was 25°. The distilled water used was passed through a mixed bed ion-exchange column before use. All the peptides were obtained commercially except for the glycylsarcosine which was a gift from W. L. Koltun and R. Roth. The *p*H was measured on a Beckman Model G *p*H meter. The spectra were recorded on a Beckman Model DU spectrophotometer, and for this purpose the nickel was added as the chloride.

Results

The titration curves for solutions of varying ratios of glycylglycine and divalent nickel ion are shown in Fig. 1. For all ratios greater than two—precipitation occurs at lower ratios—two additional equivalents are titrated in the 9 to 11 *p*H region. There is some evidence that a third additional equivalent is titrated at high ratios with a pK_3 of about 10.7. The titration curves for similar mixtures of glycylsarcosine and nickel are also

(1) This work was supported by grants from the United States Public Health Service (H-3169) and from the National Science Foundation (G-3230). Some of the work reported here is presented in the honors thesis of Michael Chamberlin in Biochemical Sciences, Harvard, 1959.

(2) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59**, 246, 257 (1955).

(3) S. P. Datta and B. R. Rabin, *Biochim. Biophys. Acta*, **19**, 572 (1956); *Trans. Faraday Soc.*, **52**, 1117, 1123 (1956); B. R. Rabin, *ibid.*, **52**, 1130 (1956); *Biochem. Soc. Symposia*, **15**, 21 (1958).

(4) C. B. Murphy and A. E. Martell, *J. Biol. Chem.*, **226**, 37 (1957).

(5) W. L. Koltun and F. R. N. Gurd, THIS JOURNAL, **81**, 301 (1959); W. L. Koltun, M. Fried and F. R. N. Gurd, *ibid.*, **82**, 233 (1960).

(6) F. R. N. Gurd, private communication. M. Fried and F. R. N. Gurd (private communication) have independently observed the titration of additional hydrogens in solutions of glycylglycine and divalent nickel. A. R. Manyak, C. B. Murphy and A. E. Martell, *Arch. Biochem. and Biophys.*, **59**, 373 (1955), observed the titration of one additional hydrogen in a 2:1 mixture of glycylglycine and nickel(II) ion. However, their evidence that the peptide hydrogen is titrated, as opposed to a proton from the hydration sphere of the metal ion, is not conclusive and even questionable as they also claim peptide hydrogen ionization for the cobalt(II) complex which is apparently not the case.[3]

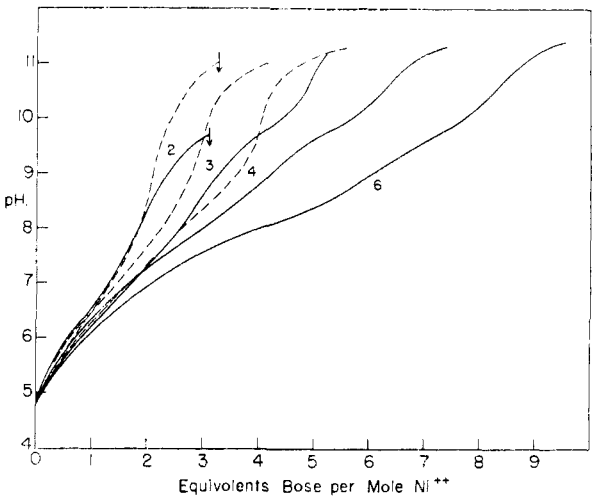

Fig. 1.—Titration curves of solutions 5 or 10 × 10^{-3} M in nickel(II) nitrate and either glycylglycine (solid curves) or glycylsarcosine (dashed curves) titrated with 0.151 N base. The numeral near the curves indicates the molar ratio of peptide to divalent nickel ion. Arrows indicate onset of precipitation.

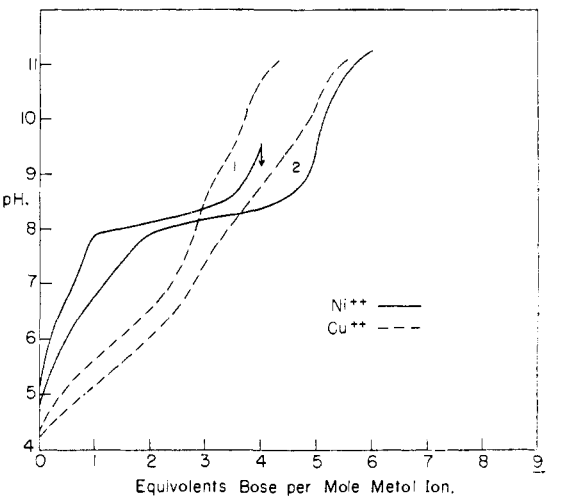

Fig. 2.—Titration curves of solutions 5 × 10^{-3} M in nickel (solid curves) or copper (dashed curves) nitrate and 5 or 10 × 10^{-3} M in tetraglycine titrated with 0.151 N base. The numeral near the curves indicates the molar ratio of tetraglycine to divalent metal ion. Arrow indicates onset of precipitation.

drawn for comparison. All the peptides studied with amide hydrogens titrated two additional equivalents of acid per mole of metal ion except tetraglycine which gave three additional equivalents. The titration curves obtained with tetraglycine and nickel(II) and copper(II) ions are shown in Fig. 2.

TABLE I

IONIZATION AND FORMATION CONSTANTS FOR NICKEL(II) WITH PEPTIDES AT 25° AND 0.16 IONIC STRENGTH[a]

pK_a is the value for the ammonium group of the peptide in the absence of Ni^{++} or Cu^{++} ions. k_1, k_2 and k_3 denote association constants between the peptide and the metallic ion. pK_1, pK_2 and pK_3 refer to acidic ionizations, either of the peptide hydrogens or, in some cases, of acid protons from the hydration sphere of the metal ion. See text.

	pK_a	log k_1	log k_2	log k_3	pK_1	pK_2	pK_3
Glycylglycine	8.15	3.93	3.25	2.0	9.35	9.95	
Glycylsarcosine	8.59	4.44	3.65	2.1	10.7		
Glycyl-L-proline	8.50	4.62	3.80	2.5	10.7		
Glycyl-L-valine	8.22	4.05	3.35	2.1	10.4	11.0	
L-Valylglycine	8.02	3.00	2.20	0.9	9.0	9.6	
Glycylglycine ethyl ester	7.76	3.65	2.95	2.0	9.2[b]	9.8[b]	
Glycinamide[c]	8.05	4.20	3.4	2.1	9.8	10.1	
Triglycine	8.01	3.70	2.90	1.6	8.25	8.45	
Tetraglycine	7.99	3.65	2.90		8.10	8.20	8.25
Tetraglycine + Cu-(II)[d]	7.99	5.05			(6.0)	6.95	9.45

[a] Except for pK_a and log k_1 for the first three peptides the results are expressed to 0.05 or 0.1 log units. [b] Estimates only due to rapid hydrolysis. [c] A constant value of pK_a was not obtained for varying ratios of acid to base. The values recorded are thus of much less accuracy than for the other compounds listed. The pK_a and log k_1 values are taken from N. C. Li and M. C. M. Chen, THIS JOURNAL, **80**, 5678 (1958), where the values of pK_a and log k_1 for other glycine peptides are in good agreement with the above. [d] Tetraglycine with copper(II) nitrate in place of nickel(II) nitrate. The method used for estimating pK_1 will yield too high a value for this complex.

In Table I are listed the ammonium ionization constant pK_a, the logarithm of the successive formation constants log k, and the ionization constants of the chelate complexes, pK_1, etc., for the compounds studied. The formation constants were determined in solutions containing high ratios of ligand-to-nickel ion to minimize the contribution of the subsequent amide ionizations. These constants were calculated by the method of Scatchard.[7] The values of log k_3 are to be taken more as an indication of the existence of a 3:1 complex than as to a precise evaluation of the strength of binding.

The ionization constants of the complexes, pK_1, etc., for all but triglycine and tetraglycine were evaluated in solutions with a 3:1 ligand-to-metal ratio. At lower ratios precipitation usually occurs and at greater ratios the ionization of uncomplexed ligand is a confusing factor. However, consistent values were obtained at the higher ratios.

A solution containing a 3:1 ratio of glycyl-DL-serine and nickel(II) ion gave a titration curve very like that of glycylglycine and therefore the constants are presumably nearly the same. A solution with glycyl-L-tyrosine also gave a similar curve until the point where the phenolic ionization occurs.

For triglycine and tetraglycine the ionization constants were determined in solutions containing equimolar amounts of ligand and nickel(II) ion. Since in all cases the constants overlap, we have analyzed the data by means of Scatchard's equation,[7] as formulated for dissociation constants.[8] For an n-valent acid H_nA, we write $\bar{h}$ as the mean number of protons dissociated per molecule of acid, and define the function pM by the relation

$$p\mathrm{M} = -\log [(\mathrm{H}^+)\bar{h}/(n - \bar{h})]$$

A plot of pM as a function of $\bar{h}$ should extrapolate to $pK_1 + \log n$ as $\bar{h}$ approaches zero, and to

(7) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, THIS JOURNAL, **76**, 3054 (1954).

(8) See J. T. Edsall, R. B. Martin and B. R. Hollingworth, *Proc. Natl. Acad. Sci.*, **44**, 505 (1958). The procedure employed here is given on pp. 515–517 of this paper.

pK_n − log n as $\bar{h}$ approaches the upper limit, n. For triglycine a value of $n = 3$ was assumed (one ammonium group and two peptide hydrogens). The pK values assigned to the peptide hydrogens—listed as pK_1 and pK_2 in Table I—then represent the second and third pK values in the scheme for a trivalent acid. Thus pK_2 − log 3 was taken as the extrapolated value of pM at $\bar{h} = 3$, and a rough approximation to pK_1 was made by assuming it equal to the value of pM for $\bar{h} = 1.5$. It is notable that pK_1 and pK_2 are closer together than would be expected on statistical grounds for equivalent and independent groups. This remarkable result indicated that a molecular rearrangement must occur as the titration proceeds; a matter discussed below in connection with tetraglycine, which exhibits the same phenomenon in even more pronounced form.

A similar procedure was adopted for tetraglycine, for which a value of $n = 4$ was assumed, and the results are illustrated in Fig. 3. The values of pM are not shown for $\bar{h}$ below 1; this region would correspond to the titration of the amino group, partly in free tetraglycine, largely in the formation of the Ni(II) complex, which forms as alkali is added. At $\bar{h} = 1$ or above, the tetraglycine is predominantly in the form of the Ni complex, but the structure of the complex alters progressively as the titration proceeds. This is shown both by the change from a blue to a yellow color (see below) and by the fact that an appreciable time is required for the pH to attain an equilibrium value after each fresh addition of hydroxyl ions. Generally it was found necessary to wait 4 or 5 minutes to make sure that the pH was no longer changing.

The value of pK_3 for the third peptide hydrogen (*i.e.*, the fourth pK value derived from Fig. 3 for a tetravalent acid) was determined by the relation $pK_3 = p$M (at $\bar{h} = 4$) + log 4. The values of pK_1 and pK_2 are more uncertain; pK_1 was approximately estimated by the relation: $pK_1 = p$M (at $\bar{h} = 1.5$) − log 1.5; and pK_2 by the approximate relation: $pK_2 = p$M (at $\bar{h} = 2.5$) + log 1.5. This method does not give precise values for pK_1 and pK_2 but has the advantage of being rapid and objective. Moreover the sign of the slope indicates immediately whether the ionizations are less than statistical; the pronounced negative slope of Fig. 3 is very striking. To obtain more accurate values a method of successive approximations would be necessary; this would be extremely tedious and insensitive for a system such as tetraglycine and nickel(II).

The first six peptides of Table I as well as glycylserine and glycyltyrosine remained greenish-blue to blue in the presence of nickel ions throughout the titrations. However, the last three compounds of Table I, glycinamide, triglycine and tetraglycine, although blue in the more acid solutions, became yellow on ionization of the amide hydrogens. For tetraglycine a definite time factor was observed in both the pH and spectrophotometric studies on the yellow solutions. After ionization of the amide hydrogens the yellow solutions gave absorption curves with maxima at 438, 430 and 412 mμ with molar extinction coefficients

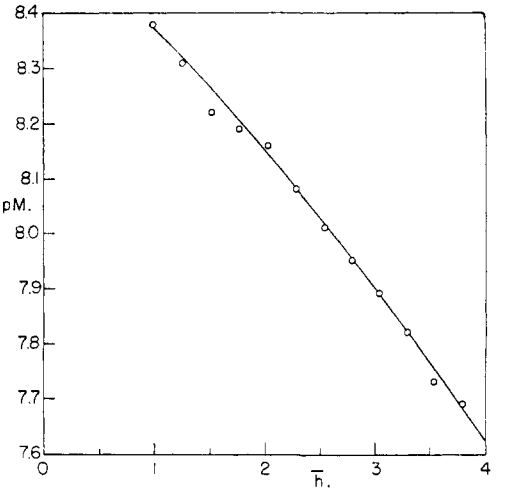

Fig. 3.—Plot of pM *versus* $\bar{h}$ for the equimolar mixture of tetraglycine and nickel(II) ion of Fig. 2. The negative slope indicates that the successive ionizations occur in less than the statistically predicted ratios.

of 61, 240 and 215 for glycinamide, triglycine and tetraglycine, respectively. The addition of sufficient acid to these basic solutions resulted in the return of the blue color present before the amide ionization.

Discussion

In general the differences between the logarithms of the successive association constants are similar for the compounds studied and the values of log k_1 run roughly parallel to the pK_a values, with an exception of L-valylglycine where log k_1 is about a unit lower than the values for the other compounds of Table I. A corresponding difference also exists for the logarithm of the first association constants of nickel(II) with glycine and valine.[9]

Unlike cupric ion–peptide complexes, in which the amide hydrogen ionization for glycylglycine occurs at about pH 4,[2-5] the additional equivalents in the case of nickel(II) ion are titrated at about pH 9. Several observations indicate that the additional equivalents of acid titrated below pH 10 are due to the amide hydrogens and not to protons from the hydration sphere of the divalent nickel ion. In Fig. 1, the apparent pK_1 for glycylsarcosine, as approximated by the pH at the first additional one-half equivalent, increases with increasing ratios of peptide to nickel(II) ion, indicative of the competition between peptide and hydroxyl anions for the binding sites. For glycylglycine, on the other hand, the apparent pK_1 is nearly constant, independent of the peptide to nickel(II) ratio. The pK_1 values listed in Table I for the peptides with no amide hydrogen available, glycylsarcosine and glycylproline, were determined in 3:1 mixtures of ligand-to-metal ion and are 1.3 pH units higher than those of glycylglycine. For glycylsarcosine and glycylproline the ionization must be from the hydration sphere of the nickel(II) ion. The lower values of pK_1 and pK_2 for glycylglycine again implicate the amide hydrogens.

(9) N. C. Li, J. M. White and R. L. Yoest, THIS JOURNAL, **78**, 5218 (1956).

The pK_1 value of glycyl-L-valine is intermediate, and it is difficult to say whether this represents the ionization of a more basic amide hydrogen than that of glycylglycine, a more acid proton from the hydration sphere of the divalent nickel ion than that of glycylsarcosine or glycylproline, or a mixture of both. The inductive effect of the valyl residue should make the removal of the amide hydrogen more difficult. In any event it is clear that for glycyl-L-valine the ionization of the amide hydrogen is significantly suppressed, with no concurrent change in metal binding capacity, by comparison with all the other compounds containing ionizable amide hydrogens. Examination of molecular models indicates that the chelation to the oxygen of the amide bond is not restricted in glycylvaline, whereas chelation to the amide nitrogen is sterically hindered. The normal log k_1 value and the high value of pK_1 are thus consistent with the postulate, originally made for cupric ion,[3] that the initial chelating point is the amide oxygen with a transfer to the amide nitrogen on titration of the amide hydrogen.

It is difficult to reconcile the observation that, at equimolar or higher ratios of peptide to nickel(II) ion, 2 additional equivalents per metal ion are titrated in triglycine and 3 in tetraglycine, with any postulate other than ionization of peptide hydrogens. If the ionizations were due to the formation of a hydroxo complex, it would not be expected that a greater number of ionizations occur for tetraglycine, for its nickel complex should be the more extensively chelated, thereby reducing the number of sites on the metal ion available for the formation of the hydroxo complex.

The successive ionization constants pK_1 and pK_2 for glycylglycine, glycylvaline, valylglycine and glycylglycine ethyl ester are approximately statistical. For glycinamide, triglycine and tetraglycine the successive ionization constants are markedly less than statistical, the ionization is accompanied by an increasing yellow color, and in the case of tetraglycine a definite time interval is required for the attainment of equilibrium. These facts imply that a profound molecular rearrangement occurs on the titration of the amide hydrogens for these three compounds; apparently a change in configuration of the nickel(II) ion from octahedral to planar. Thus after the peptide ionizations tetraglycine wraps around the four corners of the plane with a nitrogen at each corner forming an equimolar complex even in solutions with a tetraglycine to nickel(II) ratio greater than unity.

The curves of Fig. 2 graphically illustrate that the binding of tetraglycine is greater for copper than for nickel, as is indicated by the larger value of log k_1 for copper in Table I. However, because of the less than statistical ionizations of the Ni(II)–tetraglycine complex, the buffer capacity is extraordinarily high, the curves of Fig. 2 cross and the last equivalent is titrated at a lower pH for the nickel than for the copper complex, which shows a "normal" spacing between the successive pK's. Thus in the last two rows of Table I, K_3 is 16 times greater for the nickel complex, even though k_1 is 25 times greater for the copper complex. This reversal is consistent with the suggestion that a change in configuration has occurred in the tetraglycine complex of nickel upon the ionization of the peptide hydrogens. In the series diglycine, triglycine, tetraglycine, the values of pK_1 tend to increase for copper(II)[2] and decrease for nickel(II) ion.

Glycinamide apparently is also able to induce the planar configuration in nickel(II) ion with the ionization of two amide hydrogens. However, the intensity of the spectra is considerably less than for tri- or tetraglycine. All of these spectra are similar to that reported for the planar 1:2 nickel(II) complex of 2,3-dimethyl-2,3-diaminobutane where the molar extinction coefficient is 64 at 434 mμ.[10]

Glycylglycine does not give the less than statistical ionizations or the yellow color, indicating that the planar configuration is not attained in this case. Unlike the complex of glycinamide, the complex of glycylglycine has additional coördinating groups of apparently sufficient strength to maintain the octahedral field. The observation of a similar situation in glycylglycine ethyl ester indicates that these additional coördinating ligands need not be charged to maintain an octahedral configuration.

Acknowledgments.—The authors thank Dr. Walter L. Koltun and Miss Reta Roth for the gift of glycylsarcosine. We also thank Dr. Frank R. N. Gurd for interesting discussions.

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(10) F. Basolo, Y. T. Chen and R. K. Murmann, THIS JOURNAL, **76**, 956 (1954).

COMMUNICATIONS TO THE EDITOR

POTENTIAL HYPOSENSITIZING AGENTS AGAINST POISON IVY DERMATITIS. A NEW SYNTHESIS OF HYDROURUSHIOL (3-PENTADECYLCATECHOL)

Sir:

The high incidence each summer of dermatitis due to poison ivy and related members of the species *Rhus toxicodendron* continues to spur research toward improved prophylactic agents.[1] It has been established that the allergens of poison ivy are hydrourushiol and its unsaturated analogs.[2] Similar phenolic allergens are present in other members of the family Anacardiaceae, such as the oriental

(1) F. A. Stevens, *J. Am. Med. Assn.*, **127**, 912 (1945); G. E. Gaillard, *N. Y. State J. Med.*, **56**, 2255 (1956); A. M. Kligman, *A. M. A. Arch. Dermatol.*, **78**, 47 (1958); Anon., *Chem. Wk.*, **84**, 39 (June 27, 1959).

(2) C. R. Dawson, *Trans. N. Y. Acad. Sci.*, **18**, 427 (1956).