Further consideration suggested an involvement of the temperature coefficient of molar volume, and it was noted that the molar volumes of the isotopic pairs differ by a constant 0.25 (±0.02) cc./mole at both 20 and 40°, indicating that the molar volume decrement accompanying 100% replacement of protium by deuterium is little affected by temperature, as would be expected. Algebraic examination of the mathematical relations, too detailed for inclusion here, showed that since the density–temperature curve is nearly linear for these substances over a 20° span, the difference in density between 20 and 40° is proportional to the density–temperature coefficient for an individual compound, and if the difference in isothermal molar volumes for an isotopic pair is small compared to the molar volumes themselves, the approximate proportionality between the density–temperature coefficient ratios and the molecular weight ratios would be expected.

These findings suggested that the temperature coefficients of molar volume for hydrocarbon series having constant values of "x" in the empirical formula C_nH_{2n+x} might be related to the molecular weights by a simple expression. This hypothesis has been tested and the results show a convenient linear relationship which will be the subject of a separate publication.

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STATE COLLEGE, PENNA.

[CONTRIBUTION FROM THE SCHOOL OF PHARMACY, UNIVERSITY OF WISCONSIN]

Metal Chelates of Riboflavin[1]

BY WILLIAM O. FOYE AND WINTHROP E. LANGE

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A number of metal chelates of the isoalloxazines, riboflavin and 6,7-dichloro-9-(1′-D-sorbityl)-isoalloxazine, have been prepared and isolated. Evidence for chelate formation is provided by a drop in *p*H during the formation, absence of metal ions in the solution, analyses of the purified products, production of characteristic colors, decreased solubility in alkaline solution and comparison with a true salt formation. The chelates show the same relative degree of insolubility as riboflavin itself.

Since the announcement of a tumor-inhibiting action by riboflavin[2] and later by related isoalloxazines,[3] little has been done to explain this activity. A number of metal salts and chelates has also been reported to be inhibitory to tumor growth,[4] which suggested the possibility that metal chelates of riboflavin might be involved in tumor inhibition or possibly in tumor growth. To test this idea, a number of metal chelates of riboflavin and that isoalloxazine reported[3] to be most active in tumor inhibition were prepared.

Previously, Albert[5] had demonstrated the existence of metal complexes of riboflavin in aqueous solution with a 1:1 ratio of metal to riboflavin by potentiometric titration. He also reported the formation of an insoluble, non-titratable complex which he believed to have a 2:1 ratio of metal to riboflavin, but the complex was neither isolated nor characterized.

Attempts to prepare isolatable metal chelates in quantity involved first of all the use of aqueous and alcoholic suspensions of riboflavin close to neutrality. In order to obtain relatively complete complexing, it was necessary to use a procedure similar to that of Traube,[6] in which mannitol was complexed with metals in aqueous alkaline solution. Since riboflavin is soluble in aqueous alkali only at a *p*H of 9 or higher, this *p*H was maintained by simultaneous addition of alkali with metal salt to prevent precipitation of free riboflavin. The drop in *p*H which occurred without addition of more alkali, incidentally, provides good evidence for chelate rather than salt formation. In addition, no test for metal ion in solution by indicators such as dithizone and ferricyanide could be obtained until slightly more than two molar equivalents of metal salt to riboflavin had been added. At this point, a paper chromatogram of the supernatant solution showed the disappearance of the yellow riboflavin band, and only an orange or brown band of metal chelate remained.

This procedure was found to give immediate precipitation of colored metal chelates with all the metals employed, although much better yields were obtained with divalent than with trivalent metals, possibly because of greater insolubility of the former. To avoid excess metal, the reaction was carried out with approximately equimolar quantities of riboflavin and metal salt. Excess riboflavin then had to be removed, and since the chelates were insoluble in water, alkali and organic solvents and decomposed in either organic or mineral acids Soxhlet extraction with acetone was found the only means possible. Washing with water was found to remove adsorbed sodium as well.

Analyses for carbon, hydrogen and metal of the chelates, as shown in Table II, confirmed a structure containing two metal atoms per riboflavin molecule for each metal employed. The presence of water of hydration, to fill the coördination capacities of the metals, was indicated by the loss of

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(1) Abstracted from the M.S. dissertation of Winthrop E. Lange, January, 1953.

(2) G. I. Kensler, K. Sugiura, N. F. Young, C. R. Halter and C. P. Rhoads, *Science*, **93**, 308 (1941).

(3) F. W. Holly, E. W. Peel, R. Mozingo and K. Folkers, THIS JOURNAL, **72**, 5417 (1950).

(4) K. Sugiura and S. R. Benedict, *J. Cancer Res.*, **7**, 329 (1922); C. F. Geschickter and E. E. Reid, "Approaches to Tumor Chemotherapy," A.A.A.S., Washington, D. C., 1947, p. 431.

(5) A. Albert, *Biochem. J.*, **47**, 27 (1950).

(6) W. Traube and F. Kuhbier, *Ber.*, **65**, 187 (1932).

generally one molecule of water during the drying prior to analysis. The temperature of this dehydration is assumed to be the same as that at which the chelates soften, as shown in Table I. All attempts at complete dehydration resulted in destruction of the chelates.

TABLE I

RIBOFLAVIN–METAL CHELATES

Formula[a]	Yield, %	Recovered riboflavin, %[b]	Color	Dehydration temp.,[c] °C.
$\mathrm{R{-}Fe_2\cdot 5H_2O}$	96	47	Dark brown	144–148
$\mathrm{R{-}Co_2\cdot 5H_2O}$	96	46	Brown	157–163
$\mathrm{R{-}Ni_2\cdot 5H_2O}$	97	47	Light brown	105–115
$\mathrm{R{-}Cu_2\cdot 2H_2O}$	95	43	Brown	130–136
$\mathrm{R{-}Zn_2\cdot 2H_2O}$	98	48	Orange-brown	140–145
$\mathrm{R{-}Mn_2\cdot 2H_2O}$	97	41	Brown	160–168
$\mathrm{R_2{-}Hg}$[d]	91	0	Orange	220–222[e]

[a] R = $\mathrm{C_{17}H_{16}O_6N_4}$. [b] Equimolar quantities of riboflavin and metal salt were used. [c] The point at which a color change and softening were noted using a Fisher–Johns block. The temperatures are corrected. [d] Salt. [e] Melting point.

TABLE II

ANALYSES OF RIBOFLAVIN–METAL CHELATES

Formula[a]	Calculated C	H	M″[b]	1H$_2$O	Found[c] C	H	M″	1H$_2$O[d]
$\mathrm{R{-}Fe_2\cdot 5H_2O}$			19.39	3.12			20.01	3.15
$\mathrm{R{-}Fe_2\cdot 4H_2O}$	36.59	4.30			36.99	4.22		
$\mathrm{R{-}Co_2\cdot 5H_2O}$	35.07	4.48	20.58		34.90	4.05	19.99	
$\mathrm{R{-}Ni_2\cdot 5H_2O}$			20.25	3.31[e]			21.03[f]	3.62
$\mathrm{R{-}Ni_2\cdot 3H_2O}$	37.51	4.05			38.28	4.29		
$\mathrm{R{-}Zn_2\cdot 2H_2O}$			24.30	3.34			23.99	2.63
$\mathrm{R{-}Zn_2\cdot 1H_2O}$	39.15	3.45			38.97	4.13		
$\mathrm{R{-}Cu_2\cdot 2H_2O}$			23.75	3.35			23.05	2.98
$\mathrm{R{-}Cu_2\cdot 1H_2O}$	39.47	3.48			39.40	4.02		
$\mathrm{R{-}Mn_2\cdot 2H_2O}$	39.41	3.86			38.93	4.24		0.30
$\mathrm{R_2{-}Hg}$[g]			21.09				21.03	

[a] R = $\mathrm{C_{17}H_{16}O_6N_4}$. [b] M″ = divalent metal. [c] The carbon–hydrogen analyses were carried out by the Clark Microanalytical Laboratory, Urbana, Ill. The metal analyses were conducted by standard gravimetric procedures: iron according to I. M. Kolthoff and E. B. Sandell, "Textbook of Quantitative Inorganic Analysis," The Macmillan Co., New York, N. Y., 1949, p. 595; zinc according to Kolthoff and Sandell, *ibid.*, p. 373; cobalt according to W. F. Hillebrand and G. E. F. Lundell, "Applied Inorganic Analysis," John Wiley and Sons, Inc., New York, N. Y., 1929, p. 326; nickel according to Hillebrand and Lundell, *ibid.*, p. 316; copper according to Hillebrand and Lundell, *ibid.*, p. 199; mercury according to Hillebrand and Lundell, *ibid.*, p. 173. Sodium, present in the chelates to the extent of 2.0–2.6% before washing was found in the washed products in quantities of 0.05–0.10% according to the method of Kolthoff and Sandell, *loc. cit.*, p. 416. [d] This quantity represents the loss of weight on drying at 56° *in vacuo*. [e] The calculated value for two molecules of water. [f] This value is high because of the presence of iron in the nickel nitrate used. Emission spectroscopy of the product revealed iron. [g] Salt.

Martell and Calvin[7] have described a number of criteria for determining chelate formation, among which the following were shown by the riboflavin complexes: color, decreased solubility in water, drop in *p*H during formation, absence of metal ions in solution, and correct analysis of isolated products. The absence of metal ion in solution until an excess of metal had been added argues against the simultaneous formation of metal hydroxide. Presence of metal ion was determined by bringing the reaction mixture to a *p*H of 7 and adding an indicator such as dithizone. When more than two moles of metal had been added, however, metal hydroxide was formed and metal ion was detected in the solution.

Further evidence for a chelate structure is provided by the fact that a distinct salt formation was obtained on reaction with mercuric chloride. In this reaction, no drop in *p*H occurred, and the product showed a ratio of two molecules of riboflavin to one atom of mercury on analysis. Cobalt and nickel chelates were also prepared from the dichloro analog of riboflavin, and a 2:1 ratio of metal to isoalloxazine was again observed.

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Experimental

All melting points are corrected.

1,2-Dinitro-4,5-dichlorobenzene.—This compound was prepared according to the method of Hartley and Cohen,[8] except that the nitration was stirred. From 50 g. of *o*-dichlorobenzene (Eastman Organic Chemicals), after two nitrations with fuming nitric and fuming sulfuric acids, was obtained 15 g. (16%) of colorless crystals of 1,2-dinitro-4,5-dichlorobenzene, m.p. 101–103° (lit.[8] m.p. 104°). Mononitro compound, however, was removed as an oil after the product from the second nitration was recrystallized from 80% acetic acid and before it was allowed to cool. This compound gave the same yield of dinitration product after two nitrations. The added yield from more than two nitrations of either compound was found not great enough to justify the time expended.

D-Glucamine.—The method of Holly, *et al.*,[3] involving a high pressure reductive amination of D-glucose, was employed without modification, giving a 70% yield of product melting at 122–125° (lit.[3] m.p. 122–126°). This was sufficiently pure for further reaction.

2-Nitro-4,5-dichloro-N-(1′-D-sorbityl)-aniline.—The procedure of Holly, *et al.*,[3] for the condensation of 1,2-dinitro-4,5-dichlorobenzene with D-glucamine was followed except during the isolation of the product. In order to avoid formation of a gel on cooling in a Dry Ice-bath, absolute ethanol (50 ml. for 300 ml. of reaction mixture) was added at the first indication of precipitation, the mixture was removed from the bath, and the crystallization was completed as the mixture warmed gradually. After recrystallization from ethanol–water, a 55% yield of orange crystals was obtained, m.p. 191–193° (lit.[3] m.p. 193–195°).

6,7-Dichloro-9-(1′-D-sorbityl)-isoalloxazine.—The method of Holly, *et al.*,[3] was followed for the hydrogenation of the previous nitro compound, but the reaction of the reduced

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(7) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 19–75.

(8) P. Hartley and J. B. Cohen, *J. Chem. Soc.*, **85**, 865 (1904).

product with alloxan monohydrate and boric acid was found to give a better yield when stirred at room temperature for three days. After recrystallization from 18% hydrochloric acid, a 75% yield of orange product was obtained, which melted at 238–240°. The reported value[3] is 237–243°.

Metal Chelates of Riboflavin.—To a mixture of 2 g. (0.005 mole) of riboflavin (U.S.P.) and 50 ml. of water was added sufficient 5% sodium hydroxide solution to dissolve the riboflavin. An aqueous solution (10 ml.) of metal salt (0.005 mole), either ferrous sulfate, cobaltous chloride, nickel nitrate, zinc chloride, cupric chloride or manganese chloride of analytical grades, was added slowly (ten minutes) to the stirred solution along with sufficient 5% sodium hydroxide solution to maintain a *p*H of 9. After 30 minutes further of stirring and adjusting the *p*H, the mixture was filtered. The filtrate was acidified with 5% hydrochloric acid, and the unreacted riboflavin was collected and dried for further use. The residue was washed with several portions of water, extracted with acetone in a Soxhlet extractor for four hours, and dried *in vacuo*. Quantitative yields of brown product were obtained, which had characteristic softening points, as shown in Table I, but did not melt below 300°.

The formation of a mercury salt with mercuric chloride was carried out in the same manner, except that no additional alkali was needed to maintain a *p*H of 9.

Metal Chelates of 6,7-Dichloro-9-(1′-D-sorbityl)-isoalloxazine.—Metal chelates of this compound were prepared in an identical manner to that for the riboflavin chelates, using cobaltous chloride and nickel nitrate. A 94% yield of green-brown cobaltous chelate was obtained, which softened at 160–165°.

Anal. Calcd. for $C_{16}H_{12}O_7N_4Cl_2Co_2 \cdot 5H_2O$: Co, 18.11. Found: Co, 18.48.

The orange nickel chelate was obtained in 93% yield, and softened at 135–140°.

Anal. Calcd. for $C_{16}H_{12}O_7N_4Cl_2Ni_2 \cdot 5H_2O$: Ni, 17.09. Found: Ni, 18.14.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, THE POLYTECHNIC INSTITUTE OF BROOKLYN]

Synthesis in the Pyridazine Series. II. The Preparation of Pyridazines from Substituted Maleic Anhydrides. Some Properties of 4-Methylpyridazine[1]

BY R. H. MIZZONI[2] AND PAUL E. SPOERRI

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The preparation of pyridazine from hydrazine salts and maleic anhydride, previously described (ref. 3), has been extended to the synthesis of various substituted pyridazines. 4-Methylpyridazine has been obtained from citraconic anhydride and hydrazine dihydrochloride in high over-all yield; some properties of this potentially interesting substance are discussed.

The preparation of pyridazine and 3,6-dichloropyridazine from maleic anhydride was described in a recent paper.[3] In a test of the generality of this method, a number of substituted maleic anhydrides were condensed with hydrazine dihydrochloride in aqueous medium. followed by chlorination with phosphorus oxychloride and catalytic hydrogenolysis under mild conditions. This process found ready application in the case of citraconic anhydride; 4-methylpyridazine, previously unknown, was obtained in high yield. The transformation of chloromaleic anhydride into 4-chloro-3,6-pyridazinediol and 3,4,6-trichloropyridazine proceeded easily and in good yield. *cis*-Aconitic anhydride in turn was converted to 4-(3,6-dihydroxypyridazyl)-acetic acid.

These results lend further testimony to the marked effect of reaction medium on condensations of maleic anhydrides and hydrazine. The formation of substances such as N-aminomaleimides which are produced in alcoholic solution is substantially repressed in an aqueous medium of high acidity.[4] No reaction between succinic anhydride and hydrazine salts under similar conditions has been found to occur. There is apparently a driving force of some magnitude operating in the formation of the pyridazine ring.

It seemed of interest to determine whether the methyl group of 4-methylpyridazine was reactive in the sense previously demonstrated for the 3-isomer.[5] Thus, 4-methylpyridazine undergoes typical aldol-like condensations with anisaldehyde and with chloral, through the agency of a resonance-stabilized carbanion intermediate (Fig. 1).

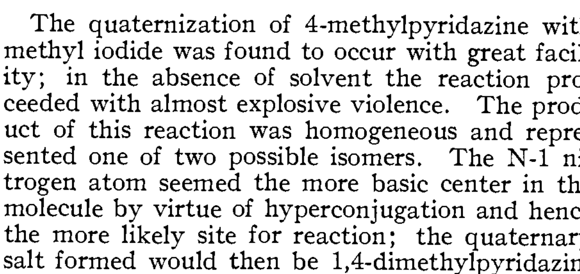

Fig. 1.

The quaternization of 4-methylpyridazine with methyl iodide was found to occur with great facility; in the absence of solvent the reaction proceeded with almost explosive violence. The product of this reaction was homogeneous and represented one of two possible isomers. The N-1 nitrogen atom seemed the more basic center in the molecule by virtue of hyperconjugation and hence the more likely site for reaction; the quaternary salt formed would then be 1,4-dimethylpyridazinium iodide, as shown in Fig. 2. In support of this proposed structure, the quaternary salt was converted to the carbocyanine iodide by the method of Hamer,[6] a process which could not occur with the other isomer.

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[1] Abstracted from the Ph.D. thesis of R. H. Mizzoni, June, 1952.

[2] Ciba Pharmaceutical Products, Inc., Summit, N. J.

[3] R. H. Mizzoni and Paul E. Spoerri, THIS JOURNAL, **73**, 1873 (1951).

[4] L. H. Flett and W. H. Gardner, "Maleic Anhydride Derivatives," John Wiley and Son, Inc., New York, N. Y., p. 130.

[5] O. Poppenberg, *Ber.*, **34**, 3257 (1901).

[6] F. M. Hamer, *J. Chem. Soc.*, 2796 (1927).