introduced methylene group is as in structure (I), its NMR pattern would be expected to be more complex than that of an AB spin system, and more in keeping with an ABC, ABX or AMX system[5]. Third, the ketonic group of lunarine is not necessary for the reaction to take place since the epimeric lunarinols I (VII) and II (VI)[1,6] also form condensation products with formaldehyde.

Lunarinol I (VII), formaldehyde and dilute acid at room temperature gives N^1, N^2-methylenelunarinol I (VIII); m.p. 195–197° (methanol-ether), $[\alpha]_D^{22}+251°$ (c 0.086, methanol), M^+ peak at *m/e* 451 (100%),and the introduced methylene group appears in the NMR-spectrum as a broadened two-proton singlet at δ 4.08. Lunarinol II, in like manner, gives N^1, N^2-methylenelunarinol II (V); m.p. 200–204° (methanol-ether), $[\alpha]_D^{22}+162°$ (c 0.15, methanol), M^+ peak at *m/e* 451 (100%), and the methylene protons in the NMR-spectrum are now just visible as an AB quartet with δ_A 4.00 and δ_B 4.15 (J = 12.0 Hz). This compound corresponds to alkaloid LBZ, the minor product of sodium borohydride reduction of N^1, N^2-methylenelunarine (IV)[1]. Fractional crystallization of the epimeric alcohols (V) and (VIII), obtained on sodium borohydride reduction of N^1, N^2-methylenelunarine (IV) gave pure N^1, N^2-methylenelunarinol I (VIII) as the major product. Examples of similar Mannich-like condensations, as reported here, in which amides are the reactive hydrogen-containing components can be found in HELLMANN[7].

The configuration of the alcoholic carbon in lunarinol I and II was established from the NMR-spectrum by the $W^1/_2$ (width at half-height) values for the α-proton. For lunarinol I, the proton is at δ 4.15 with $W^1/_2$ = 11.5 Hz, and at δ 5.06, $W^1/_2$ = 9.5 Hz for the diacetate derivative; while in lunarinol II it is found at δ 4.00, but the $W^1/_2$ value is only reliably calculated from the diacetate where it appears unobstructed at δ 5.07, $W^1/_2$ = 22 Hz. These figures, according to HASSNER and HEATHCOCK[8], indicate that, with the assumption that the cyclohexane ring is in the chair conformation, lunarinol I and II have the hydroxyl group placed axial and equatorial, respectively. Furthermore, examination of a Dreiding model of lunarine shows the bottom side (opposite the aryl-ether oxygen) is less hindered, and consequently, hydride attack from this side would produce the axial hydroxyl isomer as the predominant product[9] – a result we and others[1,6] have found experimentally.

Our evidence does not rule out the possibility that the reaction of formaldehyde and lunarine (and the lunarinols as well) may give the seven-membered ring system (perhydro-1, 3-diazepine) instead of the perhydropyrimidine. However, examination of models shows that the six-membered ring forms easily without strain, whereas the larger ring system is under strain. We are seeking experimental proof to settle this question[10].

Zusammenfassung. Für die Lunaria-Alkaloide LBX und LBZ werden die revidierten Structurformeln IV und V vorgeschlagen.

R. W. DOSKOTCH, E. H. FAIRCHILD and W. KUBELKA

Division of Natural Products Chemistry, The Ohio State University, 500 West, 12th Avenue, Columbus (Ohio 43210, USA), 22 November 1971.

[5] L. M. JACKMAN and S. STERNHELL, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd edn. (Pergamon Press, New York 1969), p. 133.
[6] P. BLADON, R. IKAN, F. S. SPRING, and A. D. TAIT, Tetrahedron Lett. *1959*, 18.
[7] H. HELLMANN, in *Newer Methods of Preparative Organic Chemistry* (Ed. W. FOERST; Academic Press, New York 1963), p. 288.
[8] A. HASSNER and C. HEATHCOCK, J. org. Chem. *29*, 1350 (1964).
[9] W. G. DAUBEN, F. J. FONKEN and D. S. NOYCE, J. Am. chem. Soc. *78*, 2579 (1956).
[10] This study was supported by a grant from the U.S. Public Health Service. We thank Mr. R. WEISENBERGER of the Chemistry Department and Dr. R. L. FOLTZ of Battelle Memorial Institute for the mass spectra.

Polyacrylamide Gel Disc Electrophoresis of Rat Bile after Intravenous Administration of $^{52}MnCl_2$, $^{64}CuCl_2$, $^{203}HgCl_2$, and $^{210}Pb(NO_3)_2$

Copper[1] and manganese[2] are excreted rapidly and in quite large amounts into the bile, whereas lead[3] and mercury[4] in small quantities only. We were interested in the possibility of explaining these differences by binding these individual metals to different bile components.

In biological systems quite a number of proteins are known to bind metals. Mercury in plasma is bound almost exclusively to proteins[5,6], manganese to transferin[7] or transmanganin[2]. In rats, approximately 90% of copper is incorporated into ceruloplasmin[8] and most of the remaining copper is loosely bound to albumin[9,10]. The protein isolated from rat liver having a molecular weight of 13,000[11] may be concerned with excretion of copper into the bile. The separation of bile components showed that there might be a relationship between lead and proteins in biliary excretion[3].

Using polyacrylamide gel disc electrophoresis, we compared the protein spectrum of rat bile with location of ^{210}Pb, ^{52}Mn, ^{64}Cu and ^{203}Hg on the electrophoreogram.

Materials and methods. Wistar rats (mean weight 200 g) with external biliary fistula were used in the experiments. Solutions of salts of metal radioisotopes were injected into the tail vein (0.075 mg of $^{52}MnCl_2$, 0.080 mg of $^{64}CuCl_2$, 0.162 mg $^{203}HgCl_2$, and 0.198 mg of $^{210}Pb(NO_3)_2$ per rat; 10–20 μCi per rat in a volume of 1 ml). The bile was collect-

[1] P. A. FARRER and S. P. MISTILIS, in *Wilson's Disease*. Birth Defects Original Articles (Williams and Wilkins Publishers, Baltimore 1968), Vol. 6, p. 14.
[2] G. C. COTZIAS, in *Mineral Metabolism: An Advanced Treatise* (Academic Press, New York 1962), p. 403.
[3] N. CASTELLINO, P. LAMANNA and B. GRIECO, Folia Medica *48*, 601 (1965).
[4] M. CIKRT, Br. J. ind. Med. *29*, 74 (1972).
[5] T. W. CLARKSON, J. GATZY and C. DALTON, Univ. Rochester AEP Report, No. *582*, 1 (1964).
[6] H. PASSOW, A. ROTHSTEIN and T. W. CLARKSON, Pharmac. Rev. *13*, 185 (1961).
[7] R. C. KEEFER, A. J. BARAK and J. D. BOYETT, Biochim. biophys. Acta *221*, 390 (1970).
[8] G. W. EVANS and R. E. WIEDERANDERS, Am. J. Physiol. *213*, 183 (1967).
[9] A. SASS-KORTSAK, Adv. clin. Chem. *8*, 1 (1965).
[10] I. STERNLIEB, Gastroenterology *56*, 1038 (1967).
[11] M. WORWOOD and D. M. TAYLOR, Biochem. Med. *3*, 105 (1969).

ed 1 h before the injection (control bile) and 3 h after application of the respective metal. The control bile and the metal-containing bile were each collected from individual rats and then placed on polyacrylamide gels[12]. Each gel was cut into 12 5-mm slices which were then dissolved in 0.2 ml of 30% H_2O_2 during 12 h at 60 °C. To the dissolved sample, 6 ml of scintillation solution, 3 ml of absolute ethanal, and 0.5 ml of hyamine hydroxide were added. The samples containing ^{52}Mn, ^{64}Cu and ^{203}Hg were measured with a Tri-Carb Liquid Scintillation Spectrometer, Packard, Model 3365. The samples containing ^{210}Pb were measured γ-spectrometrically with the peak at 47 keV (scintillation crystal NaI/Tl: 5 × 4 inches).

Results and discussion. The Figure demonstrates the spectrum of proteins in the control bile (below the diagrams). The electrophoretic separation of biliary proteins was similar to that found in human bile[13]. The 4 zones, which we numbered above the electrophoreogram, correspond to the zones named by ENGLERT et al.[13] for human biliary proteins. The column diagrams (Figure) represent the percentage of the total radioactivity placed on the gel. Each column in the diagram was obtained as a mean of 6 to 7 individual determinations.

With mercury, the radioactivity existed predominantly in the prestacking gel zone containing high-molecular bile specific protein[13]. In the case of both Mn^{++} and Cu^{++}, the radioactivity appeared mostly in the region of the pigment zone. A very small amount of manganese was found in the stacking gel near the start. Although the main quantity of Cu^{++} was found at the front of the electrophoreogram, smaller parts of it were identified in the prestacking, the albumin and the postalbumin zones, too. The maximum amount of Pb^{++} was found in the postalbumin zone, decreasing toward the front as well as toward the start. A detectable amount of lead was also measured in both the prestacking gel and the pigment zones.

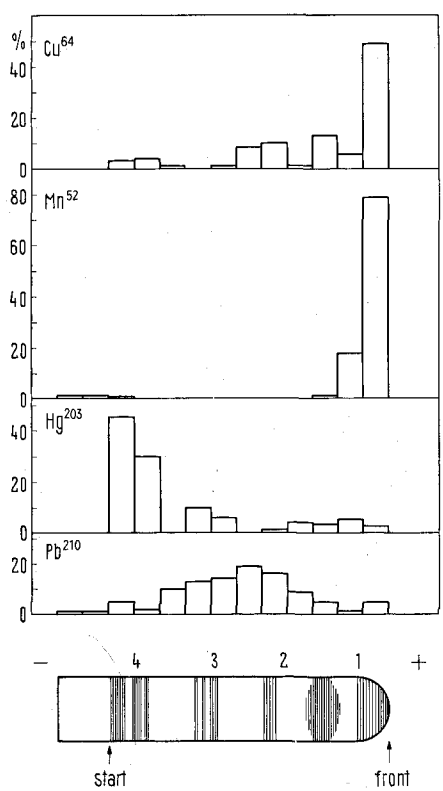

Fig. 1. Distribution of radioactivity detected on electrophoreogram of rat bile after i.v. administration of $^{52}MnCl_2$, $^{64}CuCl_2$, $^{203}HgCl_2$ and $^{210}Pb(NO_3)_2$. The values are expressed in percent of the total amount determined of studied metal radioactivity. Regions of specific bile proteins[13]: 1, pigment-prealbumin; 2, biliproalbumin; 3, biliprotein; 4, high molecular bile-specific protein.

We observed no important changes between the electrophoretic protein profile of the control bile and that of the metal-containing bile. After administration of $^{203}HgCl_2$, blood could be detected macroscopically in the bile in several cases. Changes in the central part of the electrophoreogram that could be observed in these cases cannot therefore be discussed, since these changes may be caused by the presence of plasma proteins[13].

It is evident that all four metals are bound in various extents in the prestacking gel zone, Hg^{++} possesses the greatest affinity. PASSOW et al.[6] stated that mercury was mainly bound to SH groups with favourable spheric position. Thus, it may be assumed that biliary proteins of such a type appear mostly in the prestacking gel fraction under our experimental conditions.

The cations of Cu^{++} and Mn^{++} are bound to several types of substances. Although Mn^{++} is known to be bound to proteins[14] or built into porphyrin rings[15], it is necessary to consider its binding to bile pigments and cholic acids. This possibility is in agreement with the correlation shown[16] between the amount of bilirubin in the bile and the amount of applied Mn^{++}. Cu^{++} cations are predominantly bound to proteins of small molecular weight (prealbumin zone), or possibly form complexes with bile pigments, porphyrins or bile acids as well. The binding of Cu^{++} to proteins of higher molecular weight occurs to a small extent. In this connection OWEN's[17] finding is rather interesting. He ascertained a slow ^{64}Cu incorporation into ceruloplasmin. This could be the reason for our observing low radioactivity of $^{64}Cu^{++}$ in the corresponding zones.

The more rapid excretion of Mn^{++} and Cu^{++} into the bile in comparison with that of Pb^{++} and Hg^{++} could be explained by the formation of relatively small complexes of Cu^{++} and Mn^{++}. These may penetrate through membranes more rapidly than complexes of Hg^{++} and Pb^{++} which are predominatnly bound to proteins of greater molecular weight. The distribution of the cations among the individual electrophoretic fractions of the bile is probably related to different metabolic pathways of the metals as well as to stability constants of the complexes.

Résumé. Après avoir administré i.v. à des rats les solutions des sels Pb^{++}, Hg^{++}, Mn^{++} et Cu^{++}, on a divisé leur bile par des l'élécrophorèse de disque sur gel de polyacrylamide. Les cations Hg^{++} se sont montrés surtout dans la fraction contenant des hauts polymères d'albumine spécifique pour la bile; les cations Mn^{++} et Cu^{++} furent observés surtout dans la zone pigmentée .Le maximum des cations Pb^{++} attachés s'est trouvé dans la zone de postalbumine.

M. CIKRT and M. TICHÝ

Institute of Hygiene and Epidemiology,
Department of Industrial Hygiene and Occupational Diseases,
Šrobárova 48, Praha 10 (Czechoslovakia),
19 May 1971.

[12] B. J. DAVIS, Ann. N. Y. Acad. Sci. *121*, 404 (1964).
[13] E. ENGLERT JR., E. E. WALES JR. and R. C. STRIGHT, Clin. chim. Acta *29*, 319 (1970).
[14] A. J. BERTINCHAMPS, S. T. MILLER and G. C. COTZIAS, Am. J. Physiol. *211*, 217 (1966).
[15] D. C. BORG and G. C. COTZIAS, Fedn. Proc. *18*, 470 (1959).
[16] L. S. MAYNARD and S. FINK, J. clin. Invest. *35*, 831 (1956).
[17] C. A. OWEN JR., Am. J. Physiol. *207*, 1203 (1964).