of cardiac beating the conduction time is more and more prolonged as the recovery period shortens.

The results are in general confirmatory of what is known about the cardiac effects of strophanthin and quinidine, with the possible exception of the Q-T interval modifications by quinidine. Both drugs, especially quinidine, prolong atrio-ventricular, as well as intraventricular conduction, more so at rapid rates of cardiac beating. These are presumably direct muscular effects in our experiments, unless the amputated nerve endings are also affected (?). The recovery curves are markedly depressed downward and to the right, indicating prolongation of the relative and absolute refractoriness of the atrio-ventricular as well as the ventricular musculature. The slower the recovery of conductivity, the longer the period of relative refractoriness, the greater the incidence of partial A-V and Wenckebach types of block. This is our theoretical prediction[11] as well as the actual occurrence in our data.[12] Both strophanthin and quinidine, thus, predispose to Wenckebach (and Mobitz) types of A-V block, though they are found also frequently enough in our control curves at rapid cardiac rates. These findings substantiate the known clinical effects of the two drugs.

Rapid rates of stimulation of the auricles, up to 300 per minute, usually failed to elicit paroxysmal ventricular tachycardia or fibrillation, seen in the faster stimulated ventricular preparations,[1] typically after marked QRS aberration. Ventricular ectopic beats, with bigeminy, occurred not infrequently, but runs of them were rare, nor were they apparently influenced by strophanthin or quinidine in these, as in the ventricular,[1] preparations of rabbit hearts.

Summary. Recovery curves of conductivity of the A-V node and ventricles for the isolated rabbit heart driven at various rates followed the usual logarithmic pattern. Strophanthin, and especially quinidine, caused delayed recovery and more prolonged refractoriness of both regions of the heart. Strophanthin prolonged the Q-T interval in small doses and usually shortened it in larger doses. Quinidine effects upon the Q-T interval were unexpectedly variable, contrary to the usual prolongation in thyrotron driven ventricles and in clinical cases.

[11] Decherd, G. M., and Ruskin, A., *Brit. Heart J.*, 1946, **8**, 6.

[12] Ruskin, A., and Decherd, G. M., unpublished data.

16518

Hemochromogens and Related Compounds in Liquid Ammonia.*

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A study of the reactions and reaction products of hemin and coordinating substances in liquid ammonia by means of spectroscopy and bio-assay has not been made previously. However, the senior author[1-5] has made various studies on the reactions of ammonolyzed and ammonated proteins, protein derivatives and hemin. Some of the reactions in liquid

* The authors wish to thank Armour and Company and the Wellcome Research Laboratories for their generous gifts of dried blood proteins, and the Illinois Department of Public Health for the supplies of desiccated plasma and liquid globulin.

[1] Roberts, R. G., and Miller, C. O., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 821.

[2] Roberts, R. G., and Miller, C. O., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 522.

[3] Roberts, R. G., Tweedy, W. R., and Smullen, C. H., *J. Biol. Chem.*, 1935, **112**, 209.

[4] Roberts, R. G., and Miller, C. O., *J. Am. Chem. Soc.*, 1936, **58**, 309.

[5] Roberts, R. G., *J. Biol. Chem.*, 1939, **128**, 597.

ammonia or the nitrogen system, which are to be described below, will be compared to similar ones in water or the oxygen system. Franklin[6] first demonstrated the value of comparing certain substances in regard to their classifications in the nitrogen and oxygen systems. He did not work with the hemochromogens, however.

There are certain marked differences in the behavior of hemin in the two solvents, some of which should be mentioned at this point. Hemin is soluble in liquid ammonia but reacts with it to form conjugates so that the ammonia itself is a coordinating substance. This does not exclude the formation of other hemochromogens or coordinates, however, as will be shown later. Hemin is practically insoluble in water at the neutral point or pH 7.0 but is soluble in aqueous alkali at pH 8.0 to 9.0. A search of the chemical literature has failed to show that the pH of liquid ammonia has been determined experimentally. The ionization constant of liquid ammonia, however, as calculated from its electrical conductivity is 1.9×10^{-33}. As stated by Plexkov and Monoszon[7] pure liquid ammonia ionizes as follows: $NH_3 \rightarrow H^+ + NH_2^-$ or $2NH_3 \rightarrow NH_4^+ + NH_2^-$ and $(NH_4^+)\ (NH_2^-) = 1.9 \times 10^{-33}$ at $-50°C$. The NH_4^+ ion is in greater abundance than the H^+ ion. Lykken[8] recommends that the term pH should be reserved for aqueous systems only, and that the term cG should be used for non-aqueous systems.

Experimental. The liquid ammonia used at the beginning of this work was dried over sodium by the method of Fernelius and Johnson.[9] It was found later, however, that for bio-assay or spectroscopy the conjugates or coordinates which were prepared in the anhydrous liquid ammonia made by Mathieson or du Pont could not be distinguished from the coordinates prepared in liquid ammonia which had been dried over sodium. The laborious and time consuming drying operation was, therefore, discontinued. The addition of the reactants to the liquid ammonia and the isolation of the reaction products have been described in previous publications.

A Gaertner high dispersion spectroscope was used for the spectroscopic studies of those compounds and coordinates that remained dissolved or dispersed in liquid ammonia. The liquid ammonia and solutes were contained in a small Dewar flask that was mounted directly in front of the entrance slit of the spectroscope. The vacuum flask was connected to a mercury seal by rubber tubing to permit the ammonia gas to escape and the entire apparatus was placed in a well ventilated hood. Spectrophotometric determinations were made with a Beckman quartz spectrophotometer upon ammonolyzed and/or ammonated compounds or conjugates that had been dissolved or dispersed in water, ethylene glycol, or other media with the exception of liquid ammonia itself. Franklin used the term ammono to designate ammonolyzed compounds, which in some cases were also ammonated. The term ammano as used in this paper is a more general one and means ammonia treated. Such a term is necessary especially when proteins are being considered since one often does not know the exact nitrogen distribution.

Discussion. When liquid ammonia reacts with an iron porphyrin, it is acting as a coordinating substance in the same general way that nitrogen bases act as coordinating substances to produce hemochromogens. Several of these coordinating substances (No. 1 to 16), among which is galactose, are shown in Table I. That ammano-iron porphyrin No. 1, red in liquid ammonia and absorbing at 555 mμ, might be a complex formed by ammonolysis and ammonation when hemin chloride is treated with an excess of liquid ammonia, is indicated by Kjeldahl analysis. Using 610.95 g as the gram molecular weight of hemin chloride, we found that there is an increase of 19.39 g of nitrogen per g.m.w. after the sample has remained for 24 hours over sulfuric acid in a vacuum desiccator at 22-24°C, but only 13.6 g under vacuo at 90-

[6] Franklin, *The Nitrogen System of Compounds*, Reinhold Pub. Corp., 1935.

[7] Plexkov and Monoszon, *Acta Physicochim. U.S.S.R.*, 1935, **1**, 725.

[8] Lykken, L., Symposium on pH Measurement, Am. Soc. Test. Mat., 1946, Tech. Pub. No. 73.

[9] Fernelius, W. C., and Johnson, W. C., *J. Chem. Ed.*, 1929, **6**, 444.

TABLE I.
Hemochromogens Formed in Liquid Ammonia and Dispersed in Various Solvents.

No.	Coordinating Substance	Solvent	Absorption areas and curves Wave length, $m\mu$	Reaction products and related data.
1.	Liquid NH_3	Liquid NH_3	Band 555	Brownish-black powder soluble in H_2O at pH 5.5, and in ethylene glycol.
2.	Liquid NH_3	Water	Broad shallow curve. Center 623.	Similar to hematin albumin absorption found in plasma of malaria patients.
3.	Pyridine	Liquid NH_3	Band at 540	Band at 555 disappears immediately on addition of pyridine.
4.	Ammano-pyridine	Water	Broad deep curve. Center at 540.	Failure of band at 540 to shift on change of solvent is unusual.
5.	Galactose	Liquid NH_3	Diffuse band 567-521. Sharp area 559.	Brownish-black powder. Forms red sol. in liquid NH_3 similar to ammano-iron porphyrin.
6.	Ammano-galactose	Water	Shallow curve. Center at 640.	On changing from liquid NH_3 to water the band narrows and shifts toward the red spectrum.
7.	Liquid NH_3	Ethylene glycol	Broad deep curve. Center at 600.	Ammano-iron porphyrin very soluble in ethylene glycol. Iron porphyrin is not soluble.
8.	Liquid NH_3	Ethylene glycol plus guanidine thiocyanate	Broad deep curve. Center at 500.	A small amt. of guanidine thiocyanate causes shift of 100 $m\mu$.
9.	Caffeine	Liquid NH_3	Bands α 577, β at 548.	Red powder turning to brown on exposure to atmosphere.
10.	Ammano-caffeine	Water	Wide curve. Center at 576.	Caffeine porphyrin absorbs at 570 and 540 in water.
11.	Ammano-histidine	Water	General curve. No specific absorption.	Absence of specific absorption indicates reaction between histidine and iron porphyrin.
12.	Ammano-nicotinic acid	Water	General curve. Identical with ammano-histidine.	Identity of curves of ammano-histidine and ammano-nicotinic acid indicates similar properties.
13.	Ammano-adrenalin	Water	Shallow curve. Center at 540.	Absence of specific absorption indicates coordination of iron porphyrin with adrenalin.
14.	Ammano-choline chloride	Ethylene glycol	Deep curve. Center at 260.	Choline chloride absorbs at 260. The difference in intensity indicates a reaction.
15.	Ammano-insulin choline chloride	Ethylene glycol	No specific absorptions. (Fig.1.)	Disappearance of both choline chloride and insulin absorption indicates a reaction.
16.	Ammano-egg albumin	Aqueous sol. of dried beef plasma	Curve centering at 532 and 565. (Fig. 2.)	Red powder absorptions lie midway between those obtained for haem albumin and serum hemochromogen.

100°C, and only 6.05 g after washing with water and drying at 101°C. The last increase is approximately one-half of a gram atom of nitrogen and might indicate the existence of an ammonia bridge between 2 iron porphyrin molecules.

Ammano-iron porphyrin No. 2 is soluble in water below pH 7.0, forming a brownish-amber solution which absorbs between 605-635 mμ. This absorption band resembles that of a cross between the absorption band of acid haematin described by Newcomer[10] and the absorption band of free alkaline haematin as given by Heilmeyer.[11] The latter also reported a band of similar shape centering at 623 mμ for a blood pigment obtained from clinical cases of "haematin jaundice." Any clinical comparison of the above mentioned bands is of academic interest only, at the present time.

The ammano-pyridine iron porphyrins No. 3 and 4, prepared according to the directions given by Drabkin and Austin,[12] absorbs at 540 mμ in either liquid ammonia or water. Ammano-galactose iron porphyrin No. 5, prepared by using equal weights of galactose and hemin, absorbs in a very broad band between 567 and 521 mμ with a sharp peak at 539 mμ. This range of absorption would include the class of substances named hemochromogens, such as serum hemochromogen which absorbs with the α band at 558 mμ and the β at 528 mμ (α represents absorption in the longer wave lengths of a series of bands and β in the shorter wave lengths, etc.). There is also the class of substances named haem-albumins by Fairley[13] and Keilin,[14] such as serum haem-albumins, α at 570 mμ and β at 540 mμ. In water, ammano-galactose iron porphyrin No. 6 absorbs at 640 mμ. Keilin states that only caffeine reacts with hemin to give products absorbing in the range of haem-albumins. Ammano-caffeine iron porphyrin No. 9, prepared according to the directions given by Keilin, absorbs at α 577 mμ and β at 548 mμ in liquid ammonia. In water ammano-caffeine iron porphyrin No. 10 gives a wide curve centering at 576 mμ.

An interesting theory in regard to the important role possibly played by histidine in the formation of cross linkages between protein molecules and other molecules as hemin in such compounds as hemoglobin and cytochromes has been proposed by Keilin.[15] For a comparison, histidine was allowed to react with hemin chloride in liquid ammonia and the reaction product, ammano-histidine iron porphyrin No. 11, prepared according to the directions of Keilin, was dissolved in water and studied spectrophotometrically. The absence of any specific absorption indicates that a reaction probably occurred, since the individual reactants have specific absorptions. However, nicotinic acid No. 12, prepared in a similar way to the histidine product, with proper consideration of equivalent weights, reacted in a similar way, and gave almost identical general absorption curves. The reaction of histidine in this regard is, therefore, not unique. Adrenalin No. 13, prepared in a similar manner, acts in the same way spectrophotometrically as nicotinic acid and histidine. Adrenalin conjugates give a prolongation of elevated blood pressure in dogs.

Ammano-insulin choline chloride iron porphyrin No. 15 prepared by using 20 mg of insulin, 20 mg of hemin, and 40 mg of choline chloride, is soluble in ethylene glycol but gives no specific absorption (Fig. 1), although ammano-choline chloride iron porphyrin No. 14, absorbs strongly at 250 mμ and insulin, either crystalline or amorphous, absorbs strongly at 275 mμ as has been shown by Roffo.[16] When ammano-insulin choline chloride iron porphyrin dispersed in ethylene glycol is injected subcutaneously into rabbits it gives a prolongation of lowered blood glucose lasting 5 times as long as that of insulin controls.

[10] Newcomer, H. S., *J. Biol. Chem.*, 1919, **37**, 465.

[11] Heilmeyer, L., *Deutsch. Arch. Klin. Med.*, 1932, **173**, 128.

[12] Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, 1935-36, **112**, 51.

[13] Fairley, N. H., *Brit. J. Exp. Path.*, 1940, **21**, 231.

[14] Keilin, J., *Nature*, 1944, **154**, 120.

[15] Keilin, J., *Biochem. J.*, 1943, **37**, 281.

[16] Roffo, A. N., and Francone, M. P., *Boletin Instituto de Medicina Experimental*, 1941, **18**, 1003.

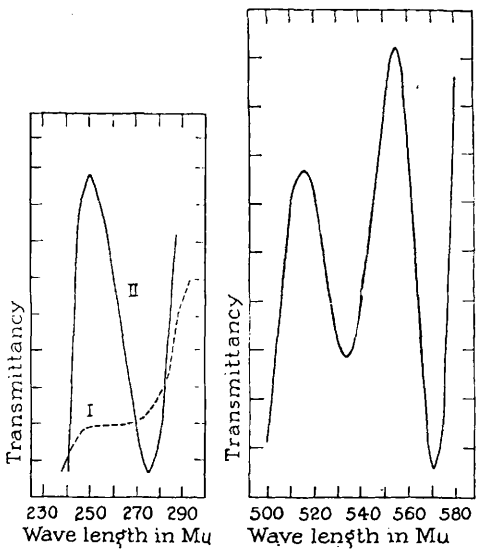

FIG. 1. (left)

Curve I, the absorption curve of Ammano-insulin choline chloride iron porphyrin. Curve II, the absorption curve of plain insulin. The thickness of the absorbing liquid was 1.0 cm in both cases. The concentration of the coordinated iron porphyrin as shown in curve I was 0.2 g in 100 cc in terms of the insulin content. The concentration of plain insulin as shown in curve II was 0.2 g in 100 cc and the transmittancy was plotted between zero and 100% for both curves.

The smoothed curves were readings on the Beckman spectrophotometer, at 5 mμ intervals, and at inflection points at even less than 5 mμ. If all these points were plotted, they would almost touch each other.

FIG. 2. (right)

The absorption curve of Ammano-egg albumin iron porphyrin. The thickness of the absorbing liquid was 1.0 cm. The concentration of the coordinated iron porphyrin was 0.1 g in 100 cc and the transmittancy was plotted between zero and 100%.

Both Fairley and Keilin state that haem-albumin can be obtained only when human serum is used to conjugate with the haem. However, ammano-egg albumin iron porphyrin absorbs at α 565 mμ and β at 532 mμ (Fig. 2). Since these wave lengths are somewhat short for haem-albumins and somewhat long for hemochromogens, the ammano-conjugate or coordinate might be considered as an ortho haem-albumin or as a meso hemochromogen, and in all likelihood represents a new class of porphyrins—the ammano-iron porphyrins.

Several other proteins form red reaction products with hemin in liquid ammonia and they probably belong in the same class of ammano derivatives as ammano-egg albumin iron porphyrin. As a rule it is difficult to get ammano protein iron porphyrins into solution and they usually lack characteristic specific absorptions. Among them are bovine beta globulin, bovine gamma globulin, castor bean globulin, bovine fibrinogen, fibrin foam, bovine albumin and human albumin. The red reaction products form as readily with the globulins and fibrins as with the albumins. Globin also forms a red reaction product and when globin and hemin are mixed in the same proportion in which they occur in hemoglobin, an α band occurs at 580 mμ (α hemoglobin 578 mμ) a β band at 528 mμ (β hemoglobin 540 mμ) and a gamma band at 465 mμ which is not found at all in hemoglobin.

Summary. 1. A study of the reactions and reaction products of hemin and coordinating substances and certain compounds and conjugates related to them has been made in liquid ammonia.

2. The nomenclature used to describe new compounds and conjugates that are formed in liquid ammonia has been adopted primarily from the nomenclature as used by Keilin for derivatives of hemin and from the nomenclature as used by Franklin in his studies of the nitrogen system of compounds.

3. A new derivative of hemin and liquid ammonia has been described, which exists only in liquid ammonia and which absorbs strongly at 555 mμ.

4. A compound has been prepared by dispersing liquid ammonia treated hemin in water. This compound absorbs at 620 mμ, which is the same spectral region as that found for some hemin compounds occurring in the blood of patients suffering from black water fever. The ammano-hemin is soluble in water at pH 5.5 and it is also soluble in ethylene glycol.

5. A new preparation has been made by combining egg albumin with hemin in liquid ammonia. This conjugate absorbs in two bands at 532 mμ and 565 mμ. These bands lie midway between those obtained by other workers for haem-albumin and serum hemochromogen. Fairley and Keilin were able to

prepare haem-albumin only when serum albumin was used.

6. Caffeine hemin can be prepared in liquid ammonia. It absorbs at 577 mμ and 548 mμ when it is dispersed in liquid ammonia.

7. Insulin reacts with choline chloride and hemin in liquid ammonia to form a conjugate in which the strong absorption band of insulin at 275 mμ is missing, yet this conjugate maintains a prolongation of lowered blood glucose of 5 times the duration obtained by insulin controls.

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Determination of Para-Aminosalicylic Acid in Blood.*

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Since para-aminosalicylic acid has been found to have a definite chemotherapeutic effect in experimental tuberculosis, a simple method for its determination in blood appears desirable. The sulfonamide method in general use[1] cannot be employed without modification. In view of several requests for a method for determination of this substance, and since the slight modification of the sulfonamide method found necessary may prove useful with other aryl amines, we are publishing this short note.

No color is obtained when a solution of p-aminosalicylic acid is subjected to the ordinary procedure for determining sulfonamides. If the acidity of the solution is increased a color is obtained. Maximum color is developed at room temperature when the solution is made about 3 N with hydrochloric acid. The results are, however, not consistent. Diazotization for one minute gives a more intense color than for 3 or 10 minutes. Obviously, either the diazo compound is unstable or a secondary reaction is occurring with the nitrous acid. Diazotization done with the solution at 1°C and made 1 N with hydrochloric acid yielded a more intense color than any other procedure which we have tried. The results under these conditions appear to be quite consistent and reproducible.

Reagents. 1. A solution of trichloroacetic acid containing 15 g dissolved in water and diluted to 100 cc.

2. A 0.1% solution of sodium nitrite.

3. An aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride containing 100 mg per 100 cc. This solution should be kept in a dark colored bottle.

4. 6 N hydrochloric acid.

5. A solution of ammonium sulfamate, containing 0.5 g per 100 cc.

6. A standard solution of p-aminosalicylic acid prepared by suspending 50 mg of the compound in water and dissolving in slightly more than the theoretical amount of sodium hydroxide, and diluting to one liter. Dilutions are made from this stock solution in order to prepare a calibration curve for the colorimeter.

Procedure. Blood or plasma is diluted 1:50 or, if very low values are expected, 1:20. For 1:50 dilution, use 1 part of blood or plasma, 39 parts of water and 10 parts of trichloroacetic acid solution. To 10 cc of the clear filtrate are added 2 cc of 6 N hydrochloric acid, and then the solution is cooled in ice (to about 1°C). One cc of the solution of sodium nitrite is added and after 3 minutes, 1 cc of the solution of sulfamate. The tubes are removed from the ice bath, and after 2 minutes, 1 cc of the solution of N-(1-naphthyl)ethylenediamine dihydrochloride is added. Read-

* This investigation has been aided by a grant from the U. S. Public Health Service.

[1] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.