From the above experiments it is seen that a non-polar liquid substance promotes the reaction between hydrogen bromide and propene; polar liquids like water, alcohol or glacial acetic acid, do not promote the addition of hydrogen bromide to propene.

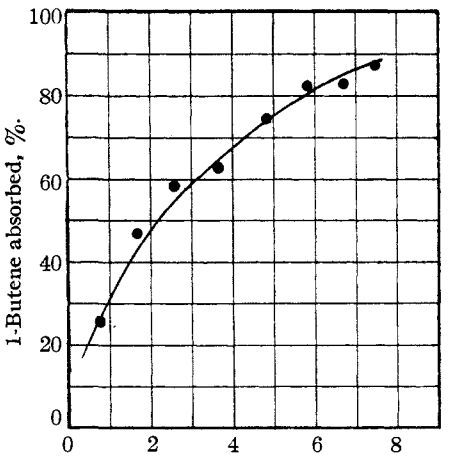

Fig. 1.—1-Butene.

The solubility of propene in the mentioned solvents was investigated. At 4° the solubility of propene in hexane, cyclohexane, benzene and tertiary butyl bromide is approximately the same. In alcohol it is 60% and in 47% glacial acetic acid solution of hydrogen bromide is about 20% of what it is in hexane.

The speed of reaction of hydrogen bromide with propene depends probably on two factors: (1) nature of the solvent, and (2) solubility of the propene in the solvent.

Although propene is soluble to some extent in alcohol and in glacial acetic acid saturated with hydrogen bromide, the reaction between hydrogen bromide and propene under our experimental conditions does not take place.

Propene is soluble to the same extent in tertiary butyl bromide and benzene as in hexane, in spite of the fact that the speed of reaction of propene with hydrogen bromide in the presence of tertiary butyl bromide and benzene is much smaller than in the presence of hexane.

Summary

Organic compounds as hexane, cyclohexane and benzene, and organic bromides promote the reaction between propene and hydrogen bromide.

Polar compounds as water, alcohol or acetic acid have no promoting effect in this reaction.

The speed of reaction between hydrogen bromide and propene depends upon the nature of the solvent used as a reaction medium.

The reaction is autocatalytic since the bromides formed are themselves catalysts for this reaction.

RIVERSIDE, ILLINOIS RECEIVED MAY 4, 1934

[CONTRIBUTION FROM THE C. F. KETTERING FOUNDATION FOR THE STUDY OF CHLOROPHYLL AND PHOTOSYNTHESIS]

Occurrence of Decomposition Products of Chlorophyll. II. Decomposition Products of Chlorophyll in the Stomach Walls of Herbivorous Animals[1]

BY PAUL ROTHEMUND, ROBERT R. MCNARY AND O. L. INMAN

The occurrence of decomposition products of chlorophyll in the digestive system of herbivorous animals (cattle and sheep) was reported in the first paper of this series;[1a] several porphyrins (phylloerythrin, rhodo-, phyllo- and pyrroporphyrin) have been found in the stomach contents of cows, phylloerythrin and probophorbide in the stomach contents of sheep under normal physiological conditions. In the meantime we ascertained the presence of phaeopurpurin 18 and of phaeophytin in cows' stomach contents by extending the hydrochloric acid fractionation to 18, 30 and 35% HCl.[2]

We have now studied the occurrence and the fate of these substances in the organism in more detail and have tried to find whether they go through the digestive system or if and where absorption takes place. Following the same procedure as for the extraction of the stomach contents, we demonstrated that the chlorophyll decomposition products mentioned above can be

(1) This article is based on a thesis submitted by Robert R. McNary in partial fulfilment of the requirements for the degree of Master of Science, Antioch College, 1933.

(1a) Rothemund and Inman, THIS JOURNAL, **54**, 4702 (1932).

(2) The formation of phaeophytin from chlorophyll under the influence of the gastric juice of dogs has been reported by Kortschagin, *Biochem. Z.*, **153**, 510 (1924).

found throughout the whole digestive tract. As to the absorption problem, it is in general agreed that the absorption of material in the stomach of mammals is relatively small, only very little water and salts, certain sugars, dextrins, proteoses and peptones being absorbed in small quantities. In certain cases of soluble or liquid substances (drugs, alcohol) the absorption in the stomach may, however, reach rather high values.

If porphyrins were absorbed from the stomach contents, they should be obtainable upon extraction of the inner lining of the stomach. The literature does not describe this occurrence, although the presence of porphyrins in different organs of the animal body under normal conditions is a well-known fact.

In our experiments the inner lining (mucous membrane) of the third and fourth stomachs (omasum and abomasum) of cows and sheep was extracted with acetone, the extract transferred into ether and subjected to fractionation with hydrochloric acid of suitable concentrations. The lower fractions contained protoporphyrin and deuteroporphyrin. Coproporphyrin, a normal product of metabolism,[3] was absent in the stomach of cows; small quantities of it were found in the lower intestinal tract and in the feces of cows. In one case hematoporphyrin was found deposited in the stomach wall around a lesion caused by a piece of iron wire swallowed with the food. The 4% HCl fraction in ether contained phylloerythrin and two other pigments; the spectrum of one of these pigments resembled the rhodoporphyrin spectrum, but was not identical with it. Phylloerythrin was found in the relatively largest amount. The higher hydrochloric acid fractions yielded a series of phaeophorbide and phaeophytin fractions of strongly phase-positive character. Pure phaeophorbide *a* and phaeopurpurin 18[4] were isolated. It may be mentioned that Marchlewski and Urbańczyk[5] as well as Fischer and Hendschel[6] found the same purpurin in the excreta of the silk worm.

The formation of phylloerythrin and other porphyrins from chlorophyll would suggest rhodoporphyrin-γ-carboxylic acid as a possible intermediate product. The presence of this acid could be demonstrated neither in the stomach contents nor in the stomach walls. The occurrence of protoporphyrin and deuteroporphyrin in the stomach walls can be explained by assuming decomposition of the blood pigment under conditions which are similar to those studied by Fischer and Lindner[7] (autolysis of meat or putrefaction of blood over a period of months).

Feeding experiments on normal rats, on rats with nutritional anemia, on guinea pigs and on rabbits were undertaken to obtain information on the biological significance of absorbed chlorophyll decomposition products. The chlorophyll derivatives found in the stomach walls had a definite erythropoietic effect when *small* doses (0.1–2.0 mg. per rat and per day for example) were administered per os. Individual differences in the behavior and reaction of the experimental animals, especially with respect to their weight curves, were noticed which we cannot account for as yet. Further experiments are in progress to study this stimulating effect of chlorophyll decomposition products on the formation of red blood corpuscles and on the increase of the hemoglobin percentage.

Experimental

We used the inner lining of the third and fourth stomach of cows. The stomachs were obtained from the abattoir shortly after the animals had been killed, taken to the laboratory and worked up immediately. The material was thoroughly washed in running water and freed from food particles by brushing with a soft brush, washed again with distilled water and finally wiped dry with a towel. In the first experiments the layers of muscle were stripped from the lining of the stomachs and the lining was then ground up in a meat chopper. This method, however, yielded a relatively large amount of pigments which are derivatives of the red blood pigment, because the method of separation of the mucous membrane from the muscular coats is by no means a quantitative one. We therefore scraped the lining from the muscular layers by means of a metal scraper. The paste thus obtained was steeped in acetone and kept in the electric refrigerator for several days at a temperature of +4°. The acetone liquor was then filtered off and the extracted pigments were transferred into ether by the addition of ether and then of distilled water. For every kg. of stomach lining 1.5 liters of acetone and about 1.5 liters of ether was used. The acetone–water layer was separated and extracted several times with small quantities of ether; these extracts were then combined with the original ether extract. The resulting ether solution was of orange-red color and showed a series of absorption bands. It was washed with distilled water until the washings were neutral to litmus and to congo paper and then subjected to hydrochloric acid fractionation. The following hydrochloric acid concentrations were used for the first fractionation: 0.35, 1.5, 4, 8,

(3) Fischer and Zerweck, *Z. physiol. Chem.*, **132**, 12 (1924).
(4) Conant and Moyer, THIS JOURNAL, **52**, 3013 (1930).
(5) Marchlewski and Urbańczyk, *Biochem. Z.*, **263**, 166 (1933).
(6) Fischer and Hendschel, *Z. physiol. Chem.*, **222**, 250 (1930).
(7) Fischer and Lindner, *Z. physiol. Chem.*, **145**, 203 (1925), and **161**, 18 (1926).

12, 20, 22, 25, 30, 33 and 38%. Each fraction was transferred into fresh ether by means of sodium acetate in the case of the lower concentrations and by partial neutralization with 10% sodium hydroxide and then addition of sodium acetate for the higher concentrations.

The 0.35% hydrochloric acid fraction in ether showed only a very faint spectrum with shadows at 610–600 and 535–520 mμ, 0.1% hydrochloric acid did not extract any porphyrin from this ether. From the 1.5% hydrochloric acid fraction two porphyrins, deuteroporphyrin and protoporphyrin, were isolated. The separation was performed by transferring from hydrochloric acid into ether with acetate, washing with distilled water until the washings were free from acetic acid and refractionation with 1% hydrochloric acid. Deuteroporphyrin entered the 1% hydrochloric acid, while protoporphyrin remained in the ether layer. When the deuteroporphyrin fraction was driven back into ether, it occasionally exhibited the protoporphyrin band at 632.5 mμ; in these cases the fractionation with 1% hydrochloric acid was repeated. The two separated porphyrins were identified by direct comparison of the spectra of their ether solutions with the solutions of material prepared from haemin; esterification of the porphyrins yielded the esters, identified by spectroscopic properties, hydrochloric acid numbers, melting points, and melting points of the mixtures with pure deuteroporphyrin and protoporphyrin dimethyl ester, respectively.

Upon standing the crude 4% hydrochloric acid fraction in ether deposited on the walls of the container a small amount of needle-shaped crystals, which were identified as phylloerythrin. From the remaining ether solution 4% hydrochloric acid removed a porphyrin, which showed the following spectrum in pyridine–ether: shadow 644.0; I. 638.0–(634.0)–630.0: shadow 624.0; II. 591.0–(587.0)–584.5 . . . III. 581.0–(577.0)–574.0; IV. 558.0–(554.5)–551.0 . . . V. 548.0–(544.5)–541.0; VI. 517.0–(503.0)–490.5; E. A. 447. Intensity: III, II, VI, V, IV, I. The rest of the ether solution, after the extraction with 4% hydrochloric acid, had this spectrum: shadow 605.5 . . . I. 590.5–(587.5)–585.0 . . . 575.0; II. 564.0–(560.0)–556.0 . . . 551.5 . . . III. 547.5–(543.0)–538.5; IV. 532.0–(524.5)–516.5; shadow 517.5; E. A. 460. Intensity: I, II, IV, III.

Due to the small quantities obtained, these two pigments were characterized by their spectroscopic properties only; crystallized material could not be prepared.

The 8% and the 12% fractions contained phylloerythrin as shown by spectra and hydrochloric acid number. Crystallized phylloerythrin was prepared from pyridine–ether. Esterification of this material yielded the well crystallized methyl ester of m. p. 264°. The mixture of this ester with phylloerythrin methyl ester from beef bile phylloerythrin did not give any depression of the m. p.

The 20% hydrochloric acid fraction exhibited positive phase test in ether solution and showed a spectrum in which the phaeophorbide spectrum was superimposed upon the phaeopurpurin 18 spectrum. The ether solution was fractionated with 15% and with 18% hydrochloric acid, each of these fractions was separately brought back into fresh ether in the usual manner and the resulting ether solutions were refractionated with 15% and with 18% hydrochloric acid, respectively. From the finally obtained 15% fraction, phaeophorbide *a* was isolated in the form of rhomb-shaped platelets, while the 18% fraction yielded phaeopurpurin in crystallized form. Phaeophorbide *a* and phaeopurpurin 18 were esterified and the free compounds as well as the esters were identified spectroscopically by direct comparison of their ether solutions with ether solutions of the corresponding material prepared from pure chlorophyll *a*. The melting point of the phaeopurpurin 18 monomethyl ester was 280° with sintering preceding the melting; the mixture of this ester with ester prepared according to Conant[8] (m. p. 273°) had a melting point of 274–275° and the same melting point was found for the mixture with ester prepared according to Stern and Klebs[9] (m. p. 272°) by extracting Conant's ester several times with acetone from a thimble. For the identification of the phaeophorbide *a* Fischer's method[10] of decomposing it to form chlorin *e* trimethyl ester was used.

In two experiments the 20% hydrochloric acid fraction was transferred into ether, the ether evaporated and the residue esterified. The ether solution which contained the methyl esters of phaeophorbide *a* and of phaeopurpurin 18 was subjected to fractionation with 15 and 18% hydrochloric acid. Esterification preceding the fractionation did not improve the separation of the two pigments.

The remainder of the ether after the 20% hydrochloric acid fractionation was still of dark green color, but evaporation to dryness showed that the amount of coloring matter in it was very small. Spectroscopically phaeophytin was predominant in this ether. The spectra of the 25, 30, 33 and 38% hydrochloric acid fractions in ether and the ether spectrum of the residue in the ether after the 38% hydrochloric acid extraction resembled each other very closely. The spectrum of the 22% hydrochloric acid extract in ether, however, was different from the spectra of the other fractions between 20 and 38% hydrochloric acid. It had the following absorption bands: I. 681.0–(674.0)–667.0; II. 665.5–(659.0)–653.5; III. 610.0 . . . 595.0–(590.5)–587.0 . . . 584.0; shadows at 573.5, 568.5, 561.5; E. A. 488. Intensity: I, II, III.

The attempts to obtain crystallized material from the ether solutions of the higher fractions failed due to the small amounts of pigments present in these fractions.

The yields in one experiment may be given here (acetone–ether method): 4 mg. of protoporphyrin, 4 mg. of deuteroporphyrin, 7 mg. of phaeopurpurin 18, 11 mg. of phaeophorbide *a*, and 15 mg. of phylloerythrin, were isolated from 15 kg. of inner lining of stomachs 3 and 4 of cows.

Summary

1. A series of pigments has been isolated from the gastric mucosa of cows under normal physiological conditions.

2. The chlorophyll decomposition products among these pigments are phaeophytin *a*, phaeopurpurin 18 and phylloerythrin.

3. The presence of protoporphyrin and of deuteroporphyrin is explained as due to the decomposition of the red blood pigment.

(8) Conant and Moyer, THIS JOURNAL, **52**, 3013 (1930).
(9) Stern and Klebs, *Ann.*, **505**, 306 (1933).
(10) Fischer, Gottschaldt and Klebs, *ibid.*, **498**, 194 (1932).

4. The chlorophyll derivatives found in the stomach walls have a definite erythropoietic effect when administered by mouth to certain animals.

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RECEIVED JUNE 22, 1934

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CINCINNATI]

The Action of Nitrous Acid on Phenyl-beta-naphtholaminomethane. II

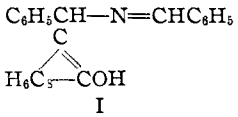
BY NZEER AHMED AND MARTHA G. HEMPHILL WITH FRANCIS EARL RAY

In a recent paper[1] it was reported that the action of nitrous acid on phenyl-β-naphtholaminomethane produced an aliphatic diazo compound. Further work has shown that the reaction is much more complicated than was at first supposed[2] and we now find that the supposed diazo compound was, in fact, a mixture of two compounds.

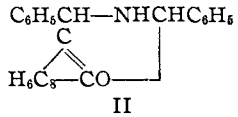
The material obtained by treatment of phenyl-β-naphtholaminomethane with nitrous acid turned red on the addition of alkali. When, however, this material was carefully recrystallized from a mixture of acetone and alcohol at room temperature a *neutral* compound, unaffected by alkali, was obtained which melted at 163°. The optical activity was due entirely to this substance.

This compound, melting at 163°, gave Liebermann's test for the nitroso group. On hydrolysis with hydrochloric acid benzaldehyde, which was identified by means of its phenylhydrazone, and the hydrochloride of phenyl-β-naphtholaminomethane were obtained.

There have been two formulas assigned to the condensation product (which melts at 150°) resulting from the reaction of benzaldehyde, β-naphthol and ammonia. One is a Schiff base or benzylidine, I, and the other is an iso-oxazine structure, II.



I



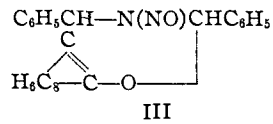
II

If formula I is correct the compound has a free naphtholic group and should methylate on treatment with alkali and methyl sulfate. This methoxy compound has been prepared by an entirely different synthesis by Ray and Moomaw[3] and melts at 98°. Even the most drastic treatment with methyl sulfate failed to convert the condensation product into this known Schiff base.

On the other hand, the condensation product (melting at 150°) reacted with acetic anhydride to give a compound melting at 170°. This compound on hydrolysis gave an acetyl derivative of phenyl-β-naphtholaminomethane. On methylating this the N-acetylphenyl-α-(β-methoxynaphthyl)-aminomethane previously prepared by Ray and Moomaw[3] was obtained. This showed that the compound melting at 170° was the N-acetyl derivative of II.

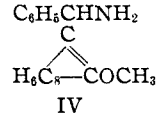
Formula II represents a secondary amine and should form an acetyl derivative but no methoxy compound. A secondary amine of this type should also form an N-nitroso derivative.

To test this hypothesis, the condensation product was treated with nitrous acid and a high yield of the pure compound melting at 163° was obtained with no trace of the salt-forming material. This synthesis, together with the decomposition products, and the absence of a free naphtholic group, establishes the constitution of this compound as the N-nitroso derivative of 1,3-diphenyl-4,2-β-naphtho-iso-oxazine, III. It also confirms II as the formula for the condensation product. The formula given in "Organic Syntheses"[4] is, therefore, incorrect.

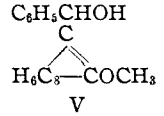


III

As was shown in a previous paper the replacement of the naphtholic group by the methoxy stabilizes the amine, IV. When, however, this methoxy amine was treated with nitrous acid the alcohol only was obtained, V.[3]



IV



V

(1) F. E. Ray, THIS JOURNAL, **54**, 295 (1932).
(2) F. E. Ray, *ibid.*, **54**, 4753 (1932).
(3) Ray and Moomaw, *ibid.*, **55**, 3837 (1933).
(4) "Organic Syntheses," Coll. Vol. I, 1932, p. 372; *cf.* Littman and Brode, THIS JOURNAL, **52**, 1655 (1930).