may be explained in terms of solvent properties. In all cases the slope in alcoholic solution is greater than in water, and the slope for the higher valence types except mercuric chloride is greater than for the simpler type. The values at infinite dilution in methanol are considerably less than in water. This general result has been justified by Vosburgh, Connell, and Butler[6] in their work on electrostriction. There is also a greater individual variation in this value among the salts in methanol than in water. For the four salts KI–KCNS–NH_4NO_3–$Ca(NO_3)_2$ in aqueous solution ϕ_v extrapolated is, respectively, 45.2–48.0–47.5–43.3, while in methanol for the same series ϕ_v is 21.9–28.2–32.6–21.0. Other differences are not interpreted so easily. The behavior of mercuric chloride is anomalous, the slope being practically zero.

Calcium nitrate in methanol shows considerably less curvature than in water. Disregarding the last point in dilute solution the curve might be drawn as a straight line. In methanol the curve for ammonium nitrate is straight within the limit of error. Gucker[3b] has shown likewise that the curve is almost linear in water solutions. The curve for potassium iodide from the results of Jones and Fornwalt shows good agreement with that of Vosburgh, Connell, and Butler. Potassium thiocyanate gives a straight line in both solvents. In addition it might be mentioned by way of completeness that the data of Jones and Fornwalt for ammonium chloride likewise yield a linear curve, although it is not shown in the figure.

Summary

1. The densities of methanol solutions of potassium thiocyanate, ammonium nitrate, calcium nitrate, and mercuric chloride have been determined for several concentrations of each salt at 25°.

2. Apparent molal volumes have been calculated for these and other salts in methanol, and the results presented graphically.

3. The curves are discussed and compared with those for the same salts in aqueous solution.

CORVALLIS, OREGON RECEIVED JUNE 14, 1937

[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES OF E. R. SQUIBB & SONS]

The Preparation of Pure *d*-Riboflavin from Natural Sources

BY R. D. GREENE AND A. BLACK

The preparation of *d*-riboflavin from natural sources has been described by a number of investigators[1–9] and more recently Karrer[10] and Kuhn[11] have reported the synthesis. Considerable differences in the biological activities of the preparations from different laboratories have suggested that probably there were variations in the degree of purity of the natural product. It has been our experience, which has been gained from work extending over three and a half years, that it is not a simple matter to make pure riboflavin. In our early work in 1935, we prepared a lot of material by following the methods of Kuhn[2] and Karrer,[3] which consisted of adsorption on fuller's earth, elution with pyridine–alcohol–water solutions, repeated readsorptions on lead sulfide and elutions with boiling water followed by precipitations. The resulting crystals appeared to be pure and more recent tests have confirmed this. Although the yields were fair, the method was rather complicated and tedious. Consequently we have endeavored to improve it and have developed a method which is based upon adsorptions on fuller's earth and the use of immiscible solvents.

Adsorption of Riboflavin.—In the concentration of riboflavin from the crude extracts of natural products adsorption steps have been used invariably by other investigators. Of the adsorbents commonly used we have tried fuller's earth, frankonite and charcoal. Charcoal seems to have the greatest adsorptive power, but removal of the riboflavin is difficult. The behavior of fuller's earth and frankonite in adsorption and subsequent removal of riboflavin is very similar. As a first step in concentration,

(1) Ellinger and Koschara, *Ber.*, **66**, 315 (1933).
(2) Kuhn, György and Wagner-Jauregg, *ibid.*, **66**, 317, 576 (1933).
(3) Karrer and Schöpp, *Helv. Chim. Acta*, **17**, 735 (1934).
(4) Booher, *J. Biol. Chem.*, **102**, 39 (1933); **107**, 591 (1934).
(5) Lepkovsky, Popper and Evans, *ibid.*, **108**, 257; *ibid.*, **109**, Proc. 54 (1935).
(6) Itter, Orent and McCollum, *ibid.*, **108**, 579 (1935).
(7) Elvehjem and Koehn, *ibid.*, **108**, 709 (1935).
(8) Stare, *ibid.*, **111**, 567 (1935).
(9) Ansbacher, Supplee and Bender, *J. Nutrition*, **11**, 401 (1936).
(10) Karrer, Schöpp and Benz, *Helv. Chim. Acta*, **18**, 426 (1935).
(11) Kuhn, Reinemund, Weygand and Ströbele, *Ber.*, **68**, 1765 (1935).

the riboflavin in the extracts of yeast or other source materials has been adsorbed on fuller's earth at a *p*H of about 4.5. The adsorption may be performed anywhere in the *p*H range of 1.0–7.0; the more acid solutions require somewhat less fuller's earth but the composition of the adsorbate is essentially the same. Adsorbates which are prepared in this way from yeast, livers and whey are satisfactory for the preparation of pure riboflavin. In the further purification of the vitamin, re-adsorption with a much smaller quantity of fuller's earth or Lloyd's reagent is advantageous. Others have reported the preparation of pure riboflavin without such a step, but we have found the separation from impurities very tedious and incomplete without it.

Removal of Riboflavin from Adsorbates.—A number of methods for removing the riboflavin from fuller's earth adsorbates have been investigated. In addition to the commonly used pyridine,[1,2] ammonia,[2] and caustic solutions,[5] we have found that triethanolamine is also effective. Barium hydroxide, because of the insolubility of the barium salt, does not remove riboflavin.[12] A 0.1 normal solution of sodium hydroxide is a very effective eluting agent, although it probably removes more of the adsorbed impurities than some of the others. It was observed very early in this work that a cold aqueous–alcohol mixture would remove some riboflavin from fuller's earth adsorbates of yeast or beef liver extract. Later investigation showed that boiling 60% ethyl alcohol was much better, although the required volume was still rather high. We have, therefore, adopted the use of a solution of 0.1 normal sodium hydroxide in 60% ethanol, which at room temperature removes as much riboflavin and less impurity than the aqueous alkali. For the elution of re-adsorbates, however, we have found boiling 60% ethanol very effective and, due to the reduced scale, the volume is not excessive.

Distribution of Riboflavin in Immiscible Solvent–Water Systems.—The literature contains few references to the use of water immiscible solvents in the fractionation of riboflavin from crude extracts. Kuhn[2] and his collaborators reported that ovoflavin from egg white was soluble in amyl alcohol. Preliminary studies of water immiscible solvents in this Laboratory showed that riboflavin was quite soluble in a number of them. Such systems, which had been employed previously[13] by us in the concentration of vitamin B_1, have been of value in the purification of riboflavin and may have further applications in the fractionation of the "B complex." This is under study at the present time and will be the subject of a later publication.

Table I shows the results of a study of the distribution of *pure* riboflavin in systems of solvents at equilibrium with neutral aqueous or saturated sodium chloride solution.

The high coefficients of phenol and the cresols, as in the case of vitamin B_1,[13] are very outstanding. Quinoline, pyridine, aniline and benzyl alcohol are also very good. It should be pointed out that the values of these coefficients may differ for crude solutions of riboflavin, due to the effect of certain impurities on the solubility. We have found that urea, which goes almost completely into the water phase in a *n*-butanol water system, decreases the distribution in the butanol. The effect of substituting saturated salt solution for water, where significant differences occur, is due apparently to the greater mutual exclusion of the two components. The effect of variations in *p*H was determined for a few solvents, as shown in Table II, and probably shows the relative solubilities of the free flavin and its salts.

TABLE I

DISTRIBUTION COEFFICIENTS OF PURE RIBOFLAVIN IN SOLVENT/NEUTRAL AQUEOUS SYSTEMS AT 25°

Solvent	Water	Saturated NaCl
n-Propanol	.....	0.27
Isopropanol	.....	.25
n-Butanol	0.67	.14
Isobutanol	.46	.09
n-Pentanol	.17	.053
Isopentanol	.19	.053
s-Butylcarbinol	.22	.042
Diethylcarbinol	.23	.053
Dimethylethylcarbinol	.58	.11
n-Hexanol	.12	.047
Methylpentanol	.09	.026
2-Ethylbutanol	.08	.031
2-Ethylhexanol	.024	.01
Benzyl alcohol	3.75	6.0
Cyclohexanol	0.54	0.11
Terpineol	0.22	0.06
Phenol	68	1000
o-Cresol	113	500
m-Cresol	170	667
p-Cresol	227	1000
Aniline	4	2.8
Dimethylaniline	0.0013	0.0012
o-Toluidine	1.25	.50
Pyridine	.....	7
Quinoline	21.3	29.2
Furfural	0.29	0.21
Phenol–BuOH 1:1	8	9
Aniline–BuOH 1:1	6.4	4
Ethyl ether	0.00016	0.00016
Petroleum ether (b. p. 35–60°)	.00002	.000005

TABLE II

DISTRIBUTION COEFFICIENTS OF RIBOFLAVIN IN SOLVENT/AQUEOUS SYSTEMS AT 25° AT DIFFERENT *p*H

Solvent	*p*H 1.0	4.0	7.0	10.0	13.0
n-Butanol	0.58	0.58	0.67	0.31	0.026
Benzyl alcohol	3.3	3	3.75	1.7	.015
Phenol	120	134	68	..	...
Aniline	....	....	4	1.4	.002
Quinoline	....	....	21.3	9.7	.12

These coefficients were obtained in the following manner: in most cases 2 cc. of a 0.01% solution of pure riboflavin in water or saturated salt solution was shaken vigorously for five minutes at 25° with 2 cc. of solvent. After separation, the layers were measured and a filtered aliquot of each was freed from solvent and adjusted to a *p*H of 7.0. The riboflavin content of the solutions was determined by fluorometric[14] comparison at suitable dilution against

(12) Narayan and Drummond, *Biochem. J.*, **24**, 19 (1930).

(13) Greene and Black, THIS JOURNAL, **59**, 1395 (1937).

(14) Supplee, Ansbacher, Flanigan and Hanford, *J. Dairy Sci.*, **19**, 215 (1936).

standard solutions of pure riboflavin. In cases where the coefficient is very low, stronger solutions of riboflavin and more solvent were used. In this study, as in all of our other work, all reasonable precautions have been taken to prevent destruction of riboflavin by exposure to light.

Isolation of Pure Riboflavin.—The preparation of fuller's earth adsorbates of riboflavin from crude extracts is so well known that further description is unnecessary. The procedure for preparing pure riboflavin, which is a combination of known features with others developed in this Laboratory, will be given in detail.

For elution, 300 g. of adsorbate containing 200–300 gamma of riboflavin per gram is stirred for twenty to thirty minutes at room temperature with 2550 cc. of a solution of 0.1 *N* sodium hydroxide in 60% ethanol. After separation by centrifuge the residue is extracted a second and then a third time with 2400-cc. portions of 0.05 *N* sodium hydroxide in 60% ethanol. The eluates are promptly adjusted to a *p*H 4.5–5.0 and evaporated *in vacuo* to a total volume of 1500 cc. After cooling, an inert precipitate is removed by centrifuging. At this point re-adsorption may be employed directly or a solvent treatment may be included. Either phenol or butyl alcohol or a mixture of these two solvents will separate the riboflavin from much inert matter. If the solvent procedure is followed, the aqueous solution, which has a volume of 1500 cc., is extracted twice with 150-cc. portions of 88% phenol. The phenol is then removed by ether extraction in the presence of 300–400 cc. of water. After such treatment, the concentrate consists of salt-free solids which are 1–2% riboflavin. In either case, the aqueous solution is diluted to 5000–6000 cc., adjusted to a *p*H of about 1, and stirred for one hour with 20–40 g. of Lloyd's reagent. The proper amount of adsorbent, which will take up about 95% of the riboflavin, varies somewhat with the original adsorbate and the degree of purification attained. The mother liquors from adsorption are difficult to examine directly for residual riboflavin but we have found that two extractions of an aliquot with four volumes of *n*-butanol yield a solution which can be tested accurately by a fluorometric method.

For elution, the re-adsorbate is stirred with ten volumes of 60% ethanol[15] at 78–80° for thirty minutes. After separation of the eluate by centrifuge the treatment is repeated until the residue contains no appreciable amount of riboflavin; five elutions are usually sufficient. The acqueous–alcoholic solution of the riboflavin is evaporated to remove alcohol and the small amount of fine flocculated earth is removed by centrifuge and washed with a little 60% alcohol to recover riboflavin. At this point the concentrate contains 4–5% riboflavin and represents a yield of 75–80% of that present in the original adsorbate. If phenol has not been used previously in the purification, the aqueous solution without further concentration (300–400 cc.) is extracted with two 50-cc. portions of phenol. The phenol is removed with ether in the presence of water and the volume of the ether-free aqueous solution is reduced to about 100 cc. In either case, the aqueous solution of this volume is extracted with 150, 100 and 100 cc. portions of *n*-butanol. Butanol is removed from these extracts by petroleum ether in the presence of water or may be evaporated *in vacuo*. Instead of the foregoing procedure, a mixture of 1:1 phenol–butanol has been used with success; it is believed, however, that the maximum purification is accomplished by the use of the individual solvents. We have found that acetone precipitation is advantageous at this point and leads to a better crystallization of riboflavin. Others[6-7] have reported successful use of this step with much more impure concentrates but our experience has not been favorable. The aqueous solution, the solids of which now contain 8 to 12% riboflavin, is evaporated to 15–20 cc. and 200 cc. of boiling acetone is added, with a trace of concentrated hydrochloric acid to improve precipitation of the inert fraction. After chilling to 0° the acetone solution is removed and the precipitate is taken up in 5 cc. of hot water and 95 cc. of boiling acetone. After chilling the mixture, the precipitate, which is now practically devoid of riboflavin, is discarded. The combined acetone liquors are evaporated nearly to dryness and precipitation with 95 and 98% acetone is employed successively, after which the riboflavin is about 20% of the soluble solids. For first crystallization we have used a solvent mixture which we find especially favorable. The 98% acetone solution is evaporated to about 2–3 cc. and to this hot aqueous solution 9 cc. of acetone and 4 cc. of petroleum ether (b. p. 35–60°) are added. The mixture is left at 0° and a first crop of crystals is obtained. A second crop is obtained from the mother liquors after removal of some insoluble material in a low volume of 98% acetone and crystallization from the above mixture on a reduced scale. In this way we have obtained 50–60% of the riboflavin from the original fuller's earth in the form of crystals of 70–90% purity. Another procedure has also been very successful for removing an additional amount of riboflavin from the mother liquors. A phenol extraction from a small amount of aqueous solution leaves behind an appreciable amount of inert material. After transferring the phenol soluble fraction to water an extraction with a small amount of butanol or preferably hexanol removes a considerable amount of inert material with only a small loss of riboflavin. Recrystallization of the combined crops from water or aqueous alcohol produces crystals which are equal by fluorometric test to samples of pure natural and synthetic riboflavin and melt at 273–274° (uncorr.). The yield is 40–50% of that in the original adsorbate. Biological tests on this pure crystalline riboflavin showed that 1.5 gamma gave an average weekly gain of 3.8 g. per rate while 2 gamma gave a gain of 8 g.

(15) Methanol may also be used.

Summary

A method for the preparation of pure natural riboflavin is presented which is based on adsorptions on fuller's earth, fractionation with immiscible solvents and acetone and crystallization from an aqueous acetone-petroleum ether mixture. Aqueous–alcohol solutions have been used for elution of the adsorbates.

A study has been made of the distribution of riboflavin in immiscible solvent–water systems. A number of these solvents offer definite advan-

tages over methods which have been used previously for the isolation of this substance. This work and earlier work with vitamin B_1 indicate that such procedures are useful in the fractionation of certain members of the "B complex."

NEW BRUNSWICK, N. J. RECEIVED JULY 19, 1937

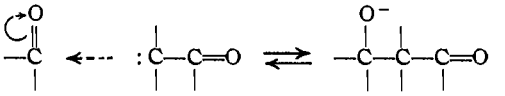
[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, DUKE UNIVERSITY]

Certain Condensations Brought about by Bases. I. The Condensation of Ethyl Isobutyrate to Ethyl Isobutyryl-isobutyrate[1]

BY CHARLES R. HAUSER AND W. B. RENFROW, JR.

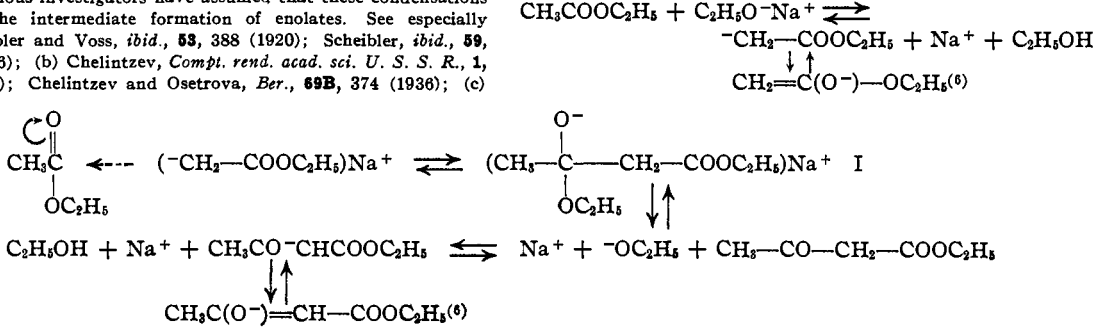
In this Laboratory a study has been made recently of certain organic elimination reactions which are brought about by bases.[2] The view has been held that these reactions are initiated or facilitated by the removal of a proton from the organic compound. This view has been applied also to certain condensations that are brought about by bases, and which may be regarded as "aldol types," such as the Claisen,[3] Perkin,[4] etc. These condensations are considered to proceed through the intermediate formation of enolates.[5] In the presence of the base a proton is removed from the group $H-\overset{|}{\underset{|}{C}}-C=O$ to form a negative enolate ion, which may be represented in two resonance forms,[6] (a) $:\overset{|}{\underset{|}{C}}:C::O:$ and (b) $\overset{|}{\underset{|}{C}}::C:\ddot{O}:$.

The condensation may perhaps be represented most simply as the reaction of (a) with the carbonyl group of a molecule which has not been converted into an enolate. The free pair of electrons of (a) are accepted and shared by the potentially positive carbon atom of the carbonyl group,[7] and the negative charge shifts to oxygen, thus



The condensation does not usually proceed to completion, however, unless a further reaction occurs. In the Perkin, the elimination of water allows the process to be completed, and in the Claisen condensation of an ester, the release of ethylate ion and the formation of an enolate of the condensation product appears to furnish the necessary condition for allowing the reaction to go to completion.

The Claisen condensation of ethyl acetate in the presence of sodium ethylate may be represented as a series of equilibria.



The negative enolate ion of ethyl acetate formed in the first step condenses with the carbonyl group of a molecule of the ester to form the negative ion of (I). This negative ion releases ethylate ion, which immediately removes a proton from the

(1) This paper embodies a part of the material presented by W. B. Renfrow, Jr., to the Graduate School of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) Hauser, LeMaistre and Rainsford, THIS JOURNAL, **57**, 1056 (1935); Hauser and Renfrow, *ibid.*, **59**, 121 (1937).

(3) See Cox, Kroeker and McElvain, *ibid.*, **56**, 1173 (1934).

(4) See Arndt and Eistert, *Ber.*, **69**, 2386 (1936).

(5) Various investigators have assumed that these condensations involve the intermediate formation of enolates. See especially (a) Scheibler and Voss, *ibid.*, **53**, 388 (1920); Scheibler, *ibid.*, **59**, 1022 (1926); (b) Chelintzev, *Compt. rend. acad. sci. U. S. S. R.*, **1**, 393 (1935); Chelintzev and Osetrova, *Ber.*, **69B**, 374 (1936); (c) Arndt and Eistert, *ibid.*, **69**, 2384 (1936); (d) Kalnin, *Helv. Chim. Acta*, **11**, 977 (1928); *Ber.*, **69B**, 2843 (1936); (e) Müller, Gawlick and Kreutzmann, *Ann.*, **515**, 97 (1934).

(6) Perhaps the enolate ion is best represented as having a structure intermediate between (a) and (b), formulated as, $(-\overset{|}{\underset{|}{C}}\!\!\circlearrowleft\!\!C\!\!\circlearrowleft\!\!O)^-$. In this connection see Waters, "Physical Aspects of Organic Chemistry," D. Van Nostrand Co., New York, 1936, p. 314. Also see Sidgwick, *J. Chem. Soc.*, 694, 1937.

(7) In this connection see Waters, "Physical Aspects of Organic Chemistry," pp. 170, 383.