A detailed study of the kinetics and mechanism of each of these reactions is now underway in this laboratory and will be reported later.

Acknowledgment.—Thanks are expressed to The James G. Boswell Foundation (Stanford Research Institute) and to The Public Health Service (Grant GM 08347-02, Division of General Medical Sciences) for financial support.

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          RECEIVED SEPTEMBER 18, 1962

THE SEPARATION OF GUANOSINE OLIGONUCLEOTIDES: USE OF UREA TO AVOID AGGREGATE FORMATION

Sir:

Separation of guanine-rich oligonucleotides by usual methods is difficult and unreproducible, presumably because of aggregation of such compounds. We wish to report that a satisfactory separation can be carried
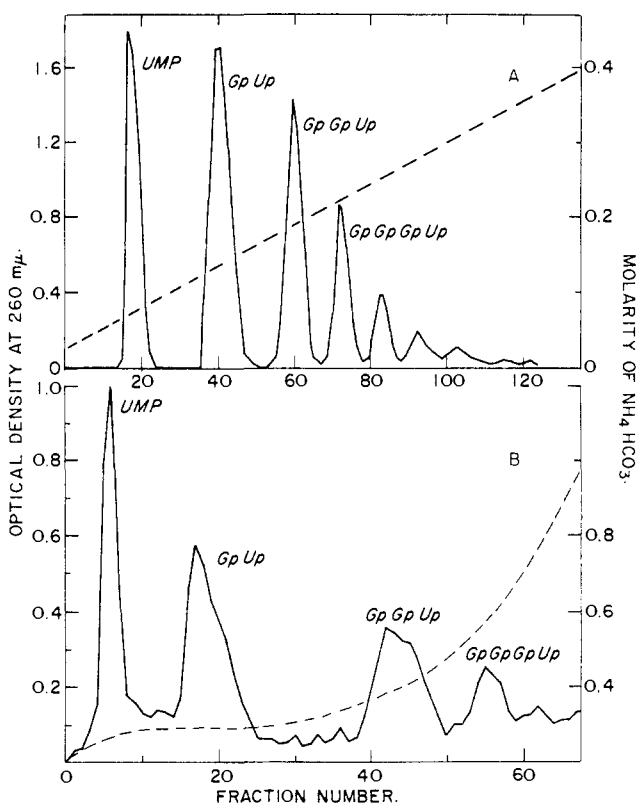

Fig. 1.—A. Separation of a pancreatic ribonuclease digest of poly GU (570 O.D. units at 260 mμ) adsorbed on a 12.8 × 2 DEAE-cellulose–bicarbonate column, eluted with a linear gradient. The mixing chamber contained 750 ml. of 0.03 M NH$_4$HCO$_3$ in 7 M urea, the reservoir 750 ml. 0.5 M NH$_4$HCO$_3$ in 7 M urea, both at pH 8.6. Samples of 8 ml. were collected at a flow rate of 2 ml./min. B. Similar to (A), 184 O.D. units at 260 mμ adsorbed on a 10 × 1 cm. column, eluted with a 600 ml. non-linear gradient[7] of NH$_4$HCO$_3$, pH 8.6, as shown, collecting 10 ml. samples at a flow rate of 0.75 ml./min.

out by DEAE-cellulose chromatography with the addition of 7 M urea, as described by Tomlinson and Tener[1] for the separation of ribonuclease digests of nucleic acids.

It has been shown[2] recently that deoxyguanosine oligonucleotides possess a secondary structure and are

(1) R. V. Tomlinson and G. M. Tener, *J. Am. Chem. Soc.*, **84**, 2644 (1962).
(2) R. K. Ralph, W. J. Connors and H. G. Khorana, *ibid.*, **84**, 2265 (1962).

capable of forming aggregates. We have independent evidence that points to secondary structure in guanosine oligonucleotides. (1) A mixture of guanine-containing oligonucleotides of average chain length equal to 6 was unusually resistant to Takadiastase ribonuclease T$_1$[3] and pork liver nuclease.[4] A preliminary heating of the substrate resulted in somewhat more rapid hydrolysis, but the rates still were very slow. (2) Removal of terminal phosphate by *E. coli* alkaline phosphatase[5] was extremely slow and incomplete for GpGpGpGp, GpGpGpUp and homologs of higher molecular weight. (3) Paper chromatograms of pancreatic ribonuclease digests of poly GU[6] showed the presence of 3'-UMP, GpUp, GpGpUp and GpGpGpUp as discrete bands, but nothing else migrated from the origin even after several days development in the tank. Material at the origin was hydrolyzed and found to have a ratio guanine/UMP of 4.2/1. This indicates that oligonucleotides containing uracil did not display the expected mobility in the solvent system. Further, when GpGpUp, GpGp, GpG or larger homologs of these were re-chromatographed, over a third of the material remained at the origin.

The use of 7 M urea with the DEAE-cellulose column appears to prevent aggregation. With urea (Fig. 1), a simple linear gradient gave sharper peaks, better resolution and better return of optical density to the baseline between peaks than was obtained without urea and using a gradient deliberately flattened[7] to allow maximum resolution of the first four peaks.

Striking differences also were noted when single compounds were chromatographed on DEAE. Thus, GpGpUp (40.2 O.D. units at 260 mμ) was applied in 7 M urea to a DEAE-bicarbonate column (2.8 cm.^{3}) and eluted, with a linear gradient of NH$_4$HCO$_3$ in 7 M urea, as a single peak. Recovery was 40.8 O.D. units (101%). By contrast, chromatography of GpGpUp in the absence of urea led to recovery of only 65% of the material in the expected elution position, and even eluting the column with high salt did not completely remove the remainder.

Similarly, a digest containing a mixture of guanine oligonucleotides with 2' (or 3') phosphate end groups, eluted in the presence of urea, yielded a series of sharp peaks, of which the first five accounted for over 90% of the starting material. Chromatography as usually carried out with DEAE[7] or Dowex-1-Cl$^-$, 2× cross-linked,[8] was quite unsatisfactory, since even a compound as simple as GpG could not be recovered quantitatively.

(3) K. Sato-Asano and F. Egami, *J. Biochem.* (Japan), **44**, 753 (1957).
(4) M. N. Lipsett, L. A. Heppel and W. E. Razzell, unpublished data.
(5) A. Torriani, *Biochim. et Biophys. Acta*, **38**, 460 (1960).
(6) Poly GU is a random copolymer of uridylic and guanylic acids.
(7) Using the varigrad of E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956); *Anal. Chem.*, **31**, 857 (1959).
(8) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).

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## CONCERNING THE STRUCTURE OF THE GRIGNARD REAGENT

Sir:

Although the Grignard reagent has been in use for over sixty years, considerable confusion exists as to the precise nature of this reagent in ether solution. The composition of the Grignard reagent has been represented most often by the equilibria[1]

$$2RMgX \rightleftharpoons R_2Mg + MgX_2 \rightleftharpoons R_2Mg \cdot MgX_2$$
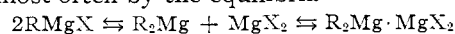
(1) M. S. Kharasch and O. Reinmuth, "Grignard Reactions of Nonmetallic Substances," Prentice-Hall, Inc., New York, N. Y., 1954.

More recently, Dessy, Wotiz, Hollingsworth and co-workers have described experiments which have indicated that the species RMgX is essentially non-existent in the Grignard equilibria. The basis for this conclusion was the lack of exchange of Mg between $Mg^{28}Br_2$ and Et_2Mg in ether solution.[2] Evidence was presented showing that an equimolar mixture of $MgBr_2$ and Et_2Mg has the same characteristics as the Grignard reagent.[3,4,5] Thus, it was concluded that the Grignard reagent is best represented by the equilibrium

$$R_2Mg + MgX_2 \rightleftarrows R_2Mg \cdot MgX_2$$
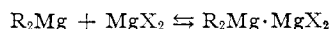
Since Grignard reagents in diethyl ether solution are dimeric (based on RMgX), and $MgCl_2$ does not precipitate from a solution of EtMgCl on standing, the above equilibrium is said to lie far to the right.

Recently we have made two unusual observations concerning the nature of the Grignard reagent which have caused us to reconsider the equilibrium proposed by Dessy: (1) the Grignard reagent,[6] although dimeric in diethyl ether, is monomeric in tetrahydrofuran, and (2) fractional crystallization of "EtMgCl" in tetrahydrofuran produced $EtMg_2Cl_3$ and Et_2Mg in quantitative yield. These observations have led directly to two conclusions: (1) in tetrahydrofuran there *is* alkyl exchange in Grignard solutions, and (2) the predominant species in solution is RMgX.

The monomeric nature of "EtMgCl" in tetrahydrofuran implies that the species in solution are (1) EtMgCl, (2) a mixture of Et_2Mg and $MgCl_2$, or (3) an equilibrium mixture of (1) and (2). The possibility of (2) seems unlikely since a 2 M solution of "EtMgCl" did not precipitate $MgCl_2$ on standing although the solubility limit of $MgCl_2$ in tetrahydrofuran is only 0.5 M. Confirmation of the lack of association of the predominant species in solution was given by the observation that both Et_2Mg and $MgCl_2$ were found to be monomeric in tetrahydrofuran. Consequently, the implication is strong that in tetrahydrofuran the Grignard reagent must be predominantly in the form originally ascribed to it by Schlenk,[7] namely, RMgX. Thus the equilibrium

$$RMgX \rightleftarrows R_2Mg + MgX_2$$

is supported with the equilibrium lying predominantly to the left. The equilibrium could be extended to include RMg_2X_3.

$$3 RMgX \rightleftarrows 3/2 R_2Mg + 3/2 MgX_2 \rightleftarrows RMg_2X_3 + R_2Mg$$
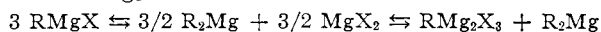
However, it is felt that the RMg_2X_3 is formed on crystallization through a combination of RMgX and MgX_2 in the tetrahydrofuran–benzene system.

The molecular association of EtMgCl and EtMgBr was determined ebullioscopically in tetrahydrofuran at the normal boiling point of the solution (66°) and at 30°. The values listed are the average values of

	Concentration, moles/liter	760 mm. (66°)	200 mm. (30°)
EtMgBr	0.1–0.3	1.01	1.04
EtMgCl	0.1–0.3	1.11	1.01

three consecutive determinations. The molecular weight of a 2 M solution of EtMgCl in tetrahydrofuran was shown to be 65 (theory, 88.8). Although molecu-

(2) R. E. Dessy, G. S. Handler, J. H. Wotiz and C. A. Hollingsworth, *J. Am. Chem. Soc.*, **79**, 3476 (1957).

(3) R. E. Dessy, J. H. Wotiz and C. A. Hollingsworth, *ibid.*, **79**, 358 (1957).

(4) R. E. Dessy and R. M. Jones, *J. Org. Chem.*, **24**, 1685 (1959).

(5) R. E. Dessy, *ibid.*, **25**, 2260 (1960).

(6) All Grignard solutions prepared in either tetrahydrofuran or ether, were prepared by the reaction of the alkyl halide with magnesium. In tetrahydrofuran the solution prepared from ethyl chloride and magnesium was identical to the solution prepared from Et_2Mg and $MgCl_2$ with respect to infrared spectra, conductance and dipole moment.

(7) W. Schlenk and W. Schlenk, Jr., *Ber.*, **62**, 920 (1929).

lar weight determinations at such a high concentration are not too accurate, it appears that "EtMgCl" even at this concentration is essentially monomeric.

The fractional crystallization of EtMgCl from tetrahydrofuran was accomplished by adding an equal volume of benzene to the Grignard solution and slowly removing the solvent mixture under vacuum. Two fractions of white crystalline solids were isolated. Magnesium, chlorine and gas evolution analyses determined the empirical formula of both fractions to be $EtMg_2Cl_3 \cdot THF$. The solute in the mother liquor was shown by analysis to be Et_2Mg.

In contrast, we have found the molecular weight of EtMgCl in diethyl ether to indicate a dimeric structure over a wide concentration range. The two most logical dimeric structures are shown as I and II. The work
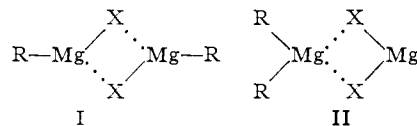
of Dessy and co-workers leads to preference of structure II over structure I.

Fractional crystallization of EtMgCl and MeMgCl from diethyl ether solution did not produce the same clear-cut results as did the experiments carried out in tetrahydrofuran. However, $MeMg_2Cl_3$ was isolated in good yield from an ether solution of the Grignard prepared from methyl chloride and magnesium, thus showing that alkyl exchange also occurs in diethyl ether. With alkyl exchange demonstrated, there is no reason to prefer the unsymmetrical structure (II) proposed by Dessy over the symmetrical one (I), especially in view of the results obtained in tetrahydrofuran. In this connection we have determined the molecular aggregation of mesitylmagnesium bromide in diethyl ether to be dimeric. If Grignard compounds in diethyl ether solution exist as the unsymmetrical dimers, a severe steric problem would arise from a magnesium atom surrounded by two mesityl groups and two bromine atoms. Thus, it would appear that the equilibrium which exists in diethyl ether is similar to that occurring in tetrahydrofuran.

We are continuing to study the relationship of structure to molecular association in Grignard solutions. Work is also in progress to further define the significance and role of the RMg_2X_3 species in Grignard solution.

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THE CHEMISTRY OF BLUENSOMYCIN. I.[1a] THE STRUCTURE OF BLUENSIDINE

Sir:

The isolation of a new basic antibiotic, bluensomycin, has been described recently.[1b] Bluensomycin purified as its hydrochloride, has the molecular formula $C_{21}H_{39}N_5O_{14} \cdot 2HCl$,[2] pK_a' 7.53, equivalent weight 659. It gave a strong Sakaguchi color test, indicative of a monosubstituted guanidino group. The hydrochloride showed infrared absorption at 3350–2950, 1710, 1655, 1610,

(1a) Bluensomycin is the generic name for antibiotic U-12,898.

(1b) M. E. Bergy, T. E. Eble, R. R. Herr, C. M. Large and B. Bannister, Second Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 31–Nov. 2, 1962, Chicago, Illinois.

(2) Analytical values for all the compounds described in this paper were consistent with the indicated formulas.