HELIX FORMATION BY GUANYLIC ACID

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In 1910, Bang[1] reported that concentrated solutions of guanylic acid formed a gel. We have also observed that concentrated solutions (25.0 mg/ml) of guanylic acid (GMP) at pH 5 are extremely viscous and, if cooled, form a clear gel. Less concentrated solutions also gel on cooling but assume a more normal viscosity at room temperature. From examination of the optical properties of the gel and investigation of the structure of fibers obtained from the gel by drying, we have concluded that, at least in the case of the 5′ isomer, the phenomenon may be explained as being due to helix formation by the guanylic acid. A possible structure is presented for this helix.

Materials and Methods.—Ultraviolet absorption was measured with a Cary Model 15 spectrophotometer, using an 0.1 mm cell. A water-jacketed cell housing was used to maintain temperature. To measure optical densities greater than 2.0, calibrated neutral density screens were placed in the reference beam. In this way, optical densities up to at least 3.5 could be accurately measured, the instrumental stray light level being sufficiently low.

Optical rotation measurements were performed on a Rudolph Model 80 spectropolarimeter, using a water-jacketed 1-cm cell. Specific rotations are uncertain by $\pm 10°$.

Fibers were obtained from concentrated solutions at pH 5 by suspending a drop of the material over the ends of a U-shaped piece of paper clip and allowing it to dry slowly at 4°C. The material dried into highly negatively birefringent fibers 5 mm long by 0.1 to 0.3 mm wide. X-ray diffraction patterns were recorded in a modified Philips fiber camera at several different relative humidities.

The materials examined were obtained from the following sources: 5′-GMP, Pabst and Schwarz: guanosine, Schwarz; mixed 2′-, 3′-GMP, disodium salt, Sigma. Since the chromatographic separation of 2′- and 3′-GMP was found to require different conditions from those previously described for hydrolysates of crude RNA,[2] the separation is described.

The commercial isomeric mixture of GMP was found by paper chromatography in the system (saturated $(NH_4)_2SO_4$:1 *M* Na Acetate:isopropanol::80:18:2)[3] to contain 5 per cent guanosine, 15 per cent 2′-GMP, and 80 per cent 3′-GMP. One hundred milligrams were dissolved in 400 ml water, brought to pH 8.2 with NaOH, and loaded on a 2.3 × 12.8 cm column of Dowex-1-formate, which had been prepared by suspending 40 gm Dowex 1, 8 per cent cross-linked, 200–400 mesh, chloride form, in 1 *M* HCOONa, packing, washing with this solution until chloride-free, and then washing with water until neutral to bromothymol blue. Five per cent of the optical density added to the column came off immediately with the guanosine peak using 0.05 *M* $HCOONH_4$-0.01 *M* HCOOH, as was expected, but less than 1 per cent of the 2′ and 3′-GMP were removed in the next step, using 0.1 *M* $HCOONH_4$-0.1 *M* HCOOH. The salt concentration had to be raised to 0.5 *M* $HCOONH_4$-0.1 *M* HCOOH before the remainder of the material came off, in two peaks. The fractions were lyophilized and identified chromatographically as 2′- and 3′-GMP respectively, with no detectable cross-contamination. More than 98 per cent of the material was recovered from the column.

The materials obtained by this method (preparation I), while they were chromatographically free of other nucleotide materials, were found to contain appreciable amounts of ammonium formate, even after lyophilization and prolonged storage *in vacuo* over KOH and P_2O_5. This contamination may have amounted to as much as 50 per cent by weight. Purification by barium precipitation proved impractical because of the relative insolubility of barium formate. Therefore, 300 mg of the 3′-GMP in 300 ml 0.01 *M* NH_4HCO_3, pH 8.6, was put on a 2.2 × 9.5 cm DEAE-cellulose column, washed free of formate with 200 ml of the same buffer, and eluted with 50 ml 0.3 *M* NH_4HCO_3. Lyophilization and subsequent storage *in vacuo* over KOH and P_2O_5

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removed the last traces of NH_4HCO_3 (preparation II). Treatment of a solution of this ammonium guanylate with solid Dowex 50- Na^+ yielded a clean preparation of sodium 3′-guanylate for study (preparation III).

Results.—1. *Gel formation:* When solutions of 5′- and 3′-GMP at a concentration of 10 mg/ml in 0.01 *M* acetate buffer at pH 5 and 0.2 *M* NaCl are cooled to 0°C, they form clear viscous gels. At pH 7 or pH 2, no gel formation is observed; the pK of the secondary phosphate of the guanylic acids is 6.0, and the protonation of the guanine occurs at pH 2.4.[4] This implies that the form which is capable of gel formation is that which has a single negative charge on the phosphate and the neutral form of the guanine. 2′-GMP does not appear to form a gel under the conditions in which gelation occurs for the 5′ and 3′ isomers. Guanosine is not soluble at this concentration; there appears to be a slight increase in viscosity of saturated guanosine solutions, but the effects are too faint to be conclusive.

2. *UV absorption studies:* Figure 1 shows the difference spectrum produced by warming a 5′-GMP gel from 1°C to 40°C. Figure 2 shows the change in optical

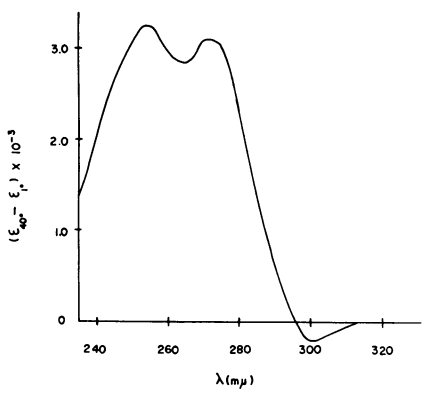

FIG. 1.—Difference spectrum between 5′-GMP at 40°C and 1°C. Conditions: 0.025 *M* GMP, 0.2 *M* NaCl, 0.01 *M* Na Acetate, pH 5.0.

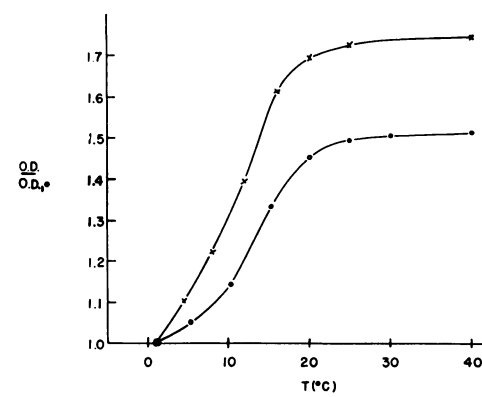

FIG. 2.—Melting curves of 3′-GMP and 5′-GMP gels. The optical density at 275 mμ is plotted. Same conditions as in Figure 1. O, 5′-GMP; ×, 3′-GMP, preparation I.

density at 275 mμ upon gradually increasing the temperature of solutions of 5′-GMP and 3′-GMP. It can be seen that there is a substantial increase in optical density on warming, whose magnitude is comparable to that found on denaturation of helical polynucleotides. By this criterion, the gels are completely "melted" at 40°C, the optical density coinciding with that of dilute solutions of GMP.

Optical density changes were found to be only moderately reproducible in matched experiments, due in large part to a considerable time dependence of the optical density in the melting region. The melting curves also showed a clear hysteresis; the steepest optical density change of a solution which is being cooled lies several degrees below that of a solution being warmed.

The 2′-GMP under similar conditions showed only a small change in optical density upon cooling (~5 per cent).

3. *Optical rotation changes upon gel formation:* The optical rotation changes upon gel formation are summarized in Table 1. It can be seen that a large change in specific rotation occurs upon cooling the 5′-GMP. The magnitude resembles that

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TABLE 1

OPTICAL ROTATION OF GUANYLIC ACIDS

5′-GMP	1°C	40°C
$[\alpha]_D$	−200°	−20°
$[\alpha]_{365}$	−1170°	−100°
3′-GMP (preparation I)		
$[\alpha]_D$	−30°	−20°
$[\alpha]_{365}$	−180°	−90°
3′-GMP (preparation III)		
$[\alpha]_D$	−60°	−20°
$[\alpha]_{365}$	−280°	−90°
2′-GMP		
$[\alpha]_D$	−20°	−20°
$[\alpha]_{365}$	−100°	−90°

(Same conditions as in Figure 1.)

observed for the formation of helical polyribonucleotide complexes, but the ordered 5′-GMP solution is laevorotatory, as opposed to the dextrorotation observed for the helical polyribonucleotides.[5]

The changes in the 3′-GMP solutions are smaller but occur with the same sense. The 2′-GMP shows no changes in optical rotation upon cooling to 1°C.

4. *X-ray diffraction studies:* X-ray diffraction patterns from fibers of the 5′ isomer and of two salts of the 3′ isomer are shown in Figure 3*a*, *b*, and *c*, respectively. Figure 3*a* is a characteristic helix diffraction pattern with a layer line spacing of 13.0 Å and a strong meridional reflection at 3.25 Å. This is indicative of a helical structure with four units per turn of the helix, each unit being spaced 3.25 Å apart along the helix axis. The equator contains a strong reflection at 24.2 Å, which may be regarded as a measure of the distance between neighboring molecules. However, because of the lack of other sharp reflections on the equator, it is not possible to assign a lattice and hence to determine the distance unambiguously. For instance, if this were the first-order reflection from a hexagonal lattice, then the intermolecular separation would be 27.9 Å. At 33 per cent relative humidity, the diffraction pattern remains essentially unchanged, but the spacing of the strong equatorial reflection is reduced to 21.7 Å.

Although the two diffraction patterns of the 3′-GMP are quite different, they both

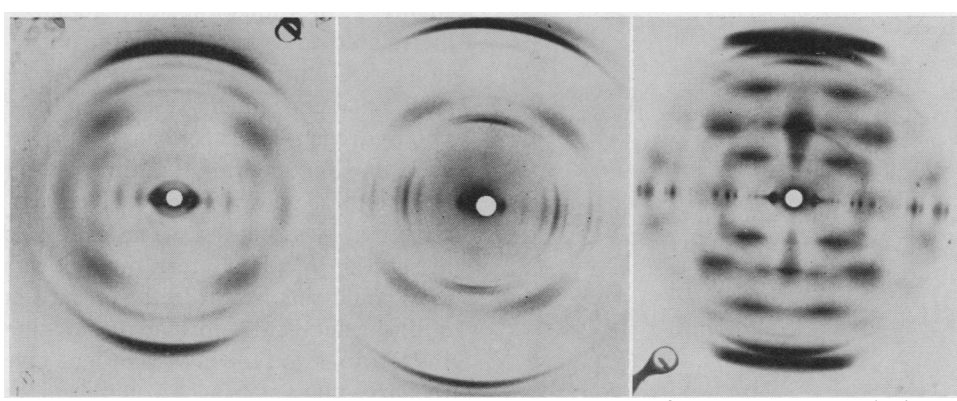

FIG. 3.—(*a*) X-ray diffraction pattern of 5′-GMP. (*b*) X-ray diffraction pattern of 3′-GMP (preparation I). (*c*) X-ray diffraction pattern of 3′-GMP (preparation II). The fiber axis is approximately vertical in all cases.

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have reflections on or very near to the meridian at a spacing of 6.73 Å. Thus, both structures have asymmetric units which repeat along the fiber axis every 6.73 Å. In this respect, the structures differ from the 5′-GMP and from the ordered polynucleotides, where the first meridional reflection occurs with a spacing of between 3 and 4 Å.

In Figure 3*b*, there are, in addition to the equator, only two layer lines, with spacings of 6.73 Å and 3.4 Å. This indicates that the structure forms an exact repeat every 6.73 Å with a unit screw rotation of 0°. The equatorial reflections may be indexed on a hexagonal lattice with a spacing of 31.4 Å.

In Figure 3*c*, the distribution of layer line intensity may be interpreted in terms of helical structure with z axis repeats of 6.73 Å and 12.1 Å for the asymmetric unit and the pitch of the helix respectively. The structure is, therefore, a helix with a 6.73 Å unit translation and $\pm 200°/n$ unit rotation, with n-fold rotational symmetry along the helix axis. The very sharp equatorial reflections may be indexed on a hexagonal lattice with $a = 25.5$ Å, and the sharpness of the reflections is consistent with a nonintegral screw.

The considerably larger intermolecular separation for the material in preparation I is presumably the direct result of the presence of large quantities of ammonium formate in this preparation. Patterns similar to Figure 3*c* were obtained for the sodium salt of 3′-GMP (preparation III) as well as for the sodium salt plus one equivalent of sodium chloride.

Discussion.—Recent work by Ralph, Connors, and Khorana[6] has shown that tri- and tetranucleotides of deoxyriboguanylic acid are capable of forming organized macrostructures of remarkable stability. It is perhaps not too surprising, therefore, to find that guanylic acid itself can also form a regular structure.

There are four ways in which two guanines may be paired to form satisfactory hydrogen-bonded dimers, as originally pointed out by Donohue[7] (structures 9, 10, 11, and 12). None of these dimers accounts for the remarkably stable structures formed by guanylic acid. If, however, two pairs of Donohue's structure 10 are brought together, they can form the hydrogen-bonded arrangement shown in Figure 4, in which the four guanines are related to each other by the operation of a fourfold rotation axis.[8] In such an arrangement, there are now two hydrogen bonds per base compared with one for each of the dimers, and one would expect this to be a particularly stable structure. The existence in solution of planar tetramers of this kind could then result in the formation of linear aggregates formed by stacking the tetramers on top of each other, since the large planar surfaces would result in strong van der Waals attractions. Such aggregates would be roughly cy-

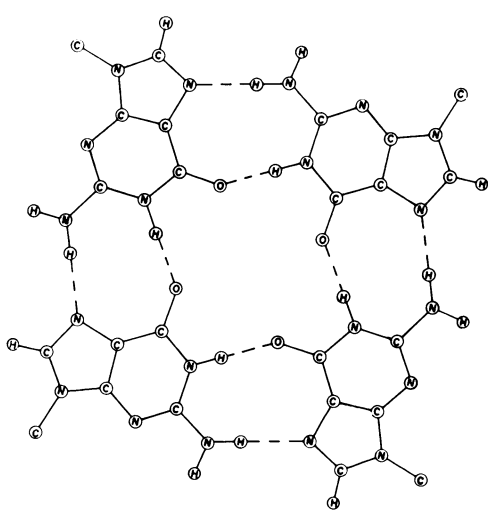

FIG. 4.—Proposed arrangement of the bases in GMP gels.

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lindrical in appearance and would contain a hole in the middle in which it might be possible to place one water molecule per tetramer.

This hypothesis has been tested in the case of 5′-GMP, whose diffraction pattern may be simply interpreted in terms of such a structure. Each group of four bases would then have a similar group above and below it with a screw rotation of $\pm 22.5°$ and an axial translation of 3.25 Å. In order to reconcile the helix repeat of 3.25 Å with a base separation of 3.36 Å, the bases have to be tipped by a small amount. Models have been constructed with these dimensions; it is clear that the model with right-handed rotation of 22.5° cannot be constructed because of close contacts between neighboring ribose-phosphate groups. With the left-handed rotation, however, such a model can be constructed, and it becomes apparent that the phosphate group is then located in a position which is very favorable for hydrogen bonding. Hydrogen bonds can be formed from the 2-amino group to one of the oxygens of a phosphate one layer below; the other oxygens can form hydrogen bonds with neighboring phosphates and with the 2′-hydroxyl group of a neighboring ribose, two layers above. Preliminary calculations of the Fourier transform of the model show a satisfactory agreement with the observed intensity distribution.

It should be noted that there exists a possible alternative structural explanation for the 5′-GMP diffraction pattern. If the bases are uniformly tilted so that the fourth pair of hydrogen bonds is used, not to close the ring, but to bond to a fifth guanine situated 3.25 Å above the first, then by proceeding in this manner a continuous connected helix can be formed. Such an interpretation would require that the Bessel function on the fourth layer line be J_1 instead of a J_0. Although the present diffraction patterns indicate that this is indeed a J_0, it is possible that the meridional minimum is obscured by the relatively poor orientation of the fiber, so that this alternative hypothesis cannot be ruled out.

In the case of the 3′-GMP, the diffraction patterns may be discussed in terms of the same general hydrogen bonding scheme. Here, the better orientation of the diffraction pattern gives one more assurance that the reflection at 3.36 Å is truly meridional, thus favoring the planar tetramer hypothesis.

However, both diffraction patterns of the 3′ isomer, i.e., with and without an excess of ammonium formate, require a repeating unit with an axial translation of 6.73 Å. This requirement may be the result of some relatively minor periodic distortion of the helix in such a way as to couple adjacent tetramers together to form a repeating unit with a thickness of 6.73 Å. If such diffraction patterns were obtained from a covalently linked polynucleotide, then it would be necessary to invoke such a hypothesis to account for the observed layer line distortion. However, when no covalent backbone exists, it is no longer obligatory to stack the bases in a regular manner such that the face of one base is in contact with the back of the next base along the helix. It is then possible for the base stacking to occur in couples formed by stacking two adjacent tetramers face to face. The next couple may then be stacked on top of this, and so on, giving rise to helical aggregate ABAB-ABAB... with a repeating unit (AB) of 6.73 Å.

We propose this mode of alternating base stacking as a possible interpretation of the observed diffraction phenomena. For the material of Figure 3*b*, containing an excess of ammonium formate, the stacking of adjacent couples occurs without rotation about the helix axis, whereas in the case of Figure 3*c* the rotation would be

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$\pm 50°$. The rotation between the two tetramers A and B is, however, not defined by the dimensional features of the diffraction patterns, and it is, therefore, not possible to verify this hypothesis in detail.

The differences between the structures of the 3′ and 5′ isomers indicate clearly that the position of the phosphate group plays an important role in determining the structure of the linear aggregates. This is presumably because of the ability of the phosphate group to form hydrogen bonds with atoms on neighboring molecules, thus adding to the stability of particular configurations, as well as being due to the electrostatic repulsion of the charge on the phosphates. The further differences between the two preparations of the 3′ isomer indicate that the structures can also vary according to the nature of the environment of the aggregate.

The large difference in optical rotation of gels of the 3′ and 5′ isomers is also consistent with the X-ray results. The 5′ isomer forms a regular helix and would be expected to give a large rotation in the helical form,[9] whereas the 3′ isomer consists of pairs of planar tetramers stacked on top of each other and would be expected to have a considerably different helix contribution to the optical rotation. The differences in optical rotation between preparations I and III of the 3′-GMP are not unexpected in light of the observed differences in the diffraction patterns.

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[6] Ralph, R. K., W. J. Connors, and H. G. Khorana, *J.A.C.S.*, **84**, 2265 (1962).

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[8] A four-stranded model containing a similar arrangement of the bases, but with one hydrogen bond per base, was considered by A. Rich (*Biochim. Biophys. Acta*, **29**, 502 (1958)) as a possible structure for polyinosinic acid.

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STREPTOMYCIN AS A MUTAGEN FOR NONCHROMOSOMAL GENES

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Stable hereditary determinants segregating in a non-Mendelian manner were first described in 1908 by Correns.[1] In the following decades, some hundred or more well-established examples of non-Mendelian heredity were reported,[2, 3] but few attempts have been made to integrate them into a general theory of genetics. The principal difficulty blocking a systematic study has been the rarity of their occurrence and their failure to respond to mutagenic agents.

The chance isolation, some years ago, of a mutant of the alga *Chlamydomonas reinhardi*, exhibiting nonchromosomal inheritance of streptomycin resistance (*sr*-500),[4] provided a new material with which to reinvestigate the role and origin

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