PROPERTIES OF AGAR: PARAMETERS AFFECTING GEL-FORMATION AND THE AGAROSE–IODINE REACTION

N. M. K. NG YING KIN AND W. YAPHE*

Department of Microbiology and Immunology, McGill University, Montréal 130, Québec (Canada)

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ABSTRACT

The effects of concentration of agarose, methyl sulphoxide, and substituted agaroses on the mechanism of gel formation have been evaluated with reference to the "Network theory of gel formation". Factors affecting formation of the agarose gel–iodine color complex were investigated, and the results suggest that the iodine molecules are incorporated between the agarose helices in the Gel II state of agarose.

INTRODUCTION

Gran[1] in 1902 observed that *Bacillus gelaticus* caused depressions (pits) in an agar medium which stained purple-brown in the area of the undigested agar, but not around the colony, when flooded with an iodine–potassium iodide solution. This agar–iodine reaction has subsequently been used as a marker to isolate agar-decomposing bacteria[2]. The agar–iodine color is associated with the gel state, but its relationship to the mechanism of gel formation is not known.

Recent advances have shown that agar consists of a mixture of related polysaccharides and that a highly purified preparation of agarose, the fraction yielding a strong gel, could be obtained by anion-exchange chromatography[3]. Rees[4,5] has postulated that the sol-to-gel transition probably involves the following stereochemical changes in the agarose molecules: from the primary structure, a random coil, in the sol state; to the secondary structure, a single helix; to the tertiary structure, double-helices, a weak Gel I; and finally to the quaternary structure, aggregates of the double-helices, a strong Gel II. Accordingly, gel formation is expected to be accompanied by a gradual stacking of the molecules, giving rise to an increase in turbidity, which can be conveniently measured at 400 nm. Thus, physical properties, such as gelling temperature and optical clarity, can be determined by a spectrophotometric method[6], which has been used to investigate the factors affecting sol-to-gel transition and the relationship between gelling of agarose and its reaction with iodine.

*To whom requests for reprints should be addressed.

RESULTS AND DISCUSSION

The absorbance *vs.* temperature curve on cooling a solution of agarose in water is shown in Fig. 1. At temperatures higher than T_1, inflection point *A* in Fig. 1, the random coil and helix conformations of agarose are probably in a dynamic equilibrium, with a greater proportion of molecules in the former state. As the temperature drops, this dynamic equilibrium is irreversibly upset by molecules in the helix conformation possibly adopting the energetically more-favorable, double-helix structure[4], in which the constituent strands may be stabilised by inter-strand H-bonding. Mismatching of the helical strands and "kinks" (due to masking groups such as sulphates) in some of the strands give rise to the more-ordered, three-dimensional network of the double helices, and this accounts for both the observed increase in absorbance and the formation of a weak Gel I. Further interactions among the double helices produce the quaternary structure which, because of its compact nature, is responsible for the rapid and steady increase in absorbance and also the formation of a strong Gel II. Such aggregation appears to have reached a near maximum at temperature T_2 (at the inflection point *B*) as indicated by the very slow increase in absorbance below that temperature. This relatively small change could also be the result of syneresis. It should be emphasized here that, although the sol-to-gel transition is expressed as a function of temperature (Fig. 1), it is also a time-dependent process. Thus, when the agarose solution was equilibrated at temperatures ranging from 37 to 40°, in the region of T_1 (Fig. 1), the absorbance *vs.* time curves (Fig. 2) were similar to the cooling curve (Fig. 1), but with increasing temperature the absorbance change extended over a longer period. This dependence of gelling on both temperature and time is in accord with the current concept that the transition from sol to gel in agarose solutions involves a nucleation step[5]. Consequently, extreme care was taken so as not to induce any nucleation.

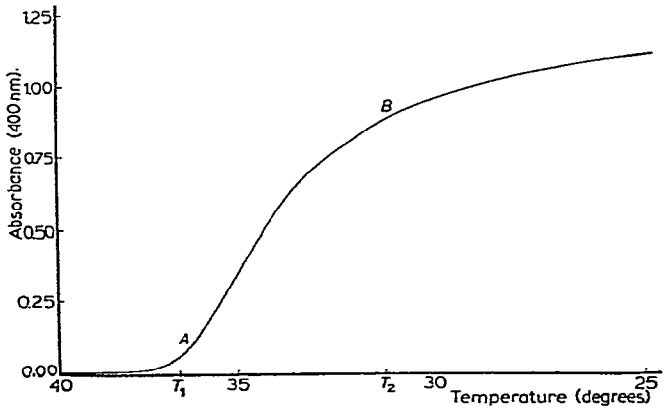

Fig. 1. Cooling curve. Absorbance at 400 nm of a 1.5% aqueous solution of agarose cooled at the rate of *ca.* 0.5°/min. T_1, inflection point *A*, is 37°; and T_2, inflection point *B*, 31°.

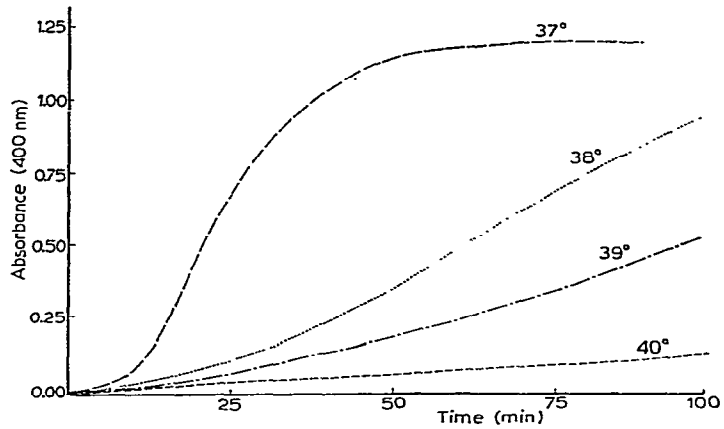

Fig. 2. Rate of gelation of a 1.5% aqueous solution of agarose equilibrated at 40, 39, 38, and 37°.

Concentration of agarose. — The absorbance *vs.* temperature curves in Fig. 3 show an increase in temperature T_1 and T_2 with increasing concentrations of agarose. In the more-concentrated solutions, the larger number of polysaccharide chains increases the chance that single helices will interact (to possibly form double helices). Consequently, in the initial conformational changes accompanying the sol-to-gel transition, the dynamic equilibrium between the random coil and the single helix structure is shifted towards the latter, so that concentrated agarose solutions gel earlier on cooling. The greater extent of chain–chain interactions in such solutions may also account for the stronger and more-opaque gels observed.

Solvent. — Agarose is partially soluble, at room temperatures, in such dipolar aprotic solvents as formamide[7] and methyl sulfoxide, but is insoluble in water. For the following Me_2SO–H_2O solutions at room temperature, *A* 1:3, *B* 1:1, *C* 3:1, a gel was obtained in *A* and *B*, and a clear, viscous solution in *C* which set to a clear gel

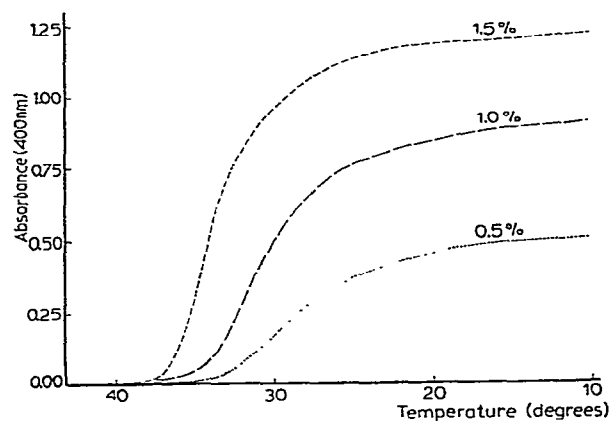

Fig. 3. Effects of agarose concentration on cooling curve. Conditions as for Fig. 1.

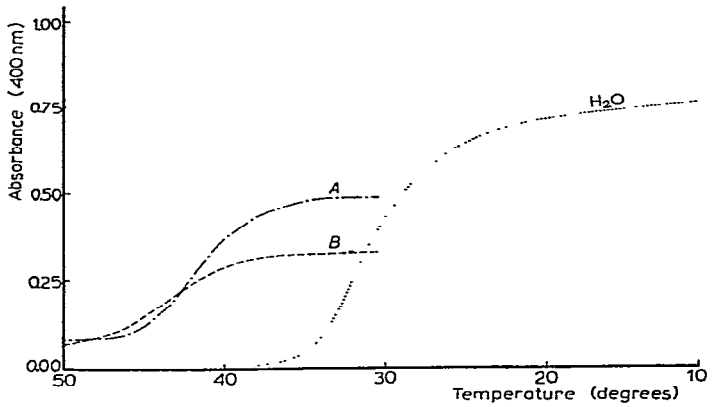

Fig. 4. Cooling curves of 1.0% solutions of agarose in Me_2SO–H_2O; *A*, 1:1; *B*, 2:1. Conditions as for Fig. 1.

below 6°. Agarose in distilled water and agarose in solutions *A* and *B* were dissolved in a boiling water-bath and the resulting absorbance *vs.* temperature curves are illustrated in Fig. 4. Temperature T_1 increased with increasing concentration of Me_2SO, but the rate of increase in absorbance over the temperature range T_1–T_2 was reduced.

The opposing effects of Me_2SO may reflect the solvation of the agarose single-helix and a weakening of chain–chain interactions within the tertiary and quaternary structures. The conditions for solvation of the agarose helix are probably analogous to those recently proposed by Banks and Greenwood[8] for amylose. Thus, in aqueous solutions, the agarose molecule probably favors the random-coil conformation because of the relatively poor solvation in the compact cavity of the agarose helix by "hydrogen-bonded clusters" of water, which are in dynamic equilibrium with monomeric water[9]. Indeed, Child and Pryce[10] concluded from n.m.r. studies that the agarose molecule in water is poorly solvated in the gel state.

In the presence of Me_2SO, the water clusters are broken, giving monomeric water which may solvate and hence stabilise the agarose helix. Consequently, the dynamic equilibrium between the random coil and the single-helix conformations is upset in favor of the latter, thus promoting the initial stage of gel formation (and hence a higher gelling-temperature). The subsequent stages of gel formation are, however, retarded since methyl sulphoxide, being a better solvent for the polysaccharide, would interfere with chain–chain interactions. In 75% Me_2SO (solution *C*), the Me_2SO effects on chain–chain interactions appeared to be the over-riding factors controlling gel formation. Thus, a clear gel was only formed at 6°.

Substituted agaroses. — The temperatures T_1 and T_2 for commercial preparations of agar and agarose are difficult to interpret, since these agars are obtained from a variety of agarophytes[3] and thus contain different proportions of essentially neutral agarose, charged agarose, methylated agarose, and sulphated galactans. Commercial

samples of agarose containing 0.5–0.7% of sulphate contain approximately equal amounts of essentially neutral agarose and charged agarose, and therefore have lower gelling-temperatures and form weaker and clearer gels than the highly purified agarose prepared by anion-exchange chromatography. On the other hand, a methoxyl content of as little as 1.9% in agarose can raise[11] the gelling temperature from 35 to 52°.

Sulphate. — The D- and L-galactose residues in agarose, when substituted with sulphate, may interfere with the formation of the secondary and tertiary structures. For example, esterification of the hydroxyl groups in D-galactose residues weakens interactions between helices because of electrostatic repulsions. The L-galactose residues of agarose may be present as L-galactose 6-sulphate, L-galactose 2,6-disulphate, 3,6-anhydro-L-galactose, or 3,6-anhydro-L-galactose 2-sulphate. The secondary, helical structure of agarose is associated with 3,6-anhydro-L-galactose which adopts a (C_4^1) chair conformation[12], in which three of the C–H bonds are equatorial, giving the agarose helical-chain greater flexibility. Sulphation at C-2 does not appear to alter this conformation or affect inter-strand H-bonding. Thus, agarose from *Gloiopeltis furcata*, which contains 3,6-anhydrogalactose 2-sulphate, gels[13]. However, replacement of the 3,6-anhydro-L-galactose with L-galactose 6-sulphate or 2,6-disulphate does not yield a helical structure, but results in what Rees[4] has described as a "kink" in the secondary structure, since these sulphated L-galactose sugars adopt an "inverted" chair conformation relative to the anhydride.

Pyruvic acid. — Pyruvic acid acetals occur at the 4,6-positions of the D-galactose residues[14]. Therefore, formation of the secondary structure (single helix) may not be affected appreciably, but the gel strength is weakened by electrostatic repulsions between helices.

Methoxyl content. — Methylated porphyran-agarose gels at approximately the same temperature as non-methylated agarose[6]. However, the gelling temperatures of some methylated agaroses increase with increasing methoxyl-content[11]. It would, therefore, appear that the gelling temperature is affected by secondary methoxyl groups since, in porphyran agarose, the methoxyl group occurs on the primary carbon-atom[15].

The higher gelling-temperature may be associated with two effects. Firstly, the presence of secondary methoxyl groups and, to some extent, primary groups may shift the dynamic equilibrium between the random coil and helical structure in favor of formation of the latter at higher temperatures. Secondly, the hydrophobic nature of the methoxyl groups may decrease the solubility of methylated agaroses, with concomitant gelation at higher temperatures. The normal gelling-temperature of methylated porphyran-agarose may reflect a balance between decreased solubility and a weakening of chain–chain interactions.

The agarose–iodine reaction. — The absorbance (610 nm) *vs.* temperature curves for a 1.5% solution of agarose in water and in an aqueous solution of iodine–potassium iodide (Fig. 5) indicate that, unlike starch, agarose only forms a complex with iodine when it is in the gel state. In Me_2SO–water solutions, no color developed

with mixtures containing 25% or more of Me_2SO; with 10% Me_2SO, color developed only at 8°. Since Me_2SO inhibits non-bonded interactions among the double helices of the agarose aggregates in Gel state II, the formation of the agarose–iodine complex in aqueous solutions of Me_2SO is in accord with the contention that the iodine molecules are probably incorporated in the Gel state II. It is possible that Me_2SO affects the reactivity of the iodine–iodide mixture. However, Smith and Smith[16] found that the starch–iodine complex developed in 47% Me_2SO, and we have shown above that Me_2SO affects the gelling temperature and optical clarity of agarose.

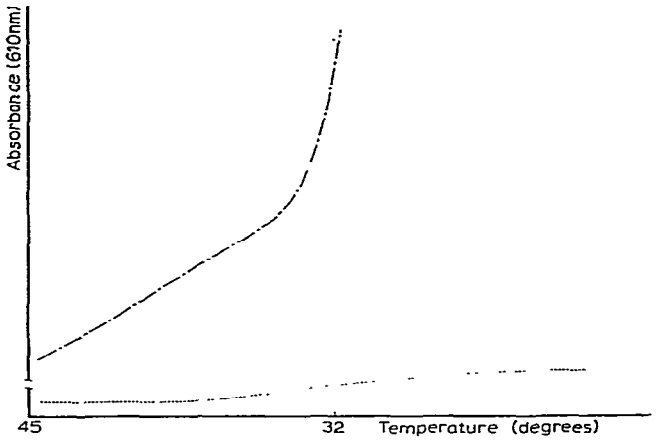

Fig. 5. Cooling curves (*ca.* 0.5°/min) at 610 nm of 1.5% agarose solutions in distilled water (·····) and in iodine–potassium iodide solution (————————). The absorbance change at 610 nm for the former is less pronounced as compared with Fig. 1, since turbidity measurements are less sensitive at higher wavelengths.

Further support for our concept of the agarose–iodine complex in Gel state II is found in that κ-carrageenan gels but does not give a color complex with iodine, under conditions identical to those for agarose. κ-Carrageenans contain D-galactose 4-sulphate which would not interfere markedly with Gel I, but would inhibit formation of Gel state II due to electrostatic repulsions. It is of interest to note that Gould *et al.*[17] have recently suggested that the blue xyloglucan–iodine complex "arises by the interaction of iodine molecules and possibly iodide ions within the interstices between aggregated xyloglucan chains".

EXPERIMENTAL METHODS

Agarose was prepared from commercial agar (Difco Bacto-Agar) by Sephadex DEAE A-50 anion-exchange chromatography as described by Duckworth and Yaphe[18]. The absorbance *vs.* temperature curves were recorded on a Gilford 2000 spectrophotometer unit, and the temperature (±0.1°) of the cuvette chamber was controlled by a circulating water bath (Lauda K-2/R, Brinkmann Instruments). In all

recordings, the temperature was allowed to drop at the rate of *ca.* 0.5°/min, and was monitored by a telethermometer (Yellow Springs Instrument Company, Inc., Ohio), with the thermistor probe inserted in another cuvette containing an agarose solution.

Agarose–iodine reaction. — The iodine–potassium iodide solution was made up of iodine crystals (40 g) and potassium iodide (60 g) dissolved in water (1000 ml). Four drops (or *ca.* 0.2 ml) of the iodine solution were added to each agarose solution (4 ml) at 55°, mixed thoroughly, and the absorbance (at 610 nm) *vs.* temperature curves recorded.

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