THE VARIABILITY IN GEL-PRODUCING PROPERTIES OF COMMERCIAL AGAR AND ITS INFLUENCE ON BACTERIAL GROWTH

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(With 1 Figure in the Text)

INTRODUCTION

It is well known that commercial agar varies considerably in gel-producing properties according to the source from which it is obtained. Chakraborty (1948) investigated a number of samples of agar from different sources and found that the setting power varied considerably. He considered that the reduced setting power of some batches was due to a reduced polysaccharide content, and he demonstrated that those samples exhibiting the best setting properties contained a high proportion of a substance precipitable by excess ethanol, which he referred to as pectin. A very slight reduction in the proportion of this precipitable substance in the agar resulted in a very considerable loss of setting power.

Prior to the last war the great majority of agar was imported from Japan where the best agar was prepared from a red sea-weed, *Gelideum corneum*. Other varieties were obtained from other *Gelideum* spp., *Gracilaria* spp., and *Euchema* spp. Commercial agar is now obtained from a variety of sources, and even in Britain a quantity of agar has been prepared from *Gigartina stellata* (Marshall *et al.* 1949).

Various methods have been used for testing the gel rigidity of these agars. The technique used for testing gel rigidity by Chakraborty was to sink the pan of a balance in a suitable vessel, fill the vessel with agar which was allowed to set, and observe the weight required to tear the pan out through the gel. Another method employed by Kizevetter (1937) depended on the breaking of a gel by gradually increasing the weight applied to its surface. Mercury was added to a metal cup, the weight being transmitted to a button resting on the agar surface. A method (Campbell, 1938) originally intended for testing more elastic gels was employed by Marshall *et al.* A metal spade was sunk into agar contained in a metal box, and the weight required to twist the spade through an angle of 10 or 20° by a system of pulleys was used as an indication of the rigidity of the gel.

The disadvantage of the above methods is that they do not take account of the fact that penetration of agar is to some extent a gradual process, and that penetration will occur over a range of different weights if they are added quickly or slowly, and that the weight required will depend on the time given for it to act. It is difficult with the methods of Chakraborty or Campbell to do many tests without much equipment, and moreover it is desirable to be able to test the actual material as required for use, in the form of poured agar plates.

A method of testing large numbers of plates was therefore devised to overcome these difficulties.

It is well known that nutrient agar plates, if they are over-dry or contain too large a proportion of agar, cause colonies of *Shigella* and *Salmonella* to develop a rough appearance. The development of a simple method of testing agar rigidity therefore, offered an opportunity of investigating the relationship of gel rigidity to colonial morphology and antigenic change in the organisms tested. The rigidity of the plates used could be estimated in parallel with observations on the colonial morphology and agglutination reactions of the organisms grown on them.

METHODS

Preparation of agar plates

Except where otherwise stated, all plates were poured at 60–70° C. to give a depth of agar of 4–6 mm. and dried in an incubator for 2 hr. at 37° C. with the lids slightly raised. Before testing for gel rigidity, each plate was left for at least 2 hr. on the bench at room temperature to ensure that thermal equilibrium was attained. The thickness of the plates was at first checked by stabbing the agar vertically with a capillary pipette, sliding the pipette sideways and withdrawing. The agar core left inside could then be measured with a pair of dividers. Plates with flat faces were used. After some practice it was found possible to pour plates of 4–6 mm. thickness repeatedly by eye.

Preparation of agar media

All media were prepared in screw-capped medical flats and autoclaved at 15 lb. for 20 min., except where otherwise stated. If allowed to set in the bottles the media were remelted by steaming and not reautoclaved.

Testing the gel rigidity

A poured plate was placed on one pan of a Beranger balance and the balance set at zero by the addition of weights to the opposite pan. Half a microscope slide 1 cm. wide and 1 mm. thick was firmly clamped in a stand. The edge (1 cm.) of the glass slide was then adjusted, so that contact was just made with the surface of the agar. By trial and error a weight sufficient to drive the slide through the agar in 3–4 sec. was added to the opposite pan. The pan was then released and the glass slide allowed to exert pressure on the surface of the agar.

A series of readings of the time taken to penetrate the agar was taken, from which a mean value could be calculated (Table 1). It was found that the slide hesitated for a period on the surface of the agar and then plunged sharply through to strike the glass bottom with an obvious click. Sufficient weight was removed from the balance to increase the time taken for penetration by about 4 sec. This process was repeated at about 4 sec. intervals until a mean time of penetration of about 20 sec. was reached. A simple graph was then constructed, from which the weight required to penetrate in 12 sec. was read off (Fig. 1). The weight required to penetrate in this time was used as a measure of the gel rigidity of the medium under test, i.e. *the 12 sec. value.*

Table 1. *Estimation of the time required in seconds for a glass slide to penetrate an agar plate where the plate is forced up against the slide by a series of weights of gradually decreasing value.*

(Medium: 2% Conray agar in digest broth A.V.L. 10/49 at pH 7·4)

Weights used	100 g.	90 g.	85 g.	80 g.	75 g.	70 g.
Readings of time	4	6	8	12	16	26
of penetration	5	7	9	9	18	28
in sec.	4	5	7	10	13	16
	3	6	8	12	16	22
	4	7	9	10	17	18
	4	7	8	10	15	25
Mean values	4·0	6·3	8·2	10·5	16·3	22·5

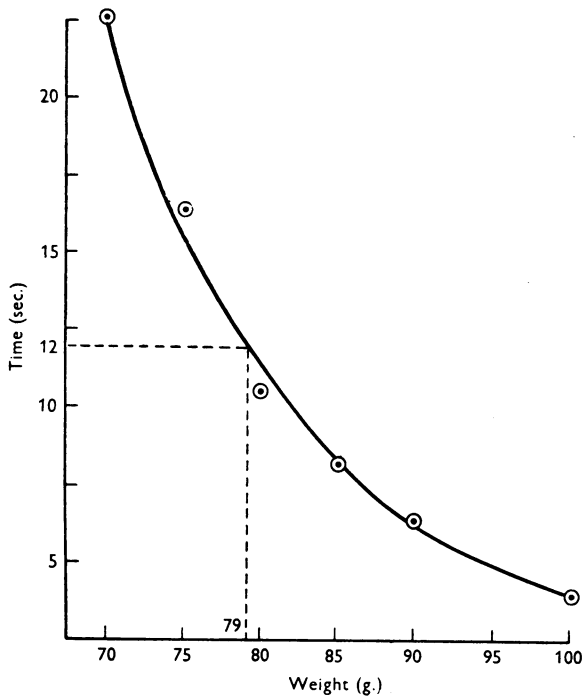

Fig. 1. Estimation of the 12 sec. value for agar gel rigidity. The mean values of the time taken by a glass slide to penetrate an agar plate plotted against the series of weights used to drive the slide into the agar, and the curve obtained used to estimate the weight required to penetrate the agar in a standard time, i.e. 12 sec.

Colonial morphology

Plates which had been inoculated were inspected after 18 hr. incubation at 37° C. with a plate microscope (× 20 magnification) using part transmitted and part direct lighting. Note was taken of the size, shape, edge and surface of the colonies, and an estimate made of the degree of curvature of the surface.

Agglutination reactions

Where it was intended to carry out tests on the plate growth, this was washed off with saline and the growth harvested. Wherever possible the tests were done with live cultures. The saline suspensions were adjusted to a concentration of 8×10^9 organisms per ml. The tests were performed in round bottom tubes (Felix, 1944). The serum dilutions were made up to 1 ml. and 0·05 ml. of the suspension added. The test was then incubated for 2 hr. at 37° C. and transferred to the refrigerator for 19 hr. and left for 3 hr. on the bench at room temperature prior to reading. The tubes were inspected by naked eye and by × 10 lens and the results recorded by a standard method (Felix, 1938).

Commercial agar agar

The specimens of agar used were drawn from various sources as shown in Table 2.

Table 2. *Varieties of agar used*

Type	Form	Trade name	Source
Japanese	Fibre	—	Stafford Allen and Sons Ltd.
Japanese	Fibre	—	Potter and Clark Ltd.
Japanese	Fibre	Kobe No. 1	W. J. Bush and Co.
American	Powder	Bacto Agar	Difco Laboratories
American	Powder	—	Conray Products Corp.
New Zealand	Powder	—	Davis Gelatine Ltd.

RESULTS

Estimation of gel rigidity

The first attempt to produce a satisfactory method of testing agar was made with a glass rod of 3 mm. diameter instead of the glass slide described previously. This was found to be unsuitable, since variation in the thickness of the agar produced considerable variation in the 12 sec. values (Table 3).

Table 3. *Large increase in* 12 *sec. values of agar plates of increasing depth using a glass rod for penetration*

(Medium: 2% Conray powder in digest broth A.V.L. 10/49 at pH 7·4)

Depth of agar plate (mm.)	12 sec. value (g.)
3·3	69·0
4·7	82·5
7·0	90·3
7·5	90·0
9·7	98·0

It was found that a circular glass rod of this dimension did not penetrate the agar by crushing it, but split the surface, producing a star-shaped fracture. Since the force required to produce such a fracture would be influenced by the thickness of the material fractured, this would account for the variation produced. A thinner rod was found to penetrate the agar without producing visible radiating fractures

in the surrounding agar. Such a small object, however, required a very small force to penetrate; consequently the difference in weight required to penetrate in 5 sec. from that required to penetrate in 20 sec. was of the order of a fraction of a gram. This was too small to be conveniently used.

The straight edge of a microscope slide was found to penetrate agar gel without producing radiating fractures in the surrounding agar and, by altering the length of the edge, a convenient size of 1 cm. × 1 mm. was found to give the most satisfactory results. It was found experimentally that the 12 sec. values over a wide range of agar depth were very close indeed (Table 4).

Table 4. *Small increase in* 12 *sec. values of agar plates of increasing depth using a glass slide for penetration*

(Allenbury agar 2·5% A.V.L. broth 10/49 at pH 7·4.)

Depth of agar plate (mm.)	12 sec. value (g.)
4·5	60·0
6·5	63·0
10·0	65·0

The repeatability of the test was demonstrated by pouring replicate plates with agar concentrations of 1, 2 and 3%. From these figures the standard error of the method at different levels of agar rigidity was estimated (Table 5).

Table 5. *An estimation of the* 12 *sec. values for gel rigidity of replicate plates at different levels of agar concentration, and the standard error of the results obtained*

(Medium: bacto-agar in Difco dehydrated broth 20·0 g./l. adjusted to pH 7·4.)

Percentage agar ...	1% (g.)	2% (g.)	3% (g.)
Plate I	22·5	68·0	124·0
Plate II	24·0	72·5	132·5
Plate III	27·0	76·3	127·0
Plate IV	22·0	70·0	123·5
Plate V	24·0	74·3	133·4
Plate VI	25·0	73·0	128·0
Standard deviation	1·65	2·72	3.80
Coefficient of variation	6·9	3·8	2·9
Standard error of mean	0·67	1·11	1·55

It was found by trial and error that the minimum drying necessary to ensure that plates prepared for testing would have a dry surface was 2 hr. This was adopted as a standard, since any increase in the time alloted for drying increased the 12 sec. value (Table 6).

After drying the plates it was found necessary to leave them at room temperature with the lids slightly open to allow thermal equilibrium to be attained, since the rigidity of the gel was materially altered by temperature (Table 7).

It was, moreover, discovered that the temperature at which the plates were poured altered the rigidity of the gel obtained. It was evident that pouring at

temperatures close to 100° C., when the agar had just been removed from the autoclave, produced a more rigid plate and pouring at low temperatures produced plates which were a good deal softer. The most constant results were obtained if plates were poured at 60–70° C. (Table 8).

Plates poured, dried, and stored in the refrigerator did not materially alter in rigidity after several days.

Table 6. *Influence of drying time on the* 12 *sec. value of agar plates*

(Medium: 1·25 % New Zealand agar batch 9/47 in Difco dehydrated broth 20 g./l. at pH 6·8.)

Drying time (hr.)	12 sec. value (g.)
2	56·0
4	60·0
8	66·5
24	74·0

Table 7. *The effect of temperature on* 12 *sec. value*

Medium	Temperature (° C.)	12 sec. value (g.)
2 % Stafford Allen agar, in digest broth, A.V.L. 10/49, pH 7·4	4	39·3
	20	31·5
	37	27·5
2 % Kobe no. 1, in digest broth, A.V.L. 10/49, pH 7·4	4	74·0
	20	61·7
	37	43·0

Table 8. *The effect of pouring plates at different temperatures*

(Medium: 2 % Kobe no. 1 in digest broth, A.V.L. 10/49, at pH 7·4.)

Temperature (°C.)	12 sec. value (g.)
98	72·0
82	61·0
66	60·5
50	55·0

Factors influencing gel strength

It was found that the composition of the diluent used to make up the agar gel had some influence on the gel rigidity. With Japanese agars, neutral distilled water gave a gel with an appreciably higher 12 sec. value than did broth. With New Zealand agar, however, neutral distilled water as a diluent produced a gel lower in value than that obtained when any nutrient medium was used as a diluent. The effect on the rigidity of agar gels of five different varieties of peptone as a diluent was tested. Made up in 1 % solution with distilled water and the pH adjusted to 7·3, they were tested with four varieties of agar, two Japanese, one American, and one from New Zealand. The various peptones did not materially alter the rigidity of any agar from the values obtained in digest broth.

The influence of pH was tested by making up 2 % agar media in digest broth

which was adjusted to pH's 6·0, 7·0 and 8·0. An appreciable difference was found between the 12 sec. values of these specimens. The lowest values were obtained at pH 6·0, and the highest at 8·0. The effect was most marked in New Zealand agar (Table 9).

Table 9. *The effect of* pH *on the final rigidity of agar media*

Medium	pH 6·0 12 sec. value (g.)	pH 7·0 12 sec. value (g.)	pH 8·0 12 sec. value (g.)
2% Kobe no. 1 agar in digest broth, A.V.L. 12/49	53·0	61·0	65·5
2% Stafford Allen agar in digest broth, A.V.L. 12/49	20·5	29·5	36·5
1% New Zealand agar batch 9/47 in digest broth, A.V.L. 12/49	38·0	48·3	64·5

The effect of dissolving agar by steaming or by autoclaving was investigated. It was found that steaming for 2 hr. was inadequate for New Zealand agar and for one Japanese type (Kobe no. 1), but adequate for the other agars tested. Autoclaving at 10 lb. for 15 min. gave maximum rigidity in all the agars tested.

The effect on colonial morphology of different agar gel rigidities

Serial concentrations (1–3%) of five commercial agars were made up with digest broth AVL 10/49 at pH 7·4 and plates poured. Each plate was then inoculated with a smooth virulent culture of *Salmonella typhi* strain TY 2. After incubation at 37° C. for 18 hr. the morphology of the colonies on the plates was examined and noted and the plates were then tested for their 12 sec. value (Table 10).

At some point in each of these five series it was noted that the colonies developed changes of morphology normally associated with S–R variation. This was first apparent as a change from a glossy to a matt surface with the appearance of a very slight irregularity of the edge of the colony. With increasing degrees of rigidity of the agar used, this change became more marked. On agars with high rigidity values the colonies were reduced in size, became 'heaped up', with a conical cross-section, and the surfaces became granular or coarsely wrinkled. As the rigidity of the agar was increased the cross-section of the colonies changed from flat, low convex to dome-shaped and finally to the conical form. Concurrently with this change in surface and cross-section, the edge passed from entire to slightly irregular and finally to a fimbriated or frilly appearance.

It was also observed that in each series the change in appearance of colonies at each concentration bore a closer relationship to the 12 sec. values at the same concentration than it did to the actual quantity of agar present. Repeat tests of this nature with the same and other strains of *Salmonella* and *Shigella* gave consistent results.

Table 10. *The influence of different agar rigidity and different types of agar on colonial morphology*

Percentage agar ...	1%	1·5%	2·0%	2·5%	3·0%
W. J. Bush, Kobe no. 1, Japanese fibre	11·0 g. (just stiff)	31·5 g.	60·0 g.	70·0 g.	106·0 g.
	S.	S.	S.R.	S.R.	S.R.
Potter and Clarke, Japanese fibre	7·2 g. (just stiff)	22·0 g.	40·5 g.	51·5 g.	72·0 g.
	S.	S.	S.	S.	S.R.
Stafford Allen, Japanese fibre	— soft, some growth in agar	15·0 g.	28·3 g.	46·5 g.	55·0 g.
	S.	S.	S.	S.	S.R.
Conray Products, American Powder	24·0 g.	56·0 g.	80·0 g.	111·0 g.	135·0 g. –
	S.	S.	S.R.	S.R.	R.
Bacto-Agar, American Powder	23·0 g.	43·0 g.	70·0 g.	100·0 g.	124·0 g.
	S.	S.	S.R.	S.R.	R.

Medium: digest broth. A.V.L. 10/49, pH 7·4.
Gel rigidity: 12 sec. value in grammes.
Test organism.: *Salm. typhi* strain Ty 2.
S. = smooth glossy surface, entire edge. Variable curvature from low convex to dome shaped.
S.R. = varying degrees of early S–R appearance, matt or finely granular surface edge slightly irregular or crenated, dome shaped cross-section.
R. = coarsely granular or wrinkled surface, very irregular or fimbriated edge, conical cross-section.

The effect of varying the type of nutrient base added to the agar

Nutrient broths of seven different varieties were made up by adding New Zealand agar in serial concentration—1·0, 1·25, 1·5 and 2·0%. In each case the pH was adjusted to 7·4, plates were poured, and the 12 sec. value estimated. It was found that the 12 sec. value rose with increasing agar content in each series, as already observed. The 12 sec. value at each level of agar concentration was, however, not significantly altered by the type of nutrient broth added. Although the appearance of a spurious S–R change was observed, as previously described with increasing agar rigidity when the plates were inoculated, the kind of nutrient base did not affect the colonial morphology at any one level of agar concentration.

One batch of tryptic digest broth, however, persistently produced flat-topped colonies having a plateau-form on cross-section. This batch of broth produced this form of colony with all the organisms tested. The appearance could be abolished by the addition to the broth of 1% of several commercial peptones or of 1% Lab Lemco. No explanation of this phenomenon is offered.

The relationship between rigidity value, colonial morphology and agglutinability

Smooth strains of *Salmonella* spp. and *Shigella* spp. were plated on various agars of different rigidity values in a series of concentrations as before. After incubation, the colonial morphology on each plate was noted, and the 12 sec. value recorded. The plates were then washed off with physiological saline and the suspensions

obtained were tested for agglutinability. It was found that the agglutinability of suspensions grown on different agars at differing concentrations was the same, irrespective of the apparent S–R variation induced by the increasing agar rigidity (Table 11).

Two cultures of *Salm. typhi* strain TY 2 were inoculated on to a series of agar plates of increasing stiffness. One culture developed apparent S–R change at a lower level of agar rigidity than did the other. On washing off the two cultures it was found that the culture giving S–R appearance at the lower concentration of agar was O-agglutinable.

Two cultures of *Salm. californica*, one of which was agglutinable to a titre considerably above that of the titre of the serum used, were tested by this method. It was found that the hyper-agglutinable culture developed an S–R appearance at a lower level of agar rigidity than did the normally agglutinable culture (Table 12).

It was noted, however, that on nutrient agar with very low 12 sec. values colonies of cultures which were hyper-agglutinable presented a smooth appearance.

Some cultures of a feeble agglutinability were also observed to develop S–R appearance at a lower level of agar rigidity than would be expected. These cultures were often associated with the development of dwarf colonies (Table 13).

Typical rough cultures, i.e. those producing a deposit and clearing in broth and auto-agglutinability in physiological saline, did not present colonies with an entire edge even on agar with a very low 12 sec. value. It was only the intermediate S–R variants which were disguised in this way. Moreover, the intermediate hyper-agglutinable cultures tended to produce the flat-spreading type of colonies on very rigid agar, whereas the normally agglutinable smooth cultures developed the heaped-up conical appearance.

Occasional variations with some batches of agar

In the course of preparation of repetitions of the above tests it was noted that the majority of batches of agar gave very steady and constant results, whereas some batches of New Zealand agar at times produced inexplicable variations. Moreover, in an occasional series of tests employing New Zealand agar, the agar was completely liquefied. This occurred most commonly with distilled water as a diluent. Experimentally this could not be associated with the pH of the distilled water, nor with any particular batch of agar. It was not possible to associate this sudden liquefaction with any particular form of cleaning agent used in the preparation of the bottles, nor with the source of the distilled water, whether from metal or glass stills. It was found that water from the reservoir of the still or plain tap water gave a perfectly good gel on all those occasions when it was used. No reason for this behaviour could be found.

The blending of agars

The possibility of blending different batches of agar to give a constant product was investigated. It was found both with New Zealand agars of different batches, having different 12 sec. values, and with Japanese and American agars from

Table 11. *The agglutinability and colonial morphology of* Salm. typhi *grown on nutrient agar plates of different gel rigidity*

Agar	Concentrations (%)	12 sec. value (g.)	Colonial appearance	Serum dilutions, *Salm. typhi.* 901 'O' 43/46/1 anti-serum: 250	500	1,000	2,500	5,000	10,000	25,000	50,000	Saline control (%): 0·85	2·5	5·0
Japanese	1·5	29·5	S.	+++	++±	++	±	(+)	{±}	—	—	—	—	—
Kobe no. 1	2·0	58·0	S.R.	+++	++±	++	±	(±)	{±}	{±}	–	–	–	–
	2·5	70·5	S.R.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
	3·0	103·0	S.R.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
Japanese	1·5	16·4	S.	+++	++±	++	±	{±}	{±}	–	–	–	–	–
Stafford Allen	2·0	30·0	S.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
	2·5	47·0	S.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
	3·0	56·0	S.R.	++±	++±	++	±	(±)	{±}	–	–	–	–	–
American	1·5	43·0	S.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
Bacto-agar	2·0	68·0	S.R.	+++	++±	++	+	(±)	{±}	–	–	–	–	–
	2·5	101·0	S.R.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
	3·0	122·5	R.	+++	++±	++	±	(±)	{±}	–	–	–	–	–
Standard suspension, batch 921				+++	++±	++	±	(±)	{±}	–	–	–	–	–

Medium: digest broth A.V.L. 10/49, pH 7·4.
Test organism: *Salm. typhi.* strain 901 'O'.
Agglutinations read after 2 hr. in the incubator at 37° C., 19 hr. in the refrigerator and 3 hr. room temperature.

+++ =complete agglutination with water clear supernatant.
++± to + =degrees of incomplete agglutination, supernatant fluid turbid.
± =weakest degree of agglutination visible to the naked eye.
(±) =trace } visible with ×10 hand-lens.
{±} =faint trace }

Table 12. *The effect of different concentrations of agar on the colonial appearance and agglutinability of two cultures of* Salmonella california

(Medium: Difco dehydrated broth 20 g./l., pH 6·8, New Zealand agar. Batch 39. Test organism *Salm. california.*)

Agar concentrations (%)	12 sec. value (g.)	Colonial appearance	Serum dilutions, *Salm. abortus-equi* 'O' 50/45/2 anti-serum								Saline controls (%)		
			20	50	100	150	200	250	300	500	0·85	2·5	5·0
Normally agglutinable culture													
1	17·5	S.	+++	++	(±)	{±}	−	−	−	−	−	−	−
1·25	26·0	S.	+++	++	(±)	{±}	−	−	−	−	−	−	−
1·5	40·0	S.	+++	++	(±)	{±}	{±}	−	−	−	−	−	−
1·75	54·2	S.R.	+++	++	(±)	{±}	−	−	−	−	−	−	−
2·0	63·5	S.R.	+++	++	(±)	{±}	−	−	−	−	−	−	−
Hyper-agglutinable culture													
1	17·0	S.	+++	+++	++	+	±	(±)	{±}	−	−	−	{±}
1·25	24·5	S.R.	+++	+++	++	+	±	(±)	{±}	{±}	−	−	−
1·5	42·6	S.R.	+++	+++	++	+	±	(±)	{±}	−	−	−	−
1·75	55·0	R.	+++	+++	++	+	±	(±)	{±}	−	−	−	{±}
2·0	64·0	R.	+++	+++	++	+	±	(±)	{±}	−	−	−	−
Control suspension: *Salm. california*			+++	++	(±)	{±}	−	−	−	−	−	−	−

Table 13. *The effect of different concentrations of agar on the colonial morphology and agglutinability of two cultures of* Shigella boyd I

(Medium: Conray agar powder in digest broth A.V.L. 12/49, pH 7·4. Test organism *Sh. boyd* I (170).)

Agar concentrations (%)	12 sec. value (g.)	Colonial appearance	Serum dilutions, *Shigella boyd I* 43/42/14 anti-serum										Saline controls (%)		
			100	250	500	1,000	1,500	2,500	5,000	10,000	25,000	50,000	0·85	2·5	5·0
Normally agglutinable culture															
1	26·2	S.	+++	+++	+++	++	+	±	(±)	{±}	−	−	−	−	−
2	74·8	S.R.	+++	+++	+++	++	+	±	(±)	{±}	−	−	−	−	−
3	108·5	S.R.	+++	+++	+++	++	+	±	(±)	{±}	−	−	−	−	−
Hypo-agglutinable culture															
1	26·5	S.R.	+++	+++	++	+	±	(±)	{±}	−	−	− −	−	−	−
2	75·6	R.	+++	+++	++	+	±	{±}	{±}	−	−	−	−	−	−
3	110·0	R.	+++	+++	++	+	±	(±)	{±}	−	−	−	−	−	−
Control Suspension: *Sh. boyd I* (170)			+++	+++	+++	++	+	±	(±)	{±}	−	−	−	−	−

different sources, and also with mixtures of all types, that blending could be satisfactorily carried out on a simple arithmetical basis. Blends with a definite value could be produced by mixing different samples in calculated proportions. It was found that, if two agars of different gel rigidity were mixed in equal proportions to a given percentage, the sum of half the 12 sec. values of each of the two agars at that given percentage, was equal to the 12 sec. value of the mixture obtained experimentally. Likewise different proportions of different agars gave experimental values when mixed that could be calculated on the same basis (Table 14).

Table 14. *Blending of agar*

Batch no. of agar	12 sec. value of 2% agar in distilled water (g.)	Concentration of constituents (%)	Percentage 12 sec. value (g.)	Sum of percentage 12 sec. value (g.)	Experimental result (g.)
Blending of New Zealand agar in equal proportions					
B. 39	50·0	50	25·0	77·8	79·0
B. 9/7	105·5	50	52·8		
Blending of New Zealand agar in different proportions. Final concentration to be 2% in a ratio of B. 39 : B 9/7 of 1 : 3					
B. 39	50·0	25	12·5	91·6	93·0
B. 9/7	105·5	75	79·1		
Blending of three different agars in equal proportions to a total of 2% in distilled water					
Kobe no. 1	65·0	33·3	21·7	63·1	59·6
Conray	90·4	33·3	30·1		
Stafford Allen	34·0	33·3	11·3		

DISCUSSION

It is widely accepted that the antigenic state of organisms of the *Salmonella* genus is reflected in the colonial morphology of these organisms on solid agar media (Arkwright, 1920, 1921, 1924; Wilson, 1930*a*, *b*). The change from the smooth to the rough form of colony has become accepted as indicative of the loss of those specific polysaccharides associated with the antigenic structure of the organisms. The principal of selection of smooth colonies in the preparation of potent vaccines and in the manufacture of standard diagnostic suspensions for agglutination tests is well established.

Among freshly isolated strains of *Salmonella* and *Shigella* there is considerable variety of colony form, but laboratory cultures selected over long periods tend to develop a uniform colonial appearance. The value of stock laboratory cultures for vaccine or suspension production is judged by the number of irregular forms they show, a high proportion of colonies showing S–R change and the production of dwarf colonies indicating an unsatisfactory culture. Since these laboratory cultures are as a whole uniform, only small S–R changes are as a rule observed. From the experiments described above it is evident that a small increase in the 12 sec. value of the solid medium used for checking the culture will change the colonial appearance in such a way as to mislead the observer into concluding that the culture is unsatisfactory for use. Moreover, the converse is also true with the result that cultures exhibiting early S–R change may be inadvertently passed as satisfactory if the agar medium used is too soft.

It is evident that strictly standard batches of agar should be used for the study of colonial morphology. That this is possible has been demonstrated by experiments in blending.

In the production of solid media for the study of colonial morphology a strict routine should be adhered to, especially with regard to processing of the medium and the pouring and drying of agar plates.

It is suggested that before a new batch of agar is brought into use it should be tested by some standard method to ensure that known stock cultures will produce on it a typical appearance.

SUMMARY

1. The rigidity of agar gel in poured agar plates can be tested by a comparatively simple and easily repeatable method.

2. Commercial agar from different sources varies in the rigidity of gel it produces at a given concentration.

3. The rigidity of gel produced by any agar at a given concentration is affected by processing and by the mode of production of poured plates and to some extent by the diluent used.

4. The appearance of a spurious S–R change in colonies of *Salmonella* and *Shigella* on media with an increasing agar content is a measure of the rigidity of the agar gel used and not directly of the agar concentration of the medium.

5. The appearance of this spurious S–R change induced by rigid agar bears no relation to the agglutinability of the organism.

6. Both increased or decreased agglutinability of cultures of *Salmonella* and *Shigella* is associated with the appearance of colonies of an apparently rough morphology at a lower level of agar rigidity than that at which spurious S–R change develops in a normally agglutinable culture.

7. Without some standardization of the rigidity of the gel used in agar plates, erroneous conclusions may be drawn regarding the antigenic state of the organisms under test.

8. Samples of agar of differing gel rigidity can be blended to form a product producing a gel of a calculated 12 sec. value.

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