Observations on the Differential Inhibition of Coliform Bacilli and Rough Variants of Intestinal Pathogens

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SUMMARY: Differential inhibition of Gram-negative bacilli of intestinal origin appears to depend upon a combined action of bile salts and electrolytes. This occurs only under certain conditions, among which aerobic incubation is one and the nature of the surface upon which the cultures are grown is another.

During the recent war desoxycholate could not be obtained in Great Britain and a modified Leifson's agar medium (1935) was prepared, substituting 'taurocholate' for desoxycholate, rosolic acid for neutral red and a peptic digest of sheep serum for the pork infusion peptone nutrient base. This medium gave adequate inhibition of *Bacterium coli* (*Escherichia coli*), while allowing good growth of smooth variants of intestinal pathogens. Rough variants of these micro-organisms were markedly inhibited.

Materials and methods

In all experiments the reaction of the media was standardized at pH 7·6. Plates were dried for 30 min. at 37° before use. The six strains of micro-organisms tested were inoculated thus: A 3 mm. loopful of a 24 hr. agar culture was suspended in 9 ml. of distilled water and well mixed; this suspension was stroked on the plates by dipping into it a T-shaped piece of 0·018 in. gauge chrome iron wire, the width of the spreading surface being 3 mm. This inoculation of a small quantity of a well-diluted culture was adopted to ensure that only a minimal amount of nutrients would be carried over from the parent growth.

The taurocholate used was the sodium tauro-glycocholate marketed by British Drug Houses Ltd., of B.P.C. standard. All quantities quoted as % subsequently refer to the weight of the substance in 100 ml. of solution, i.e. % (w/v).

The following strains of organism were used for testing the variously compounded media:

SR, a rough variant of a recently isolated Sonne III bacillus.

SS, a smooth variant of the same micro-organism.

C1, *Bacterium coli communis* from human faeces.

C2, *Bact. coli communior* from human faeces.

A1, *Aerobacter* sp. from milk.

A2, *Aerobacter* sp. from water.

The standards adopted for comparing the inhibitions of growth were: (a) 'partial differentiation' which means that strains SR, SS, A1 and A2 grew, but C1 and C2 failed; (b) 'complete differentiation' means that SS grew, but all others failed to do so.

Comparison of taurocholate and desoxycholate media

The first experiment was designed to compare desoxycholate and taurocholate in presence of citrate. Four batches of modified Leifson's agar medium (Brodie, 1942) were prepared, all containing bile salts (0.5 %) and trisodium citrate (2.5 %), but without ferric ammonium citrate and rosolic acid (as it was desired to test only the essential constituents of the medium), as follows: A, serum peptic digest + taurocholate + citrate; B, serum peptic digest + desoxycholate + citrate; C, meat infusion peptone + taurocholate + citrate; D, meat infusion peptone + desoxycholate + citrate.

The results of one experiment were that all strains grew on medium C, that media A and D inhibited all but strain SS, and that medium B inhibited all strains. From this it would appear that taurocholate is less effective than desoxycholate as a differentiating agent, but, when the basal nutrient is peptic digest of serum, taurocholate can replace desoxycholate. Other results were not always so clear-cut, though similar ones were obtained on very many occasions. Occasionally the degrees of inhibition were less marked, media A and C giving partial differentiation, medium B complete differentiation and medium D growth of SR and SS only. From this it appears that changes of the basal nutrient might alter the effect of the bile salts. The difference between the media is therefore probably quantitative, not qualitative.

Effect of electrolytes

Leifson apparently used citrate to give the desired inhibition of *Bact. coli*, but he noted that sodium acetate and even sodium chloride produced a similar effect. He suggested that citrate was suitable because it forms a salt with desoxycholate. Media containing peptic digest of serum have a sodium chloride content of about 0.9 % (Brodie, 1942), which is rather more than is present in most media, and it is probably upon this high salt content that the satisfactory results obtained with the modified Leifson's medium depends.

Sodium citrate in absence of bile salts. When sodium citrate was used in the absence of bile salts inhibition occurred with increasing citrate content but was not distributed among the strains tested in the same order as that produced by bile salts + citrate. All six strains grew in the presence of 4 % citrate; only SR and C2 failed to grow on exposure to 5 % citrate. It seems, therefore, that the bile salts in the medium do not merely enhance the action of electrolytes.

Sodium citrate, sodium aconitate and sodium fumarate. Varying concentrations of these sodium salts, representing two trivalent and one divalent organic anions, were used with a medium in which the peptic digest of serum was diluted to one-quarter the strength employed in routine work. The diluted digest was used because preliminary tests of quarter-strength digest as the basal nutrient in presence of 0.5 % taurocholate and 0.5 % NaCl yielded clear-cut partial differentiation with 2.5 % citrate and complete differentiation with 5 % citrate.

The same degree and distribution of partial differentiation was produced by 0.1 M-citrate, 0.1 M-aconitate and 0.2 M-fumarate; complete differentiation was

given by 0.2 M-citrate, 0.2 M-aconitate and 0.3 M-fumarate. As shown by Leifson, the effect of citrate is therefore not specific.

Investigation of salts in relation to their place in the Hofmeister series. The previous observations suggested that the influence of electrolytes might be largely physical and related to their position in the Hofmeister series. 'Swelling' or 'shrinking' of the agar upon which the organisms were grown, or a kindred effect upon the bacilli themselves, might be a factor determining the differential inhibition.

Table 1. *Molar concentrations of salts giving differentiation when added to 2% agar medium containing quarter-strength peptic digest of serum and NaCl (0.5%) in presence of (a) taurocholate 0.5%, and (b) desoxycholate 0.5%. Plates incubated aerobically at 37.5°*

	Concentration of salts needed for	
	Partial differentiation (M)	Complete differentiation (M)
(a) Ammonium citrate	0.057	0.1
Potassium citrate	0.066	0.12
Sodium citrate	0.1	0.2
Ammonium sulphate	0.13	> 0.3
Potassium sulphate	> 0.2	—
Sodium sulphate	0.3	0.42
Ammonium chloride	0.43	0.48
Potassium chloride	0.5	0.57
Sodium chloride	0.53	0.66
Ammonium acetate	0.125	0.166
Sodium acetate	0.33	0.5
(b) Ammonium citrate	0.04	0.06
Sodium citrate	0.08	0.12
Sodium sulphate	0.18	0.33

Various salts (Table 1) were added in varying concentrations to the quarter-strength peptic digest agar containing taurocholate 0.5% and NaCl 0.5% (which is the NaCl content of Saunders, Dorfman & Koser's (1941) synthetic medium). Table 1(a) records the molar concentrations of electrolyte required for partial and complete differentiation. Similar tests were made with desoxycholate 0.5% in place of taurocholate (Table 1(b)); the results indicate that desoxycholate differs quantitatively from taurocholate in its action.

A further test was made of meat infusion peptone agar of unknown electrolyte content as nutrient. Both taurocholate and desoxycholate were used. The results were substantially the same as those with the peptic digest of serum except that slightly higher molar concentrations were required. Since with both nutrient media desoxycholate was more active than taurocholate it is possible that both the bile salts acted in the same way. It is possible that the activity of the serum peptic digest as compared with the meat infusion peptone medium was due to its higher NaCl content. Complete differentiation was obtained in presence of 0.75 M-NaCl in quarter-strength peptic digest containing 0.5% taurocholate.

These observations suggest that there is some correlation between the position of the various salts in the Hofmeister series and their power to inhibit coliform bacilli and rough variants of the intestinal pathogens in presence of bile salts.

Effect of the nutrient medium

The two basal media so far employed were substantially the products of peptic digestion. The effect of taurocholate and citrate added to other types of nutrient was tested as follows. Nutrient agars were prepared with (1) Douglas broth; (2) tryptic digest of serum; (3) tryptic digest of casein as used for the preparation of Gladstone & Fildes (1940) 'CCY' medium; (4) acid hydrolysate of casein as prepared for the same medium; (5) yeast extract as used in 'CCY' medium; (6) the complete 'CCY' medium.

Complete differentiation was obtained in the presence of 0.5 % taurocholate and 5 % citrate with all the nutrient basal media tested except the complete 'CCY' medium which, containing sodium glycerophosphate and sodium lactate, required only 3.75 % of citrate. Provided a nutrient medium is adequate to support the growth of the strains tested, the differential effect can be obtained by adjusting the electrolyte content of the medium in the presence of bile salts. When the salt mixture of Saunders *et al.* (1941) was incorporated in agar containing 0.5 % taurocholate and 2.5 % citrate, and the NaCl content raised to 0.9 and 1.3 %, partial and complete differentiation respectively were obtained.

Fractionation of peptically digested serum

Ethanol fractionation. Various ethanol fractions of the peptically digested serum were tested in an attempt to define the distribution of any factor which might influence the differential effect. Material soluble in 70 % (v/v) ethanol-water was an excellent nutrient medium, as sharp in its differential action in the presence of 0.5 % taurocholate and 2.5 % citrate as the crude digest. The corresponding precipitate was a poor nutrient and devoid of differentiating activity. The fractions soluble in 80, 90 and 100 % (v/v) ethanol when used to compound media showed some differentiating action, but full activity was regained only when the corresponding filtrates and precipitates were again mixed.

Ash from peptic digest. The ash from a known quantity of peptically digested serum was reconstituted to the original volume. When quarter-strength peptic digest of serum was replaced in agar media by its content of ash in the presence of 0.5 % taurocholate and 2.5 % citrate, partial differentiation was obtained of the same degree as was achieved by using quarter-strength digest, 0.5 % taurocholate and 2.5 % citrate. The indication was that the differentiating power of the medium was dependent upon a suitable balance of nutrients, bile salts and electrolytes.

Surface-active agents other than bile salts

Because of their surface activity the influence of the bile salts might be due to physical rather than to physiological effects. Other surface-active agents were therefore investigated. Sodium oleate, sodium ricinoleate, sodium oleyl

sulphate, Tergitol, sodium succinate and Aerosol OT (diethylsulphosuccinate), all having surface-active anions, and sapamine, having a surface-active cation, were compared with bile salts in the presence of peptic digest of serum of routine strength (NaCl 0.9 %). With 2.5 % citrate and the surface-active agents in dilutions of 1/300–1/100, not even partial differentiation was obtained. It was noted, however, that: (a) strain SR was more susceptible than SS to ricinoleate, Tergitol, Aerosol OT and sapamine; (b) strains C1 and C2 showed some effect with Tergitol and Aerosol OT; (c) strains A1 and A2 were uninfluenced by any of the agents tested.

Similar experiments were conducted with 1/200 dilutions of the surface-active agents and different concentrations of citrate up to 5 %. Strain SR was inhibited by all the agents except sodium oleyl sulphate and sodium succinate above a citrate concentration of 2.5 %, but growth of the *Aerobacter* spp. remained maximal. It seems possible that the inhibition of SR as compared with SS may have been due to a combined effect of electrolyte and surface active agent. This does not hold for the other micro-organisms tested except that the commercial preparations Tergitol and Aerosol OT inhibited strains C1 and C2 when the concentration of citrate was increased. The action of bile salts differed from that of the other surface-active agents in that only bile salts inhibited the *Aerobacter* strains in presence of citrate.

The effect of air and the nature of the surface

In the experiments so far described the inoculations were made on the surface of 2 % agar media. The inhibition might be affected by the exposure to air or to a surface condensation of some of the constituents of the medium.

Anaerobic cultivation. Tubes of routine bile salt citrate medium (Brodie, 1942) were prepared and, when the surfaces were dry, the six cultures were investigated by stab inoculation. After 24 hr. at 37.5° the zone of visible growth extended up to and over the surface only with strain SS. With strain A2 it just reached the surface but not with the others. After 4 days' incubation A2 began to grow on the surface, and by this time strains C1, C2 and A1 had reached the surface. On the 5th day strain SR reached the surface and grew thereon, strains C1, C2 and A1 still failing to give surface growth.

Plates were made using routine strength peptic digest of serum (NaCl, 0.9 %) and 2.5 % citrate with added taurocholate (0.5 %) or desoxycholate (0.5 %). A similar set was made with meat extract peptone nutrient medium. After inoculation with the six strains of micro-organisms used, duplicate sets of plates were incubated aerobically and anaerobically. Only under aerobic conditions of incubation was the differential inhibition obtained.

Effect of varying the gel. All culture media so far employed contained agar as the gelling agent. A series of comparative tests was made with a number of different gelling agents. The quarter-strength peptic digest medium containing NaCl 0.5 %, taurocholate 0.5 % and citrate 2.5 % was solidified with: (a) agar 2 %, (b) substitute agar prepared from *Gigartina stellata* 2 %, (c) gelatin

15 %, (d) mixtures of agar and gelatin, and (e) silica gel. Aerobic incubation was used throughout.

With (a) and (b), at 22 and 37°, only SS grew well, SR, C1 and C2 were inhibited and growth of A1 and A2 was minimal. When gelatin was the solidifying agent the effect was reversed, SS growing least well of the six cultures tested. With gelatin, NaCl 0.5 % and citrate 2.5 %, in the absence of the peptic digest basal medium, the addition of taurocholate enhanced the nutrient quality of gelatin for the strains tested, whereas the inhibitory action of citrate was greater in the absence than in the presence of the nutrient base.

Table 2. *Degrees of differentiation obtained with agar, gelatin and mixtures thereof in the presence of serum peptic digest diluted 1 in 4 of that used in routine work (NaCl content adjusted to 0.5 %), 0.5 % taurocholate and 2.5 % sodium citrate. Plates were incubated aerobically at 22°*

Agar (2 %) parts	Gelatin (15 %) parts	Duration of incubation (days)	Strains tested					
			SR	SS	C1	C2	A1	A2
12	Nil	2	—	+	—	—	(+)	(+)
		8	—	+	—	—	+	+
10	2	2	5	+	—	—	+	+
		8	5	+	—	—	+	+
8	4	2	—	+	—	+	+	+
		8	9	+	±	+	+	+
6	6	2	3	+	+	+	+	+
		8	±	+	+	+	+	+
4	8	2	—	+	+	+	+	+
		8	—	+	+	+	+	+
2	10	2	—	—	+	+	+	+
		8	—	—	+	+	+	+
Nil	12	2	—	—	+	+	+	+
		8	—	—	+	+	+	+

Figures indicate number of colonies.

— indicates no growth.

±, ±, (+) to + indicate minimal to maximal growth.

Mixtures of agar and gelatin in presence of the other constituents yielded interesting results. These are shown in Table 2. Agar alone gave almost complete differentiation, gelatin allowed only the coliforms to grow; intermediate degrees of differentiation depended upon the amounts of each of the gelling agents present.

The findings when gelatin was used show that the influence of oxygen on differential inhibition was not manifested with all gelling agents.

Silica gel was prepared from commercial water-glass after the method of Anderson & Macsween (1942), but using hydrochloric in place of phosphoric acid for neutralization, and incorporated with quarter-strength peptic digest containing 0.5 % NaCl. Citrate (2.5 %) alone inhibited only strains SR and C2, a result comparable to that obtained with agar and the same salts. Taurocholate 0.5 % in the absence of citrate in silica gel inhibited all strains tested.

To obtain complete differentiation, it was found necessary to add only quarter-strength peptic digest of serum and 0.06 % taurocholate to the silica. It would appear that the high electrolyte content of commercial water-glass influences the differential inhibitory action of bile-salt media in which it is the solidifying agent. By adjusting the amount of bile salt, media prepared with it give the same differential inhibition of the micro-organisms tested as do agar media.

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