FORMATE LACTOSE GLUTAMATE: A CHEMICALLY DEFINED MEDIUM AS A POSSIBLE SUBSTITUTE FOR MACCONKEY BROTH IN THE PRESUMPTIVE COLIFORM EXAMINATION OF WATER

BY R. D. GRAY

Director, Public Health Laboratory, Newport, Mon.
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INTRODUCTION

The urge of water bacteriologists to find a synthetic replacement for MacConkey broth (bile-salt lactose peptone water) sprang from no criticism of its optimum performance but rather from the desire to match this, if possible, by a consistently reproducible medium.

Having described his bile-salt media (MacConkey, 1900; MacConkey & Hill, 1901; MacConkey, 1904, 1905), MacConkey (1908) stated that 'bile-salt broth is quite as good as any other medium for water analysis'. This opinion was amply confirmed at that time and is widely held now, 50 years later. During this period MacConkey broth, virtually without modification, has been in general use here and elsewhere and is still the standard medium officially recommended in this country (Ministry of Health, 1956) for the primary isolation of coliform bacteria from water. The experiments of Olsen (1952), moreover, showed that for promoting coliform growth and for milk analysis, MacConkey broth equalled in performance the three best of the other ten media tested; and the comparative study by Jebb (1959) at least suggests that in the presumptive coliform test of water MacConkey broth is in no respect inferior, but in some respects superior to the two standard American media, lactose broth and lauryl tryptose broth (American Public Health Association, 1955) and to Teepol broth (Jameson & Emberley, 1956).

Long experience with MacConkey broth, however, has exposed the weakness, for quantitative purposes, of a medium containing such variable biological constituents as bile salt and peptone. Different peptones vary considerably in their ability to promote gas-production, and the inhibitory power of bile salts on the non-coliform flora can vary even more widely. It is possible by standardization procedures (Burman, 1955) to ensure that in any one laboratory the medium maintains a fairly uniform performance; but, though such precautions should always be taken, no such uniformity can be expected in different laboratories. This disadvantage of variability, although particularly notable in bile-salt media, applies in some degree to all peptone-containing media. It is considerations such as these, and also the rising cost of the biological ingredients, that have prompted bacteriologists to seek a chemically defined medium.

Burman & Oliver (1952) showed that the glucose glutamic-acid medium devised by Folpmers (1948) for anaerobic use could be employed aerobically in the same way as MacConkey broth for the presumptive coliform examination of water

samples. In a series of trials they compared MacConkey broth with (1) Folpmers's medium, and (2) a modification they introduced whereby the glucose was replaced by lactose. They found that both these glutamic-acid media produced a slightly higher yield of *Escherichia coli* (*Bacterium coli*, type I), but that in the lactose modification the results were slow to appear and the total number of presumptive positive tubes was reduced. They concluded that, whereas both glutamic-acid media were slightly superior to MacConkey broth as regards the yield of *Esch. coli*, the lactose modification was less satisfactory because *Esch. coli* grew more slowly and the other coliform organisms grew less readily in it than in MacConkey broth.

A more extensive investigation reported by the P.H.L.S. Water Sub-committee (1958) confirmed Burman and Oliver's findings and further indicated that the glucose glutamic-acid medium, besides producing the highest yield of *Esch. coli* and of other true coliform organisms, gave rise to an excessively large number of 'false positive' tubes, i.e. presumptive positive tubes from which no true coliform organisms could be isolated. The lactose glutamic-acid medium produced an equally increased yield of *Esch. coli* with very few false positive reactions, but all the results appeared more slowly and the number of true coliform organisms other than *Esch. coli* was seriously reduced. Although the comparisons revealed a hitherto suspected, yet unproved, imperfection in MacConkey broth—that to some slight extent it suppressed both *Esch. coli* and other true coliform organisms—neither of the synthetic media could be recommended as a completely satisfactory substitute; the glucose medium introduced too many false positive results and the lactose medium proved too inhibitory to true coliform organisms other than *Esch. coli*. The Water Sub-committee suggested in their report (1958) that further modifications should be sought, either to render the glucose medium more inhibitory to the non-coliform flora or, preferably, to make the lactose glutamic acid less inhibitory to true coliform organisms. The present communication describes an examination of certain modifications of the lactose medium with this object in view.

MATERIALS AND METHODS

These refer particularly to the investigations described under Section A. Certain modifications were introduced for the experiments set out under Section B; these are indicated in the appropriate places of the text.

Choice of media

The following four media were chosen for a comparative trial: (1) MacConkey broth; (2) formate lactose glutamate (pH 7·5); (3) lactose glutamate (pH 7·5); (4) glucose glutamic acid (pH 6·0).

MacConkey broth was inevitably included as the standard medium in general routine use in this country. Glucose glutamic acid (pH 6·0) i.e., Folpmers's medium, was chosen because in the Sub-committee trials it had given the highest yields both of *Esch. coli* and of other coliform organisms; moreover, the heavy excess of false positive reactions produced by it had been found at only two (London and Newport) of the six participating laboratories. Lactose glutamic acid (pH 6·0),

i.e. Burman and Oliver's modification of Folpmers's medium, was omitted since there was already enough evidence that it is too inhibitory, but the inclusion of lactose glutamate (pH 7·5) would, it was felt, be useful in indicating whatever advantage was conferred by the formate in the formate lactose glutamate medium.

Preparation of media

Since the comparisons were to be made on four of the five 10 ml. portions of each water sample examined, the four media were made up at double strength and distributed in 10 ml. volumes in $6 \times \frac{3}{4}$ in. test-tubes containing inverted inner (Durham) tubes. The remainder of each routine examination was to be conducted with MacConkey broth and this was distributed also at double strength in 50 ml. volumes and at single strength in 5 ml. volumes according to the usual custom.

MacConkey broth (single-strength and double-strength) was prepared as officially recommended (Ministry of Health, 1956). The peptone (Evans's) had been chosen from previous trials for its optimal gas production, and the bile salt (Ward and Blenkinsop sodium tauroglycocholate, Batch No. 18952) had been adopted by the Public Health Laboratory Service as possessing adequate inhibitory power.

The glutamic-acid media (double-strength) were prepared along the lines described by Burman & Oliver (1952), viz:

	Glucose glutamic acid	Lactose glutamate	Formate lactose glutamate
Glucose	20 g.	—	—
Lactose	—	20 g.	20 g.
L(+) glutamic acid	10 g.	10 g.	10 g.
Sodium formate	—	—	0·5 g.
Potassium phosphate (K_2HPO_4)	6 g.	6 g.	6 g.
Ammonium lactate 50 % (w/w) solution	20 ml.	20 ml.	20 ml.
Tap water	1000 ml.	1000 ml.	1000 ml.

The glutamic acid was dissolved in the water and neutralized with sodium hydroxide, the lactate and phosphate were added and the whole was heated by steaming. After filtration, the glucose was added and the medium adjusted to pH 6·0, or the lactose (with or without the formate) was added and the medium adjusted to pH 7·5. Brom-cresol purple (1 % alcoholic solution) was added as indicator in the amount of 2 ml./l. After distribution the medium was sterilized by steaming for 45 min. on each of two successive days.

Sterilization of media

Since lactose in the concentration of 2 %, even in the presence of peptone but particularly when peptone is absent, is rather easily hydrolysed by heat, strict precautions were taken during sterilization. The double-strength MacConkey broth was autoclaved at 10 lb. for 10 min. only. Particular care was taken to ensure that the glutamic-acid media received no more than 45 min. steaming on each of the two successive days. Batches of the lactose media were tested by inoculation with salmonellae to confirm the absence of glucose.

Water samples and procedure

During the comparative trial all samples of water arriving at the laboratory for routine examination were set up as follows: 50 ml. into 50 ml. of MacConkey broth (double-strength); 10 ml. into 10 ml. of MacConkey broth (double-strength); 10 ml. into 10 ml. of formate lactose glutamate (double-strength); 10 ml. into 10 ml. of lactose glutamate (double-strength); 10 ml. into 10 ml. of glucose glutamic acid (double-strength); 10 ml. into 10 ml. of MacConkey broth (double-strength); 5×1 ml. (and smaller volumes) into five (or more) tubes of 5 ml. of MacConkey broth (single-strength).

The routine coliform examination was carried through in the ordinary way for assessing the hygienic quality of the water, but particular attention was devoted to the first four of the five 10 ml. tubes which will be solely considered here.

After inoculation, the tubes (without prior warming in a water-bath) were transferred to the 37° C. incubator. They were inspected after approximately 18, 24 and 48 hr. incubation. Tubes showing production of acid and gas (if necessary after gentle tapping or shaking) were recorded as presumptive positives.* These were at once further tested by: (1) subculture in MacConkey broth at 44° C. for 24 hr. (for gas production); (2) subculture in tryptophan peptone water (Taylor, 1955; Burman, 1955) at 44° C. for 24 hr. (for indole production); and (3) plating on to MacConkey agar for incubation at 37° C. for 24 hr. (in order to provide colonies for any subsequent testing).

Any presumptive positive tube giving a positive indole reaction and a positive fermentation test at 44° C. was regarded as containing *Esch. coli*. Occasionally the 44° C. gas test was positive while the 44° C. indole test was negative. In such instances the indole test was repeated at 37° C. by subculture from the MacConkey plate; a positive result was accepted as proving the presence of *Esch. coli* (see Burman 1955) and a negative result (after several days) interpreted as indicating one of the other true coliform organisms (e.g. an 'Irregular'). When no fermentation occurred at 44° C., several colonies (if there were any) from the MacConkey plate were subcultured into lactose peptone water. If, after 48 hr. incubation at 37° C., the lactose peptone water showed acid and gas, the presumptive positive tube was considered to contain true coliform organisms other than *Esch. coli*. If there were no colonies on the MacConkey plate or if, after 48 hr. incubation, the lactose peptone water failed to show acid and gas, the presumptive positive reaction was classified as a false positive reaction, i.e. a presumptive positive tube from which no lactose-fermenting coliform organisms could be isolated.

* In accordance with standard practice the minimal criterion for a 'positive' MacConkey tube was the presence of acid, together with a concavityful of gas in the Durham tube. Experience had shown, however, that with the synthetic media a reliable minimal criterion was the appearance of effervescent gas within the Durham tube (if necessary after shaking) even without detectable acid.

Section A. Formate lactose glutamate (pH 7·5)

EXPERIMENTAL

Folpmers adjusted his medium to a pH of 6·0 in order to inhibit extraneous bacteria, principally certain butyric acid organisms, which otherwise produced fermentation. When Burman and Oliver replaced the glucose by lactose they made no other change in the medium, and the comparisons described by them and by the Water Sub-committee all refer to glutamic-acid media adjusted to pH 6·0. It seemed possible that this low pH, although doubtless necessary for the glucose medium, might be unnecessary, and perhaps disadvantageous, in the lactose modification. Preliminary experiments with pure cultures of *Esch. coli* and of other coliform organisms showed that the lactose glutamic-acid medium adjusted to pH 7·5 (lactose glutamate) was noticeably less inhibitory; gas was produced earlier and in larger volume by all the *Esch. coli* and by most of the other coliform cultures. There remained, however, a few coliform strains which readily fermented MacConkey broth but failed to ferment the lactose glutamate medium in 48 hr. It appeared, therefore, that this medium would require some further component to promote gas production by these strains.

The first addition tried was sodium citrate (0·2 %), since citrate utilization is a distinctive property of the intermediate-aerogenes subgroup and Bardsley (1934) had shown that preliminary enrichment in citrate medium increased the yield of these organisms from human faeces. The only result was a *slowing* of the gas production.

It was then questioned whether the necessary help might come from some alteration in the salt content, or from the addition of a growth factor or of some further amino acids (Pinsky & Stokes, 1952; Gest, 1954), and the strains were tested in the lactose glutamate medium to which one or more of the following ingredients had been added in the concentration listed: (*a*) sodium chloride, 0·5 %; (*b*) magnesium sulphate, 0·2 %; (*c*) potassium phosphate (K_2HPO_4), 0·2 % (final concentration 0·5 %); (*d*) nicotinic acid, 0·001 %; (*e*) nicotinamide, 0·001 %; (*f*) L-arginine, 0·3 %; (*g*) L-aspartic acid, 0·2 %. These substances were tried singly and in various combinations but without any discernible benefit.

Finally, because of the observations of Stark & England (1935) who devised formate ricinoleate broth, sodium formate (0·5 %) was added and a material improvement resulted; gas was produced by all the organisms tested though not as quickly or abundantly as in MacConkey broth. The appearance of acid was masked and delayed by the buffering action of the formate. With lower concentrations of formate (0·2, 0·1, 0·05 and 0·025 %) there was no appreciable loss in gas production, but progressively less interference with the appearance of acid. With as little as 0·01 % of formate there was a noticeable improvement over the plain lactose glutamate medium, but the amounts of gas were about half those produced by the higher concentrations.

The lowest effective concentration (0·025 %) of formate was then tried in conjunction with the ingredients listed above, singly and in combination, but no further benefit could be detected. It was decided, therefore, to add sodium formate

(0·025 %) alone and to submit this medium (formate lactose glutamate) to a prolonged trial in comparison with other media in the presumptive coliform examination of routine samples of water.

The decision to use so low a concentration of formate was made for several reasons. The primary object of this trial was to determine whether the suppressive effect of lactose glutamic acid on intermediate-aerogenes organisms could be overcome without incurring an undue penalty in false positive reactions. At the higher pH the medium appeared to be reasonably satisfactory for *Esch. coli* and the commoner coliform organisms. All that seemed to be required of the formate, therefore, was the stimulation of the less active coliform organisms. By using the lowest concentration shown to be effective with the test coliform strains, it was hoped to achieve the desired result with the minimum masking of acid production and the minimum risk of stimulating false positives. In this latter connexion there was encouragement in the observations of Gest (1954) that added formate is converted to CO_2 and H_2 only by gas-producing strains and only in the presence of a fermentable carbohydrate and of certain amino acids of which glutamic acid is singly the most effective. Provided lactose was not hydrolysed during preparation of the medium, the formate should stimulate only the lactose fermenters to produce gas and not affect the glucose fermenters which, apart from the sporing anaerobes (P.H.L.S. Water Sub-committee, 1953), appear to be the commonest cause of false positive reactions in MacConkey broth.

RESULTS

Over a period of 13 months, 1273 routine samples of water received at this laboratory were examined in the manner described. Of the total, 795 were samples of chlorinated and 478 of unchlorinated water. Their behaviour in the 10 ml. tubes of the four different media is shown in Table 1, where it will be seen that 912 samples (726 chlorinated and 186 unchlorinated) gave completely negative results and 43 further samples (of which 8 were chlorinated) gave identical positive results in all four media.

The results given by these 43 samples are set out in Table 2. Thus, 35 of the samples yielded *Esch. coli*, 33 (including 6 chlorinated samples) from 18 hr. presumptive positive reactions; and 8 samples (of which 2 were chlorinated) yielded other true coliform organisms from 48 hr. presumptive positive reactions.

There were, therefore, altogether 955 samples, each of which had given identical results in all four media; and the comparisons are based on the remaining 318 samples (61 of them chlorinated) which did not produce the same results in all four media. Since the number of chlorinated samples in this category was relatively small and their contribution of positive reactions smaller still, it was not considered necessary to show these separately from the results given by the 257 samples of unchlorinated water. Both types of water displayed similar trends in the four media and these can be seen in the combined results for the 318 samples which are shown in Table 3.

Comparison between the media after 48 *hr. incubation*

The total yield of *Esch. coli* from MacConkey broth was noticeably lower than that from each of the synthetic media: 106 as compared with 132–138. In the total yield of true coliform organisms (including *Esch. coli*) MacConkey broth, formate lactose glutamate and glucose glutamic acid gave closely similar results: respectively 198, 202 and 198. On these counts, therefore, the formate and the glucose media can be regarded as superior to MacConkey broth in that they gave an equally high true coliform yield which included a significantly increased yield of *Esch. coli* ($P < 1/1000$). In this series they also both displayed the further advantage that they produced fewer false positive reactions (7 and 16, respectively) than did MacConkey broth (23).

Table 1. *Behaviour of* 1273 *samples of water in different media*

Type of water	Total no. of samples examined	No. of samples which gave negative reactions in all four media	No. of samples which gave identical positive reactions in all four media	No. of samples which did not give the same results in all four media
Unchlorinated	478	186	35	257
Chlorinated	795	726	8	61
Total	1273	912	43	318

Table 2. *Behaviour of the* 43 *samples of water which gave identical positive results in all four media*

Type of water	Total no. of samples	False presumptive positive results in all media	No. of samples producing *Esch. coli* from all media at			True coliform organisms other than *Esch. coli* from all media at		
			0–18 hr.	18–24 hr.	24–48 hr.	0–18 hr.	18–24 hr.	24–48 hr.
Unchlorinated	35	0	27	1	1	0	0	6
Chlorinated	8	0	6	0	0	0	0	2
Total	43	0	33	1	1	0	0	8

The plain lactose glutamate medium, although producing no false positive reactions, was inferior to the other media in that it yielded a significantly lower total of true coliform organisms: 166 as compared with 198–202 ($P < 1/100$). Since this total included the full yield of *Esch. coli*, the deficit of some 32 isolations represented a loss of other true coliform organisms. It would appear, therefore, that this medium still resembles Burman and Oliver's lactose glutamic-acid medium in being too inhibitory to true coliform organisms other than *Esch. coli*.

Rapidity of appearance of the reactions in the four media

It is evident from Table 3 that the initial appearance of gas was considerably delayed in the synthetic media as compared with MacConkey broth. After 18 and 24 hr. incubation, MacConkey broth produced the greatest number of presumptive

Table 3. *Reactions produced by the* 318 *samples of water which did not give the same results in all four media*

Medium	No. of presumptive coliform reactions				No. of false positive reactions				No. of true coliform organisms (including *Esch. coli*)				No. of *Esch. coli*				No. of true coliform organisms other than *Esch. coli*			
	0–18 hr.	18–24 hr.	24–48 hr.	Total	0–18 hr.	18–24 hr.	24–48 hr.	Total	0–18 hr.	18–24 hr.	24–48 hr.	Total	0–18 hr.	18–24 hr.	24–48 hr.	Total	0–18 hr.	18–24 hr.	24–48 hr.	Total
MacConkey broth	114	15	92	**221**	1	1	21	**23**	113	14	71	**198**	100	4	2	**106**	13	10	69	**92**
Formate lactose glutamate (pH 7·5)	22	74	113	**209**	0	0	7	**7**	22	74	106	**202**	21	71	46	**138**	1	3	60	**64**
Lactose glutamate (pH 7·5)	14	69	83	**166**	0	0	0	**0**	14	69	83	**166**	13	68	51	**132**	1	1	32	**34**
Glucose glutamic acid (pH 6·0)	15	63	136	**214**	0	0	16	**16**	15	63	120	**198**	14	61	59	**134**	1	2	61	**64**

Table 4. *Rapidity of appearance of the reactions produced by the* 361 *samples of water which gave positive results*

Medium	No. of presumptive coliform reactions appearing by			No. of false positive reactions appearing by			No. of true coliform reactions appearing by			No. of *Esch. coli* producing a presumptive positive reaction by			No. of true coliform organisms other than *Esch. coli* producing a presumptive positive reaction by		
	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
MacConkey broth	147	163	264	1	2	23	146	161	241	133	138	141	13	23	100
Formate lactose glutamate (pH 7·5)	55	130	252	0	0	7	55	130	245	54	126	173	1	4	72
Lactose glutamate (pH 7·5)	47	117	209	0	0	0	47	117	209	46	115	167	1	2	42
Glucose glutamic acid (pH 6·0)	48	112	257	0	0	16	48	112	241	47	109	169	1	3	72

coliform reactions, of true coliform organisms and of *Esch. coli*. Moreover, in all three categories the 18 hr. yield from MacConkey broth was greater than that given at 24 hr. by any of the other media. By 24 hr., however, the lag in the synthetic media had been largely overcome, and according to the number of reactions of all types the media were arranged in the following order: (1) MacConkey broth; (2) formate lactose glutamate; (3) lactose glutamate; (4) glucose glutamic acid.

Between 24 and 48 hr. the synthetic media all produced their extra yield of *Esch. coli*, and by 48 hr. the formate and glucose media had matched MacConkey broth in the yield of true coliform organisms. It was also during this second period of 24 hr. that, in all media, most of the true coliform organisms other than *Esch. coli* and practically all the false positive reactions produced fermentation; and it was during this same period that the lactose glutamate medium fell behind the other synthetic media by producing fewer true coliform reactions.

In the comparisons shown in Table 3 the differences between the media were heightened by excluding all similarly behaving samples. For gauging the relative efficiency of the media in routine work it is preferable to observe (by compilation from Tables 2 and 3) the total numbers of positive reactions produced by the 361 samples of water which gave positive results. The cumulative figures for each medium are shown in Table 4. It can thus be seen that the total numbers of true coliform reactions produced in MacConkey broth and formate lactose glutamate by 18 hr. were respectively 146 and 55; but by 24 hr. the respective isolations were 161 and 130 (including respectively 138 and 126 *Esch. coli*). In other words, the disparity at 18 hr. was considerable, the formate yield of true coliform reactions being only 38 % that of MacConkey broth; but by 24 hr. this disparity had been very greatly reduced, the formate yield of true coliform reactions becoming 81 % (and of *Esch. coli* 91 %) that of MacConkey broth. If, therefore, the formate medium were to replace MacConkey broth in the routine eleven- or fifteen-tube test one might reasonably expect any serious discrepancy in the 18 hr. results to be reduced to 20 % by 24 hr. In other words, a significant delay would operate in practice only during the 6 hr. between 18 and 24 hr.

Section B. Formate lactose glutamate (pH 6·7)

EXPERIMENTAL

Towards the end of the comparative trial described above, some pilot tests showed that if pure cultures of *Esch. coli* were inoculated into tubes of single-strength MacConkey broth, lactose glutamic acid (pH 6·0) and formate lactose glutamate (pH 7·5), fermentation at 44° C. occurred much more slowly in the formate tubes than in the tubes of the other media. With the usual inoculum (one loopful of a fermented 37° C. MacConkey broth culture) the 44° C. MacConkey tubes usually showed a distinctly positive reaction by 6 hr., and effervescence could be detected at that time in a large proportion of the lactose glutamic-acid tubes (cf. Taylor, 1955), but a similar change did not usually develop in the formate tubes until after some 12–18 hr. incubation.

Subsequent tests at 44° C. made with lactose glutamic-acid medium: (*a*) to

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which rising concentrations of sodium formate (nil to 0·5%) were added, and (*b*) adjusted to a pH ranging from 6·0 to 7·5, suggested that for early development of both acid and gas the optimal conditions were a pH of 6·7 and the formate concentration of 0·025%. The formate concentration was not critical at 44° C. as between 0·025 and 0·05%, but the optimal pH range was 6·5–7·0 (cf. Pinsky & Stokes, 1952, who found that maximal adaptation of unadapted cells of *Esch. coli* occurred at pH 6·0–7·0).

Since the delay in the results obtained at 37° C. with formate lactose glutamate (pH 7·5), and previously with lactose glutamic acid (pH 6·0), obviously arose through failure of *Esch. coli* to develop quickly, it was considered that the intermediate pH (6·7) might permit speedier results in the routine presumptive test at 37° C. A small number of water samples (the coliform density of which was expected to lie between 25 and 160 organisms per 100 ml.) were given the routine eleven-tube test simultaneously in the three media—MacConkey broth, formate lactose glutamate (pH 7·5) and formate lactose glutamate (pH 6·7)—and it was at once clear that the last-named medium approached much more nearly the 18 hr. performance of MacConkey broth.

It was, therefore, decided to compare MacConkey broth and formate lactose glutamate (pH 6·7) by a multiple-tube test on every sample of water received at the laboratory.

At this stage the suggestion was made (S. B. Thomas, personal communication, 1958) to replace the tap-water basis of the synthetic medium by deionized water. The resulting improvement of this step was sufficiently great to indicate that the tap water in the new laboratory (which had been occupied for the previous 18 months) must contain some inhibitory agent. A full analysis, kindly undertaken by Dr E. Windle Taylor, revealed that water drawn from the tap in the medium room contained 1 mg./l. of Cu″. Although preliminary tests could detect no difference between MacConkey broth made with the tap water and with deionized water, it was decided that both media (and, in fact, all the laboratory media) should in future be made with deionized water.

(Since the new laboratory tap water had been in use during the trial already described, it is probable that the failure of the synthetic media to reach parity with MacConkey broth by 24 hr. was caused by the presence of copper.)

The MacConkey broth (single-strength and double-strength) was therefore prepared with deionized water in place of tap water. In the first series of tests under this heading the bile salt was identical with that used under Section A—sodium tauroglycocholate (Ward & Blenkinsop, Batch No. 18952). In the second series a much less inhibitory preparation was used—sodium taurocholate (Hopkin & Williams, Batch No. 35949). The formate lactose glutamate (single-strength and double-strength) was prepared as formerly except that the tap water was replaced by deionized water and the pH was adjusted to 6·7 instead of 7·5. As recommended by Burman & Oliver (1952), the single strength glutamic-acid medium was dispensed in 5 ml. quantities in $6 \times \frac{1}{2}$ in. test-tubes in place of the usual $6 \times \frac{5}{8}$ in. tubes.

Both chlorinated and unchlorinated waters (50 ml. and 5×10 ml. for chlorinated and 5×10 and 5×1 ml. for unchlorinated specimens) were examined in the first

series of these trials, but in the second series, with the MacConkey broth containing the less inhibitory bile salt, chlorinated waters were not examined because so few of these had in the first series provided any information of value.

RESULTS

First series

During a period of $2\frac{1}{2}$ months 279 consecutive samples of water received in this laboratory were examined by the multiple-tube method described. As may be seen in Table 5, 205 were samples of chlorinated and 74 of unchlorinated water, and a total of 201 (which included 195 chlorinated waters) gave completely negative results in both media. The remaining 78 samples (of which 10 were chlorinated) gave positive results in either or both media. This figure includes 5 samples of unchlorinated water which gave identical positive results in all ten tubes of both media (4 samples yielded *Esch. coli* at 18 hr. and 1 sample an intermediate-aerogenes organism at 48 hr.).

Table 5. *Behaviour of* 279 *samples of water examined by multiple-tube technique in MacConkey broth* (*Ward and Blenkinsop, Batch No.* 18952) *and formate lactose glutamate* (pH 6·7)

Type of water	Total no. of samples examined	No. of samples which gave negative results in all tubes of both media	No. of samples which gave positive results in either or both media
Unchlorinated	74	6	68*
Chlorinated	205	195	10
Total	279	201	78

* Including 5 samples which gave identical positive results in all 10 tubes of both media, i.e. 4 yielding *Esch. coli* at 18 hr. and 1 yielding an intermediate-aerogenes organism at 48 hr.

The cumulative results for positive tubes associated with these 78 samples are shown in Table 6, where it can be seen that at every stage of incubation the synthetic medium produced (with fewer false positive reactions) equal or increased total coliform and *Esch. coli* yields. At 18 hr. the results for both media were closely similar, but thereafter the advantage was progressively on the side of the synthetic medium. Inspection of the figures shows that the advantage arose from the greater readiness with which the formate medium yielded *Esch. coli* (the increased isolations at 18, 24 and 48 hr. were respectively 6, 37 and 50), whereas MacConkey broth failed on this occasion to produce a compensatory increase in other true coliform organisms. The formate medium, therefore, outran MacConkey broth in the total yield of true coliform organisms by fifteen isolations after 24 hr. and forty-five isolations after 48 hr. incubation. As compared with the ultimate yield from MacConkey broth the extra isolations from the formate medium represented an increase in total coliform organisms of 12 % and in *Esch. coli* of 27 %.

Statistical analysis of the results given by the 68 samples of unchlorinated water showed no significant differences between the two media after 18 hr. incubation, but indicated that the formate medium produced significantly increased yields of *Esch. coli* after 24 and 48 hr. and of total coliform organisms after 48 hr. ($P < 1/100$).

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Second series

Because the comparison just described indicated that the MacConkey broth had had a suppressive effect on true coliform organisms (but principally on *Esch. coli*) and since the bile salt used was known to be a particularly inhibitory brand, it was decided to conduct a final multiple-tube comparison between the synthetic medium and MacConkey broth containing a less inhibitory bile salt. Throughout the Water Sub-committee trials the brand used in Newport had been Hopkin and Williams's sodium taurocholate (which had been abandoned thereafter only because of the expense). This known satisfactory bile salt would, it was thought, release the MacConkey broth from any question of over-inhibition. Standardization tests on the batch (No. 35949) currently available showed it to be much less inhibitory than the one previously used. Its inhibitory power was, in fact, so low that it would not normally have been accepted for routine purposes (the agar medium, for example, readily grew staphylococci), but this property rendered it particularly suitable for the projected comparison.

During a period of 6 weeks 57 consecutive samples of unchlorinated water received at this laboratory were examined by the ten-tube method described. As can be seen in Table 7, 56 of the samples gave positive results in either or both media and this figure includes 3 samples which gave identical positive results in all ten tubes of both media (all yielded *Esch. coli* at 18 hr.). Table 7 also shows the cumulative totals of positive tubes yielded at each stage of incubation by each medium. Once again the results at 18 hr. were closely similar and thereafter the advantage was on the side of the synthetic medium, the extra isolations by 48 hr. representing an increase in total coliform organisms of 17 % and in *Esch. coli* of 13 % over the ultimate yield from MacConkey broth. In so far as the relatively small numbers permit of any conclusion, it would seem that the replacement of an inhibitory by a much less inhibitory bile salt, although reducing the specific suppression of *Esch. coli*, has not materially altered the suppressive effect of MacConkey broth on true coliform organisms.

DISCUSSION

Water bacteriologists in this country are accustomed to a presumptive coliform test not heavily encumbered by false positive reactions and it has been recognized (Ministry of Health, 1956) that the use of bile salt, as in MacConkey broth, to suppress the non-coliform flora may also slightly inhibit the coliform organisms themselves. The extent of this inhibition can be measured only by comparing MacConkey broth with other media, as in the present and previous studies, but it would appear that some inhibition is a constant characteristic of MacConkey broth, and that certain samples of bile salt, even though carefully standardized beforehand, may seriously suppress *Esch. coli*, the most important of the organisms sought.

Recognizing the unpredictability of bile salts and of other inhibitory agents, water bacteriologists are now tending to seek a medium which does not at all

Table 6. *Rapidity of appearance of the reactions produced by the 78 samples of water which yielded positive tubes*

Medium	No. of presumptive coliform reactions appearing by			No. of false positive reactions appearing by			No. of true coliform reactions appearing by			No. of *Esch. coli* producing a presumptive positive reaction by			No. of true coliform organisms other than *Esch. coli* producing a presumptive positive reaction by		
	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
MacConkey broth (W. and B. sodium tauroglycocholate No. 18952)	169	218	395	0	1	10	169	217	385	162	186	187	7	31	198
Formate lactose glutamate (pH 6·7)	170	232	437	0	0	7	170	232	430	168	223	237	2	9	193

Table 7. *Rapidity of appearance of the reactions produced by 57 samples of unchlorinated water*

Total no. of samples examined	No. of samples which gave negative results in all tubes of both media	No. of samples which gave positive results in either or both media	Medium	No. of presumptive coliform reactions appearing by			No. of false positive reactions appearing by			No. of true coliform reactions appearing by			No. of *Esch. coli* producing a presumptive positive reaction by			No. of true coliform organisms other than *Esch. coli* producing a presumptive positive reaction by		
				18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
57	1	56*	MacConkey broth (H. and W. sodium taurocholate No. 35949)	134	160	240	0	6	17	134	154	223	127	133	134	7	21	89
			Formate lactose glutamate (pH 6·7)	135	164	268	0	0	8	135	164	260	130	152	152	5	12	108

* Including 3 samples which gave identical positive results in all ten tubes of both media, i.e. each yielded *Esch. coli* at 18 hr.

inhibit the coliform flora (World Health Organization Report, 1958). For similar reasons the Americans have long preferred simple lactose broth for their primary medium. This, however, produces a proportion of false positive reactions too high to be acceptable in this country, and the comparison by Jebb (1959) does not suggest that it produces a greater yield of true coliform organisms or of *Esch. coli*. Jebb's results also suggest that the other standard American medium (lauryl tryptose broth) and Teepol broth (Jameson & Emberley, 1956) have, as regards drinking water, no advantage over MacConkey broth in coliform or *Esch. coli* yield but merely tend to produce more false positive reactions.

It would seem, therefore, that peptone media must either unduly reduce the specificity of the presumptive coliform test or else contain some ingredient which, to a variable and unknown degree, inhibits the coliform flora. Moreover, although less so than bile salt, peptone is a variable and expensive constituent.

If, therefore, a synthetic medium could be found which did not inhibit the coliform organisms and did not unduly stimulate other organisms, it was to be hoped that the presumptive coliform test might at the same time become more specific, more accurate and more consistently reproducible. Folpmers's glucose glutamic acid was favourably described by Burman & Oliver (1952) and, except for false positive reactions, by the Water Sub-committee (1958), but the original lactose modification was found to be too inhibitory in both these investigations.

The studies reported here represent relatively small-scale investigations of a limited number of water supplies in Monmouthshire and adjacent areas. Only provisional conclusions can, therefore, be drawn from the results, particularly where they differ significantly from those obtained by the Water Sub-committee.

In the single-tube comparisons, the most noteworthy differences from the Sub-committee's results were: (1) that none of the synthetic media yielded more true coliform organisms than MacConkey broth; (2) that, despite this, the increased yield of *Esch. coli* from *all* the synthetic media was substantial (24–30 %) as compared with that (8–10 %) noted by the Sub-committee; (3) that glucose glutamic acid did not on this occasion produce an excess, far less a heavy excess, of false positive results, and (4) that the lag in the synthetic media was still present at 24 hr. The use of a different bile salt may explain the disparity in the *Esch. coli* isolations, particularly since this was reduced from 27 to 13 % by changing the bile salt in the subsequent multiple-tube tests. The other effects may be, in part at least, attributable to the presence of copper which significantly affected only the synthetic media. This cannot, however, wholly explain the changed behaviour of glucose glutamic acid.

In the P.H.L.S. Water Sub-committee's Report (1958) the incidence of false positive reactions from glucose glutamic acid was shown to vary very much from laboratory to laboratory—in two it was negligible, in another two it was about 10 % and in a further two (of which Newport was one) it was as high as 25 % as compared with 5–10 % from MacConkey broth. A possible explanation for these differences is the changing flora of natural waters—previously this laboratory encountered a high proportion of waters containing glucose-fermenting non-

lactose-fermenting organisms (a cause of false positive reactions), whereas few such waters were found in the present series.

The single-tube comparisons indicated that the inhibitory effect of lactose glutamic acid, which still remained when the pH was raised to 7·5, could be largely removed when this measure was combined with the addition of sodium formate, and the performance of formate lactose glutamate (pH 7·5) was at least as good as that of glucose glutamic acid, both synthetic media showing some advantages over MacConkey broth.

Further investigation suggested that the optimum pH for formate lactose glutamate was not 7·5 but 6·7 which should permit greater advantages of speed. The medium, so adjusted, produced early results as quickly as MacConkey broth (with all the uncertainty of an unstandardized medium) and yielded by 48 hr appreciably more isolations of true coliform organisms and *Esch. coli*.

Important considerations for the water bacteriologist are the high cost of bile salts for MacConkey broth and the difficulty of standardization. Formate lactose glutamate is a chemically defined medium which is at least two and a half times cheaper than MacConkey broth.

There remains one disadvantage of the synthetic medium which must be mentioned. Acid and gas formation in the tubes of MacConkey broth can be recognized at a glance. With the synthetic medium a small proportion of the minimally positive tubes cannot be recognized until effervescence is produced by shaking the tubes. This aspect requires further study, but it would appear from the investigations detailed in this communication that, with the reservations to which attention has already been drawn, formate lactose glutamate medium adjusted to pH 6·7 can be recommended for replacement of MacConkey broth in the presumptive coliform test of water. Wider and more extensive trials are obviously desirable before this claim can be substantiated, but meanwhile the following recommendations for preparation and use are offered.

Formate lactose glutamate (pH 6·7)

Double-strength medium: Lactose, 20 g.; L(+) glutamic acid, 10 g.; Sodium formate, 0·5 g.; Potassium phosphate (K_2HPO_4), 6 g.; Ammonium lactate 50 % w/w solution, 20 ml.; Brom-cresol purple (1 % alcoholic solution), 2 ml.; Deionized (or distilled) water, 1000 ml.

Stock double-strength solution

Dissolve the glutamic acid in approximately 90 % of the water (which is kept hot in the steamer) and neutralize with (5N or N) sodium hydroxide. While the bulk is cooling, add the formate, phosphate and lactate together with the remainder of the water. Adjust the pH to 6·7 and filter through Whatman filter-paper. Distribute in 500 ml. amounts in sterile bottles which are autoclaved at 10 lb. for 10 min.

For use

To each 500 ml., as required, add 10 g of lactose. (For single-strength medium now dilute with an equal volume of deionized water). Check the pH and adjust,

if necessary, to 6·7. Add the brom-cresol purple solution. Distribute into bottles and tubes and sterilize by steaming for 45 min. on each of two successive days.

The final medium is absolutely clear. The first sign of a potential positive reaction during incubation is the development of opalescence (which is quite readily distinguishable from the clarity of negative tubes). Such opalescent tubes (even in the absence of any detectable acid) should be gently shaken for several seconds. The appearance of effervescent bubbles of gas emanating from the bottom of the tube and collecting in the Durham tube can be accepted as the earliest reliable sign of a positive reaction (which several hours later will usually show definite acid and an accumulation of gas in the Durham tube). In routine examination, however, the majority of positive tubes—from 18 hr onwards—show accumulated gas with at least some acid change, and it is only occasionally that a tube need be shaken to distinguish a positive from a negative reaction.

SUMMARY

During a period of 13 months, 1273 consecutive samples of water received at this laboratory were submitted to a modified presumptive coliform test which included single tubes of four different media. These were: (1) MacConkey broth, (2) formate lactose glutamate medium (pH 7·5), (3) lactose glutamate medium (pH 7·5), and (4) Folpmers's glucose glutamic acid (pH 6·0). 955 samples gave exactly the same results in all four media, but the remaining 318 samples produced differences which enabled comparisons to be made between the media.

As compared with MacConkey broth, the three glutamic-acid media produced between 24–30 % more isolations of *Esch. coli*, and the formate and the glucose media produced at least the same total number of true coliform organisms (including *Esch. coli*) with appreciably fewer false positive reactions. The lactose glutamate medium gave no false positive reactions but, through the suppression of coliform organisms other than *Esch. coli*, reduced by 16 % the total coliform yield. MacConkey broth gave the largest early (18 and 24 hr.) yield of positive reactions, but the results at the end of 24 hr. showed the formate medium to be not far behind MacConkey broth and appreciably ahead of the glucose medium.

Further experiments with the formate lactose glutamate medium adjusted to different pH's, ranging from 6·0–7·5, indicated that a medium of pH 6·7 provided optimal conditions for the early development of both acid and gas. It was therefore decided to test this observation more fully with routine samples. During a period of 4 months, 279 unselected consecutive samples of water (of which 78 gave positive results) and 57 consecutive unchlorinated water samples (56 of which gave positive results) were examined by a multiple-tube technique in both MacConkey broth and formate lactose glutamate medium (pH 6·7). In the former series the MacConkey broth contained the inhibitory bile salt previously used; in the latter series this was replaced by a relatively non-inhibitory bile salt. In both series the formate medium yielded an appreciably increased total of coliform organisms (12 and 17 %) including an increased total of *Esch. coli* (27 % in the former and 13 % in the latter series), and in both series also the results at 18 hr. were abreast with, and at 24 hr. ahead of, those obtained in MacConkey broth.

With certain reservations, it is considered that formate lactose glutamate medium (pH 6·7) can be offered as a suitable alternative to MacConkey broth for the presumptive coliform test of water. Wider trials of this medium would be required before this claim could be fully substantiated.

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