NEUTRAL-RED IN THE ROUTINE EXAMINATION OF WATER.

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The use of neutral-red culture media has recently been suggested for the detection of *B. coli* in water supplies. Rothberger [1] grew a number of organisms on media containing various organic dyes, and found that while some species were able to bring about a reduction of the colouring matter in the medium, others produced no change. This difference in reaction was particularly marked in the case of *B. typhosus* and *B. coli* in media containing neutral-red. *B. coli* caused the reduction of the neutral-red to yellow with green fluorescence, whilst *B. typhosus* produced no change of colour beyond an occasional fading of the red. After testing a number of races of *B. coli* and *B. typhosus*, Rothberger proposed neutral-red media for the differentiation of the two organisms. Scheffler [2] confirmed in the main the work of Rothberger, and further observed that the reaction is by no means specific for *B. coli*. In addition to a number of bacterial species not commonly found in water, which gave the reaction, Scheffler found 3 of 13 organisms in spring and river waters, and 8 of 18 intestinal organisms from man which, though not belonging to the colon group, gave the neutral-red reaction.

The experiments of Makgill [3] showed that neutral-red media form a delicate test for *B. coli* in pure culture in water, but that the presence of other organisms tends to delay the reaction. Savage [4] examined 44 waters from various sources, using neutral-red media and made at the same time a search for *B. coli*. From the 34 waters with which a positive neutral-red reaction was obtained, organisms identified as *B. coli*

were isolated in 31 cases[1]. From none of the 10 negative cases was *B. coli* isolated. Savage concludes that in the series of waters examined, the error of assuming that the yellow colour and fluorescence were due to the presence of *B. coli* was about 5%, and that the test gave approximately accurate results, even if further isolation of this bacillus had not been proceeded with.

If similar results could be obtained with ground and surface waters in general, we should have at our command an additional and valuable method for the detection of *B. coli* in water supplies. In the course of the recent Streams Examination for the Chicago Sanitary District an opportunity occurred for further determining the value of neutral-red in the routine examination of water.

Methods.

Neutral-red agar and bouillon were prepared by adding to neutral agar[2] or bouillon 0·5% dextrose, and 1% of a 0·5% aqueous solution of neutral-red (Grübler's). In the routine examination, broth was generally employed. In testing the neutral-red reaction of organisms in culture, both broth and agar shake cultures were used. Dextrose broth for the fermentation tubes employed in parallel tests was made by adding 1% dextrose to neutral sugar-free broth. Dilutions of the waters were prepared by means of small 150 c.c. flasks containing known amounts of sterile tap water.

All cultures were kept at 37° C. The dextrose fermentation tubes were examined for gas after 24 and 48 hours, allowed to cool, and the CO_2 absorbed with a 2% solution of NaOH. The appearances in neutral-red tubes were recorded after 24 and 48 hours, 5, 7, and 10 days. Tubes in which the reaction was delayed beyond 3 days gave positive results later in comparatively few cases.

[1] Although it is recognized that organisms occur in water which are intermediate between *B. coli* and allied groups, and that one group passes by a series of intermediate forms into another, nevertheless, ultimate clearness and precision are not likely to be attained by a too liberal extension of the limits of the true *B. coli* group. To this end, a somewhat detailed study must be made of the cultural characteristics of organisms which upon superficial examination appear similar to *B. coli*. Exception may well be taken on this score to the criteria employed by Makgill and Savage for the differentiation of *B. coli* from other water bacteria, particularly as regards the incomplete data given concerning fermentation tests.

[2] Except where otherwise stated, media were prepared according to the "Procedures Recommended for the Study of Bacteria." *Reports and Papers of the Amer. Pub. Health Assoc.* 1898, vol. XXIII. p. 60.

In the following experiments, made in connection with the daily examination of river waters, neutral-red determinations were made with a number of dilutions of each water, controlled in exact parallel by similar determinations with the dextrose fermentation tube. It has been shown [5] that when the dextrose fermentation tube yields approximately 33 % of CO_2, *B. coli* is almost invariably present. Table A illustrates the method of comparison employed.

TABLE A.

No. of Sample	Amount of water used	Gas-Production Dextrose Fermentation Tube				Neutral-Red		
		24 hours	48 hours	Proportion of H to CO_2	Interpretation B. coli present	Reaction in Original Tube	B. coli Isolated	Reaction of Culture Isolated
183	·001 c.c.	no gas	arm clear	—	–	–		
	·01 c.c. (*a*)	,,	arm turbid	—	–	–		
	(*b*)	,,	,,	—	–	+	–	+
	·1 c.c. (*a*)	,,	5 %	—	–	+	–	+
	(*b*)	10 %	30 %	3 : 2	+	+		
	1 c.c.	15 %	25 %	3 : 1	+	+		
196	·001 c.c.	no gas	arm turbid	—	–	–		
	·01 c.c. (*a*)	,,	,,	—	–	+	–	+
	(*b*)	,,	10 %	—	–	+		
	·1 c.c. (*a*)	10 %	20 %	3 : 1	+	+		
	(*b*)	25 %	50 %	2 : 1	+	+		
	1 c.c.	65 %	75 %	2 : 1	+	+		
241	·001 c.c.	no gas	arm clear	—	–	–		
	·01 c.c. (*a*)	,,	,, turbid	—	–	+	–	+
	(*b*)	,,	,, ,,	—	–	+	–	+
	·1 c.c. (*a*)	,,	,, ,,	—	–	+		
	(*b*)	,,	,, ,,	—	–	+		
	1 c.c.	5 %	10 %	3 : 1	+	+		
	2 c.c.	95 %	95 %	3 : 2	+	+		
251	·001 c.c.	no gas	arm clear	—	–	–		
	·01 c.c. (*a*)	,,	,,	—	–	–		
	(*b*)	,,	bubble	—	–	–		
	·1 c.c. (*a*)	,,	arm turbid	—	–	+	–	+
	(*b*)	,,	,,	—	–	+		
	1 c.c.	45 %	55 %	3 : 2	+	+		
	2 c.c.	95 %	100 %	2 : 1	+	+		

As will appear from the table, such dilutions of the waters were employed that *B. coli* was almost always found in the lowest and rarely in the highest dilution. It will further be noted that from those neutral-red tubes, giving a positive reaction when the fermentation tubes of the corresponding dilution were negative, a careful

search failed to show the presence of *B. coli*, but other organisms were present which in culture gave the neutral-red reaction, thus accounting in each case for the reaction of the original tube. The results obtained with 45 waters examined in the same manner by means of both the fermentation tube and the neutral-red methods are summarized in the table below (Table B).

TABLE B.

Dilutions		·001 c.c.	·01 c.c.	·1 c.c.	1 c.c.	2 c.c.	Totals	% +
Dextrose Fermentation Tube Determinations	+	1	10	36	39	14	100	35 %
	−	44	80	54	6	1	185	
Neutral-Red Determinations	+	0	20	54	45	15	134	47 %
	−	45	70	36	0	0	151	

With each method there are shown 285 determinations in exact parallel, with 35 % positive with the fermentation tube, and 47 % positive with the neutral-red method. In so large a number of determinations, this excess of positive results with neutral-red could hardly be due to chance. I have carefully considered the possibility that the excess might be due to the failure of *B. coli* to give a characteristic gas-production in the dextrose tube; but a large number of determinations made in connection with the present and previous [5] work show that the dextrose fermentation tube gives a fairly accurate test for the presence of *B. coli*. Nor can it be objected that the dextrose reaction is too rigorous a standard for the reason that it excludes organisms giving no gas in dextrose, but otherwise like *B. coli*; for an examination of Table C will show that of 30 organisms (Group V) which do not liquefy gelatin and give no gas with dextrose, only two give the neutral-red reaction. A closer examination of individual tubes and of the neutral-red reaction of a number of cultures of water bacteria will indicate more clearly the source of the excess of positive neutral-red determinations.

Twenty-two neutral-red tubes, which gave positive reactions when the fermentation tubes of the corresponding dilutions were negative, were examined for the presence of *B. coli*. In the case of 2, *B. coli* was found, and from 19 of the remaining 20, organisms not *B. coli* were isolated, which however gave the neutral-red reaction. The organisms giving the reaction in 15 of the above 19 tubes, liquefied gelatin and gave no gas in dextrose broth.

The reactions of 132 cultures of organisms isolated in the course of

the work were observed on the various routine culture media, and on neutral-red agar and bouillon. These cultures all grew at 37° C. and are classified below, taking into consideration their reactions on all the routine media, but with special reference to their growth on those mentioned under the several groups. In the arrangement of these groups, an endeavour has been made to place together those organisms closely allied, although it is of course recognized that Groups V and VI may each contain organisms differing more or less widely in their relationships.

GROUP I. *B. coli.*

(1) Coagulation of milk with formation of acid; (2) non-liquefaction of gelatin and casein; (3) gas in dextrose, lactose, and saccharose bouillon, or in dextrose and lactose, with gas formula showing excess of H (gas not absorbed by NaOH solution).

GROUP II. *B. enteritidis?*

(1) Non-coagulation of milk, with production of alkali; (2) non-liquefaction of gelatin, and casein; (3) gas in dextrose bouillon with excess of H; (4) no gas in lactose or saccharose.

GROUP III. *B. proteus.*

(1) Coagulation of milk, with formation of acid; (2) liquefaction of gelatin and casein; (3) gas in dextrose and saccharose bouillon with marked excess of H; (4) no gas in lactose bouillon.

GROUP IV. *B. cloacae.*

(1) Coagulation of milk, with formation of acid; (2) liquefaction of gelatin; (3) gas in dextrose and saccharose bouillon with excess of CO_2, and in lactose bouillon with varying gas ratio.

GROUP V.

(1) No gas in sugar media; (2) non-liquefaction of gelatin.

GROUP VI.

(1) No gas in sugar media; (2) liquefaction of gelatin.

The reactions of the organisms of these groups on neutral-red media are summarized in Table C.

TABLE C.

Group	I	II	III	IV	V	VI
+ Reaction Neutral-Red	26	2	15	14	2	30
− Reaction Neutral-Red	1	0	1	0	28	12

It is evident that organisms common in river water other than *B. coli* give the neutral-red reaction under the conditions of the test. Scheffler and Rosenberger [6] have pointed out that the neutral-red reaction, essentially that of reduction, is, as one would expect from its nature, not specific for *B. coli*. In view of the evident non-specificity of the reaction, the marked excess of positive reactions with neutral-red compared to the corresponding positive determinations with the fermentation tube, is easily accounted for. Although neutral-red gives approximately accurate determinations when only *B. coli* is present, the results obtained in the examination of a water for *B. coli* by the neutral-red method alone, are likely to be misleading, the tendency obviously being to give too high an estimate of the number of the organism present. We must conclude, therefore, that in the routine examination of water, the neutral-red reaction, when used alone, cannot be depended upon for the diagnosis of *B. coli*, since the reaction is given under the conditions of the test by a number of other common water organisms which no classification, however liberal, would place in the colon group.

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