EIJKMAN RELATIONSHIPS OF THE COLIFORM AND RELATED BACTERIA

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In 1904 Eijkman found that cultures of *Escherichia* produced gas from glucose broth at 46°C. while *Aerobacter* did not. Attempts to confirm Eijkman's findings were so contradictory that for some time the test was practically abandoned. Recent work by Levine *et al.* (1934), Wilson *et al.* (1935) and Hajna *et al.* (1939) has revived interest in this test. The introduction of a highly buffered tryptose lactose broth by Perry (1939) seems to have solved many of the difficulties encountered in the test.

Work with the Eijkman test has been confined for the most part to the coliform bacteria. Moreover, most investigators have been concerned only with the production of gas from carbohydrates at high temperatures. The purpose of the present investigation was to determine the ability of several genera of the Family Enterobacteriaceae to grow, to produce acid, or to produce gas in the modified Eijkman medium at 45.5°C.

The tests were done in a constant level water bath maintained at 45.5°C. with a variation of less than 0.1 of one degree. The water level was at least one inch above the level of the medium in the tubes. Difco dehydrated Eijkman broth was used throughout these experiments with brom-thymol-blue indicator added. Cultures were rapidly transplanted to the Eijkman broth in groups of twelve, and since it took approximately three and one-half minutes to bring the temperature of the medium to 45.5°C. not more than several minutes elapsed between the time of inoculation of any Eijkman tube and the time it reached temperature. When large numbers of cultures were tested, each group of twelve tubes was brought to temperature in a small bath and then transferred to the test bath so that the temperature in the test bath remained constant.

A great majority of *Escherichia* cultures produced from 40 to 60 per cent gas in 12 to 24 hours. Inverted shell vials 45 x 10 mm. were used in the sugar broths. In the early part of our work on the coliform organisms gas volumes were measured by holding

the tube against a scale. After several thousand determinations had been made it was found that estimation without the scale was sufficiently accurate for our purposes since for the most part the same individual read gas volumes. Some cultures produced only 20 per cent gas in 48 hours. Frequently such cultures gave 20 per cent gas in 6 hours with no increase up to 48 hours. A few *Escherichia* cultures produced less than 20 per cent gas in 48 hours. On the other hand a few *Aerobacter* cultures produced similar small gas volumes in the same period of time. Therefore the production of 20 per cent or more gas was recorded as an Eijkman positive reaction and smaller amounts as Eijkman doubtful. All cultures giving an Eijkman doubtful reaction were tested in the same medium at 37°C. but in no case was a doubtful reaction at 45.5°C. accompanied by a similar low gas volume at 37°C. The following reactions were recorded usually at 6, 12, 24 and 48 hour intervals: no (visible) growth, growth, slight acid, strong acid, Eijkman doubtful and Eijkman positive.

Serratia cultures generally produce only acid from glucose and fail to ferment lactose at 37°C. *Erwinia* cultures ferment both carbohydrates producing only acid, a bubble of gas or moderate gas volumes. Except for gas volumes many *Erwinia* cultures possess the biochemical characteristics of *Aerobacter*. From grains, *Erwinia-Aerobacter*-like cultures producing no more than a bubble of gas and *Aerobacter* cultures giving large gas volumes (60 to 100 per cent) may be isolated in about equal proportions. From feces, however, *Aerobacter* cultures producing large gas volumes are generally isolated. To test the Eijkman relationships of such cultures 14 *Serratia* isolated from widely different sources, 24 *Erwinia* cultures[1] known to be pathogenic for plants, 24 selected *Erwinia-Aerobacter*-like cultures isolated from six different grains and 24 *Aerobacter* cultures isolated from feces and selected for large gas volumes were tested at 45.5°C. Because of the inability of *Serratia* to ferment lactose, glucose was substituted in the medium.

Two tubes of Eijkman medium were inoculated from a 24-hour agar slant of each culture: one with a small inoculum from a straight needle which imparted no turbidity to the medium and the other with a loopful of the slant culture. Reactions were recorded after 6, 24, and 48 hours. To test the viability of the

[1] These 24 *Erwinia* cultures, all of the soft-rot group, were obtained from Dr. Perry Elrod of the Bacteriology Department, Ohio State University.

organisms after 24 hours at 45.5°C. a loopful of each of the heavily inoculated tubes was transplanted to standard broth. These were incubated at 37°C. and the presence of growth recorded after 6, 24 and 48 hours. The results of this experiment are tabulated in Table 1.

None of the *Serratia* showed any growth from the small inoculum. Because of the turbidity from the large inoculum it could not be determined whether growth had occurred but no acid was produced by any culture. Three of the *Serratia* cultures transplanted to broth (from the heavily inoculated tubes after 24 hours at 45.5°C.) showed growth after 48 hours at 37°C. Acid was produced at 45.5°C. by 3 of the *Erwinia* cultures from the heavy inoculum and by 2 of those from the small inoculum. These three cultures in the Eijkman medium at 37°C. produced about 30 per cent gas. Four of the *Erwinia* cultures showed growth in the broth in 48 hours. Marked differences in growth at 45.5°C. resulted from the small and heavy inoculums with the grain and fecal cultures. With the small inoculum, 14 of the grain and 11 of the fecal cultures gave no acid whereas with the large inoculum all produced acid. All of the grain and fecal cultures were viable after 24 hours at 45.5°C. Table 1 indicates that cultures producing low gas volumes isolated from grains are much more closely related in their Eijkman reactions to *Aerobacter* than to *Erwinia*.

Using small inoculums, 3059 normal coliform cultures including representatives of the different groups and types isolated from milk, water, soil, grains, dust, hides and feces were tested at 45.5°C. in Eijkman lactose broth. The results are recorded in table 2.

Table 2 shows that of 183 type 1 cultures 64.2 per cent produced no visible growth, 32.8 per cent gave visible growth and 2.7 per cent produced slight acid. The large number of cultures showing no growth compared with the other types of normal coliforms might indicate the more primitive nature of type 1.

Of 652 type 2 cultures, 23.6 per cent showed no growth, 50.3 per cent showed growth and 20.4 per cent produced slight acid. Twenty cultures produced a strong acid reaction and while such a reaction does not constitute an "Eijkman exception" they seemed worthy of further study. To determine the constancy of their Eijkman reactions and the possibility of acclimatization to 45.5°C. such cultures were plated on eosin-methylene-blue agar from the Eijkman broth tube usually after 24 hours at 45.5°C.

After incubation at 37°C. the largest and darkest colony from each plate was fished to an agar slant. These cultures were again tested for their Eijkman reaction, and replated, etc. 8 times. Of the 20 cultures so tested, 11 varied in their reactions from growth to strong acid, 5 produced strong acid on each test while 4 on one or more of the tests produced not more than 10 per cent gas. On 2 or 3 of the platings the IMVIC reactions of these cultures were redetermined but no change was found.

TABLE 1*

Eijkman reactions of Serratia, Erwinia, and selected Aerobacter cultures

CULTURES	NUMBER	SMALL INOCULUMS				HEAVY INOCULUMS				BROTH CULTURES INOCULATED FROM 24 HOUR EIJKMAN TUBES AND INCUBATED AT 37°C.	
		0	G	A	A^2	0	G	A	A^2	0	G
Serratia	14										
6 hours		14				14?				14	0
24 hours		14				14?				13	1
48 hours		14				14?				11	3
Erwinia	24										
6 hours		24				21?		3		24	0
24 hours		22		2		21?		2	1	20	4
48 hours		22		1	1	21?		1	2	20	4
Grain *Aerobacter*	24										
6 hours		9	15			7?		16	1	15	9
24 hours		7	7	10		1?		11	12	0	24
48 hours		6	8	9	1			8	16	0	24
Fecal *Aerobacter*	24										
6 hours		7	11	5	1			13	11	16	8
24 hours		4	7	8	5			2	22	1	23
48 hours		4	7	7	6			1	23	0	24

* Numbers rather than percentages are given because of the small numbers of cultures used.

0, no growth; G, growth; A, slight acid; A^2, strong acid.

Fifteen type 2 cultures producing less than 20 per cent gas were tested as just described. Eleven varied from growth to 20 per cent gas on the different tests. Two produced gas on every test, one of these 50 per cent on one of the eight tests and the other 50 per cent on two of the eight tests.

Two type 2 cultures produced from 40 to 60 per cent gas on each of nine tests. In the speed and amount of growth and volume of gas these 2 cultures could not be distinguished from

the majority of *Escherichia*. From the type 2 cultures producing strong acid or small amounts of gas it was always difficult and sometimes impossible to recover viable organisms after 48 hours at 45.5°C. but with the 2 consistently positive cultures viable organisms could easily be obtained after 48 hours and on one occasion after 4 days.

TABLE 2*

Eijkman reactions of coliform bacteria from agar slant inoculations

(48 hour readings)

GROUP	TYPE	INDOLE	METHYL RED	VOGES-PROSKAUER	CITRATE	CELLOBIOSE	CULTURES	NO GROWTH	GROWTH	SLIGHT ACID	STRONG ACID	EIJKMAN DOUBTFUL	EIJKMAN POSITIVE
								per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	1	+	−	+	+	+	183	64.2	32.8	2.7	0	0	0
	2	−	−	+	+	+	652	23.6	50.3	20.4	3.0	2.3	0.3
	3	−	−	+	+	A	15	33.3	53.3	13.3	0	0	0
A	4	−	−	+	+	−	13	30.7	53.8	15.3	0	0	0
	5	−	−	+	−	+	7	14.2	85.7	0	0	0	0
	6	−	−	+	−	A	16	18.7	62.5	18.7	0	0	0
	7	−	−	+	−	−	8	37.5	37.5	12.5	12.5	0	0
	8	−	+	−	+	+	394	28.9	54.6	14.7	1.2	0.2	0.2
	9	−	+	−	+	A	220	19.0	56.8	23.6	0.4	0	0
B	10	−	+	−	+	−	109	22.0	54.1	18.3	5.5	0	0
	11	−	+	−	−	+	9	11.1	44.4	22.2	22.2	0	0
	12	−	+	−	−	A	11	27.3	54.5	9.0	9.0	0	0
	13	−	+	−	−	−	114	0	0	0	0	0.9	99.1
	14	+	+	−	+	+	81	3.7	90.1	4.9	1.1	0	0
	15	+	+	−	+	A	51	19.6	64.7	9.8	5.8	0	0
C	16	+	+	−	+	−	27	25.9	48.1	7.4	11.1	0	7.4
	17	+	+	−	−	+	57	3.5	5.2	0	0	0	91.2
	18	+	+	−	−	A	19	0	0	0	0	0	100.0
	19	+	+	−	−	−	1073	0	0	0	0.8	1.3	97.9

* Percentages rather than numbers are given because of the large number of cultures used.

All the type 2 cultures producing either strong acid or acid and gas were inoculated into two tubes of Eijkman medium: one with a small inoculum from an agar slant, the other with a loopful of an 18- to 24-hour broth culture. Except in the case of the two cultures consistently producing 40 to 60 per cent gas, smaller volumes were produced by the loop method and on some occasions

cultures producing gas from the slant inoculation produced only acid from the loop inoculation. Hajna *et al.* (1939), using the loop method and air incubation, found 2.8 per cent of 180 indole-negative *Aerobacter* producing gas at 46°C. while from slant inoculations and water bath incubation we found 2.4 per cent of 711 similar cultures produced gas at 45.5°C.

Cultures producing acid, and acid and gas, when plated from the Eijkman bath and incubated at 37°C. produced colonies ranging in size and color from very small colorless to large black-centered colonies. Small colorless colonies grew readily in lactose broth at 37°C. but required from 2 to 3 days to produce acid and gas. When plated after gas was formed only uniform, black-centered colonies were produced. Occasionally, large white colonies appeared which required from 5 days to 3 weeks for the production of gas from lactose at 37°C. In every case tested these slow-lactose-fermenting variants could be trained back to rapid fermentation though in some cases many weeks of plating and selecting were required. Such cultures grew with mucoid consistency and in some cases marked encapsulation resulted. A similar mucoid condition for *Eberthella typhosa* grown at high temperatures has been reported (Stuart 1924).

Of 59 cultures, types 3 to 7 inclusive, none produced gas and only one (type 7) produced strong acid.

Of the 5 type 2 cultures consistently producing a strong acid reaction in 24 hours, three were from soil and two from grains. Of the 17 cultures producing gas on one or more tests, two were from water and the remainder including the two Eijkman positive cultures were from human feces. Immune serums were prepared against one of the cultures isolated from each of these sources. An antigenic analysis by agglutination and absorption tests with the various cultures and antiserums as previously described (Stuart *et al.* 1940) showed that (a) the 3 soil cultures were probably identical (agglutinating to titer and removing all aggutinins upon adsorption), the water cultures were closely related to, but not identical with, the soil culture (agglutinating to titer and reducing but not completely adsorbing the homologous agglutinins), the grain and some human cultures were related (agglutinating to low titers) while other human cultures were not related to the soil culture; (b) the 2 grain cultures were probably identical, the 2 water, 3 soil and some human cultures were related to the grain culture while other human cultures were not related; (c) the 2 cultures isolated from water were probably

identical, the cultures from soils were closely related to the water culture, some of the human cultures were related while the grain cultures and other human cultures were not; (d) no culture was related to the human culture selected for the production of immune serum. In a previous antigenic analysis (Stuart *et al.* 1940) of 103 type 2 cultures tested in 9 type 2 antiserums no culture was found identical with or even closely related to the 9 cultures used to produce the antiserums. In the present work, an analysis of 22 cultures possessing unusual ability to grow at 45.5°C. showed that 4 cultures (18.2 per cent) were probably identical with and 5 cultures (22.7 per cent) closely related to one or another of the 4 cultures used to produce the antisera.

Since cellobiose has not been generally used for differentiating the intermediate section, our types 8, 9, and 10 will be discussed collectively as indole-negative "*Citrobacter.*" Of 723 "*Citrobacter*" cultures tested the per cent showing no growth, growth, slight acid and strong acid reactions does not appear to vary significantly from type 2 cultures. One "*Citrobacter*" culture produced a bubble of gas. The culture was plated and retested as previously described. The Eijkman reaction varied from slight acid on one test to 20 per cent gas on another. One culture was Eijkman positive producing about 30 per cent gas. When plated from the Eijkman tube this culture produced from small colorless to large black colonies and in addition large white colonies. Black colonies were Eijkman positive and when replated produced black and white colonies. Large white colonies required 17 days to produce acid and gas at 37°C. Cultures established from a black colony were called 8E+ and from a white colony 8E−. After serial plating and selecting, the ability to ferment lactose rapidly was restored to 8E−. Cultures 8E+ and 8E− were tested several times after normal lactose fermentation was restored to the latter. Culture 8E+ was consistently Eijkman positive and on plating from the Eijkman tube produced black and white colonies. Culture 8E− varied in its reaction from growth to slight acid at 45.5°C. In the biochemical and IMVIC reactions the parent and variant cultures appeared to be identical. To eliminate any question of contamination and to determine antigenic relationships, rabbits were immunized with each culture. Reciprocal differential adsorption (Stuart *et al.* 1940) showed that the parent and variant cultures were antigenically identical. It would seem that the high temperature was involved in the production of the slow-lactose-fermenting

variant since numerous attempts to isolate the variant from young and old lactose tubes at 37°C. failed.

Despite the small number of types 11 and 12 cultures tested they appear to be Eijkman negative. On the other hand, of 114 type 13 cultures, all were positive. Hajna *et al.* (1939) tested 1374 *Escherichia* including 30 "indole-negative" cultures. Of the total number 5 were Eijkman negative so all the indole-negatives may have been positive or at most only 5 could have been negative. Wilson *et al.* (1935) and Topley and Wilson (1937) reported indole-negative *Escherichia*, "*Escherichia* type II probably of nonexcretal origin," as Eijkman negative. Not having used cellobiose, Wilson's type 13 cultures may have been type 11 or 12 or both which do appear to be Eijkman negative. Phelps (1940) using cellobiose found 9 type 13 cultures, all isolated from one sample of oysters, negative. Although nonfecal, Eijkman negative type 13 cultures have been reported, their habitat must be quite circumscribed. Considering the findings of Hajna *et al.* (1939) and the present work it would seem that type 13 coliform cultures are for the most part fecal in origin (Stuart, Baker *et al.* 1940) and Eijkman positive. From a practical standpoint the use of cellobiose to distinguish between types 11, 12 and 13 is unimportant since, apart from grains, of over 10,000 coliform cultures isolated from the various sources the average distribution of types 11 and 12 was slightly over one-tenth of one per cent.

There is some evidence that even our Eijkman positive type 13 cultures are less "specialized" than type 19. Of 560 type 19 cultures inoculated on citrate agar slants and left at room temperature for 8 weeks only 7 or 1.2 per cent produced colonial growth on the medium whereas of 43 type 13 cultures 19 or 44.1 per cent showed either colonial or continuous growth in the same period of time. From 2 of the type 13 and 2 of the type 19 cultures showing colonial growth, citrate negative and citrate positive variants were established (Parr 1938, Stuart, Baker *et al.* 1940). The citrate positive variants were carried by serial transplants at irregular intervals on citrate agar, the citrate negative parent cultures on standard agar for 9 months. At the end of this time the parents and variants were still positive at 45.5°C.

Types 14, 15 and 16 (usually classed as indole-positive "*Citrobacter*") were, except for 2 type 16 cultures, Eijkman negative. The 2 positive type 16 cultures produced only one or two colonies on citrate agar in 5 days and may have been type 19 cultures producing citrate positive variants.

Type 17 appears to be Eijkman positive. The 5 exceptions could have been citrate negative variants of type 14, a shift that has been previously observed (Griffin *et al.* 1940). All type 18 cultures were Eijkman positive.

Of 1073 type 19 cultures, 1050 were Eijkman positive. Fourteen were Eijkman doubtful, producing generally from a bubble to 10 per cent gas. Growth was relatively sparse with such cultures and at the end of 48 hours at 45.5°C. a trace of the green color of the indicator could usually be distinguished. Plating and retesting from 3 to 5 times showed little variation in gas production. Plates inoculated from the Eijkman broth showed intergrading black colonies as previously described but no large white colonies. In control tests at 37°C. from 40 to 50 per cent gas was produced in Eijkman broth. The normal growth of such cultures seemed to be definitely inhibited at 45.5°C. All such cultures were isolated from human feces.

Nine type 19 cultures produced strong acid but no gas in from 12 to 24 hours at 45.5°C. Growth appeared to be quite comparable to cultures producing 50 per cent gas in the same time. No change was observed in the Eijkman reaction of the 9 cultures in any of 8 consecutive platings and testings as previously described. Eosin-methylene-blue plates streaked from the 9 cultures were quite comparable to similar plates streaked from positive cultures. Three gas-producing and 3 non-gas-producing cultures were inoculated into tubes containing 25 ml. of Eijkman medium and incubated at 45.5°C. As soon as a gas bubble appeared in the known positive cultures and at varying intervals until gas production was complete, the viable count was determined for all six tubes. No significant difference was found in the viable count of the gas-producing and non-gas-producing cultures. These non-gas-producing strains grew and produced lactase at 45.5°C. but were deficient in dehydrogenase. Since Ziegler *et al.* (1941) have shown that the dehydrogenases of lactic and formic acids are only partially inactivated at 53.5 and 77°C. respectively, the production of dehydrogenase by the 9 strains must have been inhibited at 45.5°C.

Three of the non-gas-producing strains were isolated from water and 6 from human feces. In an antiserum prepared from a water strain the 2 other water strains and all 6 fecal strains agglutinated to varying titers but failed to reduce the homologous titer of the serum upon adsorption. In an antiserum prepared from a fecal strain, 4 of the fecal strains agglutinated to titer

and completely removed the homologous agglutinins. The one remaining fecal strain and the 3 water strains agglutinated in this antiserum but failed to reduce the homologous titer. Of the 9 cultures tested 4 (44.4%) were homologous with one of the 2 antiserums whereas in previous work 7 (0.63 per cent) of 113 type 19 cultures were homologous with one or another of 10 type 19 antiserums (Stuart, Baker *et al.* 1940). This again, as with type 2 cultures, emphasizes the homogeneity of cultures reacting abnormally in the Eijkman test as compared with coliform cultures in general.

Two hundred and thirty-five aberrant coliform cultures were tested at 45.5°C. in Eijkman broth containing glucose. Among these cultures were microaerogenic, papillae-forming and anaerogenic strains (Stuart, Baker *et al.* 1940). A few strains tentatively classed as non-lactose-fermenting coliforms because of their ability to produce acid and gas in sucrose or salicin or both and to produce indole were also included. With one exception the reactions of these cultures were quite comparable to the corresponding types of normal coliform cultures. Type 2 aberrant cultures with two exceptions were Eijkman negative, type 8 were all negative, whereas type 19 aberrant cultures with one exception were positive. The only marked difference between normal and aberrant coliforms occurred in type 16 cultures. Of 27 normal type 16 cultures, 7 failed to grow, 13 showed growth but no acid, 5 produced acid and 2 were Eijkman positive, whereas of 18 aberrant type 16 cultures 2 showed no growth, 3 produced strong acid, one was Eijkman doubtful and 12 were positive. Eleven of the 12 positive cultures failed to ferment lactose and sucrose, fermented salicin slowly and gave a few scattered colonies on citrate agar in 4 or 5 days. These cultures, which will be discussed in a subsequent paper, appear to be true "paracolon" as designated by Topley and Wilson (1937). The remaining 7 aberrant type 16 cultures were wholly comparable in their Eijkman reaction to the normal type 16 cultures.

Twenty-three strains of *Proteus morganii*, 42 of *P. vulgaris* and 106 of *P. mirabilis* were tested at 45.5°C. in glucose Eijkman medium. *Proteus* organisms produce only small amounts of gas under any conditions. Some of our cultures required 48 hours at 37°C. to produce 10 per cent gas; others produced only a bubble of gas in 48 hours. It was, therefore, decided to group cultures producing any gas or strong acid in 48 hours at 45.5°C. as positive and all others as negative. Of the *morganii* cultures

4.4 per cent were Eijkman positive, 9.5 per cent of the *vulgaris* cultures were positive whereas 97.1 per cent of the *mirabilis* cultures were positive. Other biochemical tests tend to group *vulgaris* with *mirabilis* rather than with *morganii* (St. John-Brooks *et al.* 1939, Rustigian *et al.*, 1941. It will be recalled that *morganii* was until recently classed as a *Salmonella*.

To complete a survey of the Eijkman characteristics of the Family Enterobacteriaceae, 30 strains of *Eberthella typhosa*, 45 strains of *Salmonella*, and 37 strains of *Shigella* were tested at 45.5°C. in Eijkman glucose broth. With these cultures two tubes

TABLE 3*

Eijkman reactions of Eberthella, Salmonella and Shigella from agar slant and broth inoculations (48 hour readings)

	CULTURES	AGAR SLANT INOCULATION						BROTH CULTURE INOCULATION					
		0	G	A	A^2	D	+	0	G	A	A^2	D	+
Eberthella typhosa	30	1			29			3	7	11	9		
Salmonella group A	1				1						1		
group B	12				9	2	1	1			5	4	2
group C	13				7	5	1				5	4	4
group D	8				8			1		1	6		
group E	6				2	4					1	2	3
other groups	5				1	4					2	2	1
Shigella sonnei	17				17						17		
alkalescens	4				4						4		
dispar	1				1						1		
paradysenteriae (Hiss, Strong, Flexner, etc.)	15	6	4	4	1			14			1		

* Numbers rather than percentages are given because of the small numbers of cultures used.

0, no growth; G, growth; A, slight acid; A^2, strong acid; D, Eijkman doubtful; +, Eijkman positive.

of Eijkman broth were inoculated, one with a small inoculum from a 24-hour agar slant and the other with a loopful of a 24-hour standard broth culture. The results of these tests are shown in table 3.

Table 3 shows that with *E. typhosa* marked variations resulted from the size of the inoculum. Of 30 cultures inoculated from agar slants 29 produced strong acid and one failed to grow; inoculated from broth only 9 produced strong acid, 18 grew sparsely and 3 failed to grow. The presence or absence of Vi antigen does not appear to affect the Eijkman reaction of *E. typhosa*. Of 45 *Salmonella* cultures, including representatives of

the different groups, all produced strong acid or gas when inoculated from an agar slant. When inoculated from broth, 2 failed to grow, one grew sparsely while the remainder produced strong acid or gas. Tubes inoculated from slants showed growth much sooner than tubes inoculated from broth, but tubes inoculated from broth produced gas more frequently and to a greater volume than the corresponding cultures inoculated from slants. The slower growth resulting from broth inoculations appears to enhance gas production with *Salmonella* cultures, while quite the reverse appears to be true with coliform cultures. Group D cultures, including *E. typhosa*, definitely appear to be less heat resistant than cultures from the other groups particularly from broth inoculations.

Twenty-two *Shigella sonnei*, *alkalescens* and *dispar* strains and 15 strains of *paradysenteriae* (Hiss, Strong, Flexner, etc.) were tested. The first 22 strains produced strong acid from both slant and broth inoculations, some in from 6 to 12 hours. A number of the *sonnei* strains were tested several times and on only two occasions did a transplant fail to grow at 45.5°C. On the other hand, of 15 *paradysenteriae* strains, only one culture produced strong acid from either inoculum or showed growth from the broth inoculation. The *paradysenteriae* cultures were retested with the inoculums described and with a loopful of a 24-hour agar slant culture with the same results. The one culture, a Flexner Z strain, producing strong acid in from 6 to 12 hours from either inoculum, was checked biochemically and serologically and found to be a true *paradysenteriae* culture.

DISCUSSION

The ordinary biochemical activities, especially the production of gas from carbohydrates, of the different genera of the Family Enterobacteriaceae tend to increase from *Serratia* through *Erwinia* to *Aerobacter* then decrease through *Escherichia*, *Proteus*, *Salmonella* to *Shigella*. A similar condition appears to be true of the Eijkman relationships of these genera. Heavily inoculated *Serratia* cultures produced no acid and were for the most part nonviable after 24 hours at 45.5°C. A few *Erwinia* produced acid in 48 hours and were viable after 24 hours, whereas 100 per cent of the selected fecal *Aerobacter* cultures produced acid and were viable after 24 hours at 45.5°C.

Aerobacter and intermediate cultures seldom produced gas at

45.5°C. while *Escherichia* seldom failed to do so. The exact position of *Proteus* is difficult to determine since some were Eijkman positive and some were Eijkman negative. Of the more restricted *Salmonella* many produced acid and some produced gas but they were more sensitive to heat than *Escherichia*. A somewhat similar relationship was found in the genus *Shigella* wherein the less circumscribed *alkalescens* and *sonnei* produced strong acid from broth inoculums while *paradysenteriae* for the most part failed to grow at 45.5°C.

From *Serratia* to *Shigella* the normal habitats of these organisms generally become more and more restricted as do their metabolic requirements. Similar gradations in other characteristics of these organisms can be found. We believe these trends in specialization should be recognized by making *Serrateae*, *Erwineae*, and *Escherichieae* Tribes I, II, and III respectively in the Family Enterobacteriaceae.

Considering the coliform group, including aberrant coliforms, the temperature range at which these organisms grow and produce acid and gas from lactose or glucose is remarkable. Some pseudomicroaerogenic type 2 cultures producing acid and gas vigorously at 20°C. show no visible growth when inoculated into lactose broth preheated to 37°C. (Stuart *et al.* 1940). Normal *Aerobacter* cultures produce acid and gas at 37°C. but vary in their reactions at 45.5°C. Most of them grow and a number produce acid but gas production is exceptional. On the other hand the great majority of *Escherichia* produce gas more rapidly and more abundantly at 45.5°C. than at 37°C.

The Eijkman test, like all other tests, when applied to the coliform group shows no absolute lines of demarcation between the different sections (*Aerobacter*, intermediates and *Escherichia*) but only general lines of division with occasional intergrades not easily classified in any rigid system of taxonomy. The exceptions to the Eijkman rule are not sufficiently numerous to detract measurably from the value of the test for practical purposes, while the exceptions themselves make the test a valuable tool in the systematic study of the coliform and related groups of bacteria.

In previous investigations on the coliform bacteria (Stuart, Griffin *et al.* 1938, Stuart, Wheeler *et al.* 1938, Griffin *et al.* 1940, Stuart, Baker *et al.* 1940, and Stuart, Mickle *et al.* 1940) 3 groups of 6 types each and one additional type were established. While the 19 types were of great value in a detailed study of the coliform and related

bacteria they were never intended for routine work. Our investigations show that a simplified grouping of the coliform organisms similar to that of Topley and Wilson (1937) is adequate for practical purposes. It should be pointed out that the proposed grouping is one of convenience and carries no taxonomic recommendations.

Simplified grouping of the coliform bacteria

	INDOLE	VOGES-PROSKAUER	GROWTH ON CITRATE	EIJKMAN REACTION	PROBABLE ORIGIN
Escherichia coli					
Variety I	+	−	−	+	Fecal
Variety II	−	−	−	+	Fecal
Intermediate					
Variety I	−	−	+	−	Nonfecal
Variety II	+	−	+	−	Nonfecal
Aerobacter aerogenes					
Variety I	−	+	±	−	Nonfecal
Variety II	+	+	+	−	Nonfecal

In the simplified grouping the methyl-red test is omitted since we agree with Levine (1941) that it is unreliable unless the time and temperature are carefully controlled and under any conditions it gives no information not furnished by the Voges-Proskauer test. It will be noted that *Escherichia coli*, variety II is of probable fecal origin and Eijkman positive as the present and previous work shows. Eijkman negative cultures of this type which apart from grains are seldom encountered can be safely considered as nonfecal types 11 and 12. The probable origin of the great majority of strains of intermediate, varieties I and II is nonfecal but it must be admitted that cellobiose negative strains (types 10 and 16) have been isolated more often from feces than from any other single source (Griffin *et al.* 1940). The proposed grouping also takes into account the fact that a few *Aerobacter aerogenes*, variety I strains fail to grow on citrate agar.

In pure culture work the Eijkman test is doubtless the best single test that can be applied to determine the probable origin of coliform bacteria. In routine water analysis and similar work if an Eijkman bath is available the determination of the probable origin of cultures isolated from the completely confirmed test can be accomplished most conveniently by the Eijkman test. Otherwise the use of indole, V. P. and citrate tests and on a very few occasions cellobiose will give the same information.

SUMMARY

1. The more primitive members of the Family Enterobacteriaceae, *Serratia* and *Erwinia*, were frequently killed by a temperature of 45.5°C. in 24 hours even from heavy inoculums while *Aerobacter* was not.

2. In their Eijkman characteristics the intermediates were much more closely related to *Aerobacter* than to *Escherichia* because *Aerobacter* and intermediates seldom produced gas from lactose at 45.5°C. while *Escherichia* seldom failed to do so.

3. *Salmonella* strains usually grew and produced acid, and some produced gas at 45.5°C.

4. In the genus *Shigella* the less pathogenic *alkalescens* and *sonnei* produced acid readily at 45.5°C. while the *paradysenteriae* strains for the most part failed to grow.

5. It is suggested that a trend toward specialization be recognized by making *Serrateae*, *Erwineae* and *Escherichieae* Tribes I, II, and III respectively in the Family Enterobacteriaceae.

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