DISSOCIATION IN BRUCELLA ABORTUS: A DEMONSTRATION OF THE RÔLE OF INHERENT AND ENVIRONMENTAL FACTORS IN BACTERIAL VARIATION

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Brucella abortus exhibits the phenomenon of dissociation, that is, changes in colony form, culture characteristics, cell morphology, immunological reactions, biochemical reactions, and virulence. Henry (1933) has presented a detailed description of dissociation in the genus *Brucella*, which usually involves changes from the antigenically active smooth (S) type to intermediate (I), and to antigenically inactive rough (R), brown (Br), and other types.

This type of dissociation resembles variations which are common in many bacterial species. Hadley (1927, 1937) has published general reviews of bacterial dissociation and interpreted such changes as due to normal cyclogenic development. Others (e.g., Mayer, 1938; Reed, 1940; Dubos, 1945) have tried to explain the changes which occur during dissociation as being due to spontaneous hereditary changes (mutations), with a subsequent selection of mutants which can best persist in any given environment. Many investigators (e.g., Hinshelwood and Lodge, 1944) have postulated a direct influence of the environment upon such spontaneous changes. Criticism of Hadley's "ontogenetic" theory has arisen from experimental work which has demonstrated the lack of linked character variation, such as agglutinative behavior and cell form, in the change from the S type to the R type (Humphries, 1944). Such criticism is further substantiated by recent work which provided substantial proof for the existence of undirected, spontaneous hereditary changes (mutations) in bacteria (Demerec, 1945; Demerec and Fano, 1945; Anderson, 1944; Luria and Delbrück, 1943; Gray and Tatum, 1944; Roepke, Libby, and Small, 1944; Severens and Tanner, 1945).

Whereas the last-mentioned investigators focused their attention mainly on the mutational step per se, another line of approach to the problem of bacterial variation was provided by studies on differences between dissociating populations (clones) started from single cells and maintained under standard conditions. Thus Braun (1945), in a preliminary report on factors controlling bacterial dissociation, furnished data demonstrating the existence of inherent differences between clones in regard to dissociation percentages,[2] evidence suggestive

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[2] Previously, in describing methods for the comparative estimation of the percentage of dissociated cells within a population after 10 days of growth in broth, the name "dissociation rate" was suggested for the percentage of dissociation observed (Braun, 1945). Since the term "rate" may be misleading in this connection, it is now proposed to use the term "dissociation percentage" generally for the amount of dissociation observed after various periods of growth, and to call the dissociation percentage in 10-day-old broth cultures the "dissociation index" of a given strain.

of spontaneous appearance of dissociated types and their subsequent establishment within a population under the control of inherent and environmental factors. A more detailed account of the work which led to the detection of these inherent factors and an evaluation of their role in controlling dissociation will be presented here. A number of environmental effects superimposed upon the action of these inherent factors will be described and an interpretation of dissociation in terms of these results will be attempted. The order in which the data were actually obtained will be used as the order of presentation.

MATERIAL AND METHODS

The strains used throughout this work originated from *Brucella abortus*, strain 19, cultures, which have been periodically distributed by the United States Bureau of Animal Industry. They are numbered 19-1, 19-2, 19-3, etc., according to the date of their distribution. The strain most extensively used in the present work, namely, 19-9, was received at this station on October 26, 1942; it was kept on potato agar slants and was periodically transferred and checked for purity. A rough strain was isolated from old saline suspensions of 19-9 S early in 1943. This strain, called 19-9 R, was also kept on potato agar slants and was transferred and checked for purity periodically. To date, no dissociation has occurred in these stock cultures.

The percentage of dissociation (see footnote 2) was tested by suspending morphologically identical colonies in beef infusion broth at 37 C. During the early experiments an arbitrary number of colonies was suspended in a small amount of saline, and equal amounts of this suspension were inoculated into each tube of broth. Later on one picked colony was inoculated into each tube of broth, or one picked colony was suspended in saline. The suspensions were then adjusted according to density, and equal amounts of these adjusted suspensions were inoculated into the broth. The amount of broth in each tube was generally adjusted to 5 ml. Platings were made at various times after the start of the broth cultures. One loop of broth was streaked on 2 per cent glycerol, 1 per cent glucose agar plates; the plates were incubated at 37 C for four days and then checked for dissociated colonies under the low power of a dissecting microscope with an obliquely transmitted lighting arrangement (Henry, 1933). If dissociated colonies were present, at least 100 colonies of each plate were counted and classified in the region where colonies were well separated; and the percentage of dissociated colonies was estimated from such counts. Although the presence of *different* types of dissociated colonies was roughly estimated, no attempt was made actually to count the relative number of rough (R), brown (Br), intermediate (I), and other types among the dissociated colonies. That is, if a broth culture had been started with smooth colonies, the percentage of all nonsmooth colonies on the test plate was determined. If a broth culture had been started with rough colonies, the percentage of all nonrough colonies was counted.

When buffered broth was used the broth was prepared with McIlvaine's standard buffer solutions instead of water. The pH was usually tested with the

help of indicators, except in certain experiments in which small changes of pH were checked with a Coleman pH meter.

The isolation of single cells was performed according to the method of Johnstone (1943). This method proved to be entirely satisfactory once adequate experience had been gained in identifying single cells on the agar surface. The most important factor in this identification is correct lighting and use of the substage optical system.

Viability counts were made by the appropriate dilution of culture samples and the counting of colonies which grew on plates with 8 per cent cooked horse blood agar (chocolate medium) after the plates had been streaked with 0.1 ml. of the diluted sample. Total counts were made on samples from appropriately diluted cultures with the help of a Petroff-Hausser counting chamber and dark-field illumination at a magnification of 320×.

TABLE 1

Percentage of dissociated colonies on plates made at various intervals after start of broth cultures with different pH

pH OF BROTH	BROTH INOCULATED WITH	PERCENTAGE OF DISSOCIATED COLONIES AFTER					
		2 days	6 days	13 days	17 days	26 days	35 days
6.6	S	none	1	10	50	50	50
	R	1	5	5	5	10	no growth
7.2	S	none	1	1	2	2	2
	R	none	none	none	none	none	none
8.6	S	none	none	none	none	none	none
	R	none	none	none	none	none	none

RESULTS

Effect of the pH of Broth on Dissociation

The differential effect of broth with different pH values on dissociation was first noted when one batch of broth was accidentally made too alkaline (pH 8.6). When S or R type organisms were suspended in this alkaline broth, no dissociation occurred for as long as 35 days after the broth cultures were started, whereas normally dissociation became apparent a few days after the start of a broth culture. Subsequently, two other batches of broth were prepared, one with a pH of 7.2 and one with a pH of 6.6. Suspensions (Gates 4) were made of 19-9 S and of 19-9 R, and 0.1 ml of either the S or the R suspensions was added to 10 ml of broth. Plates were made from these broth cultures at frequent intervals after inoculation. The resulting (estimated) percentages of dissociation, in a series of tests for each batch of broth, are summarized in table 1. These tests showed that slight acidity of the media (pH 6.6) favored dissociation, whereas little dissociation occurred at pH 7.2 and none at pH 8.6.

In another set of experiments the effect of the pH of the media on dissociation

was further confirmed. This time 1 ml. of buffer solution of a definite pH (McIlvaine's standard buffer solution) was added to 2 ml of unbuffered broth. The addition of solutions of pH 3 and pH 4 inhibited growth of both S and R types. Considerable dissociation was observed in tubes to which a buffer solution of pH 5 had been added, much less in tubes to which solutions of pH 6 and pH 7 had been added, and none when a buffer solution of pH 8 was added.

On periodical inspection of changes in the pH of unbuffered broth after inoculation with S type cells, it was found that active growth of the bacteria increases the pH. The pH of 20 tubes of unbuffered broth was 6.8 on the day of inoculation; 2 days later all tubes showed a pH of 7.4; 4 days after inoculation the pH was 7.6; and it was 8.0 when tested on the sixth day. It remained at 8.0 until the cultures were discarded 2 weeks later. Regardless of whether

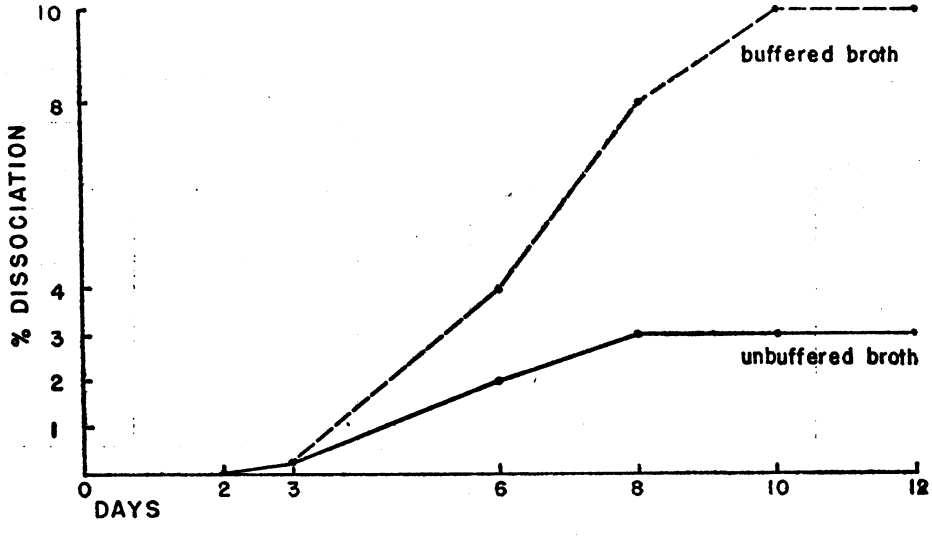

FIG. 1. PERCENTAGES OF DISSOCIATION OF IDENTICAL STRAINS AFTER VARYING PERIODS IN BUFFERED OR UNBUFFERED BROTH

the initial pH was 6.8, 7.0, 7.2, or 7.4 in subsequent tests, the pH of the unbuffered broth always increased to 8.0 a few days after inoculation with *Brucella abortus*.

The change in daily percentage of dissociation parallels this change of the pH in unbuffered broth, i.e., dissociation proceeds more rapidly during the period when the pH is low and reaches an equilibrium when the pH of the medium approaches its 8.0 equilibrium (figure 1). Similar tests with buffered broth later revealed that, under conditions of constant pH, dissociation percentages increase at a more constant rate, reaching a maximum point of dissociation after about 10 days (figure 1).

Since it was known that the optimum pH for the growth of *Brucella abortus* is 6.8 (Huddleson, 1943), a relationship between growth rates and percentage

of dissociation was immediately suspected at this point, and this was later experimentally confirmed (table 7).

Whereas these results provided proof concerning the effect of the pH of the environment upon dissociation percentages, further tests, utilizing different strains, soon indicated that the pH acts only as a modifying, environmental factor on dissociation percentages, whereas other factors, presumably inherent, determine the potential range of dissociation percentages for each strain. This was first recognized when three different smooth strains were subjected to growth in broth of identical pH. All three strains originated from the 19-9 S

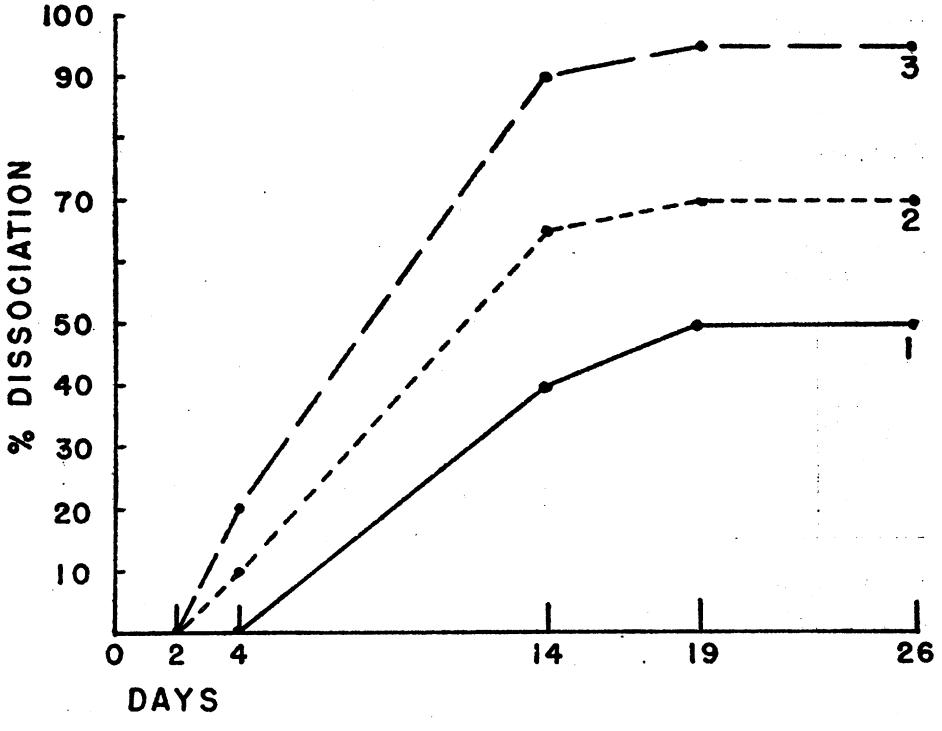

FIG. 2. PERCENTAGES OF DISSOCIATION OF THREE SMOOTH STRAINS AFTER VARYING PERIODS IN BROTH

culture. Strain 1 represents the original 19-9 S culture, i.e., an S type which has been stable for at least 1 year. Strain 2 represents an S type which was isolated from an experimentally produced I type which had reverted to S 6 months previously, and strain 3 had an origin similar to strain 2 but had reverted to S only 2 months previously. Suspensions of equal density of each of these three morphologically similar strains were inoculated into broth with a pH of 6.8, and the percentage of dissociated cells was ascertained 2, 4, 14, 19, and 26 days after inoculation. The results are represented graphically in figure 2. Although growing in identical environments, the three morphologically identical strains

showed clearly different dissociation percentages: strain 3 showed the highest dissociation percentages during all tests, strain 2 was characterized by consistently lower dissociation percentages, and strain 1 exhibited not only the lowest percentages of dissociation but also a later onset of dissociation.

In another test, one S strain showed 50 per cent dissociation after 10 days of growth in buffered broth of pH 6.6 and 2 per cent dissociation after 10 days in buffered broth of pH 7.4, but another morphologically similar strain showed 1 per cent dissociation after 10 days at pH 6.6 and none at pH 7.4.

Like results were obtained with two morphologically similar rough strains, both of which had been obtained by dissociation of 19-9 S after prolonged growth in broth, but they had been isolated at different times. Table 2 presents the results of two different tests, one with unbuffered broth with an initial pH of 7.2 and one with unbuffered broth of pH 6.8. Aside from illustrating the constant differences in percentage of dissociation between the two strains at pH 7.2 as well as pH 6.8, these results again demonstrate the differences which are

TABLE 2

Percentage of dissociated colonies on plates made at various intervals after start of broth cultures from two rough strains; illustrating the effects of strain differences and of the pH of the environment upon degree and onset of dissociation

pH OF BROTH	R STRAIN USED	PERCENTAGE OF DISSOCIATED COLONIES AFTER								
		2 days	4 days	5 days	7 days	8 days	12 days	15 days	17 days	25 days
6.8	1		none		none		none			
	2		none		none		20			
7.2	1	none		none		none		none	none	30
	2	none		none		none		none	none	70

found in onset and percentage of dissociation if broths of different pH are used, regardless of the potential tendency for dissociation of any given strain.

Inherent Factors Controlling Percentages of Dissociation

In order to obtain more information on those apparently inherent factors which determine the potential range of percentage of dissociation of a strain, it was first necessary to establish a standard set of conditions in which the degree of dissociation of different cultures could be compared, since it had been shown that dissociation percentages were subject to modifications by changing environmental conditions such as pH. Buffered broth of pH 6.8 was chosen as the standard experimental environment, and the percentage of dissociation on the tenth day of growth in this broth (each tube inoculated with one colony) will be called the "dissociation index" of a strain, i.e., the percentage of dissociated colonies observed on plates made from 10-day-old broth cultures.

A convincing proof for the existence of inherent factors controlling dissociation percentages was obtained by the utilization of a new technique of single cell isolation, first described by Johnstone (1943). The following procedure

was used: A single cell was isolated on an agar-covered slide, and, after its growth into a colony, cells from this colony were streaked on a 2–1 agar plate. Colonies arising from these daughter cells of a single isolated cell were then picked, and each colony was suspended individually in a tube of broth. After 10 days, samples from each broth culture were streaked on 2–1 agar plates, and the plates were checked for percentages of dissociated colonies 4 days later.

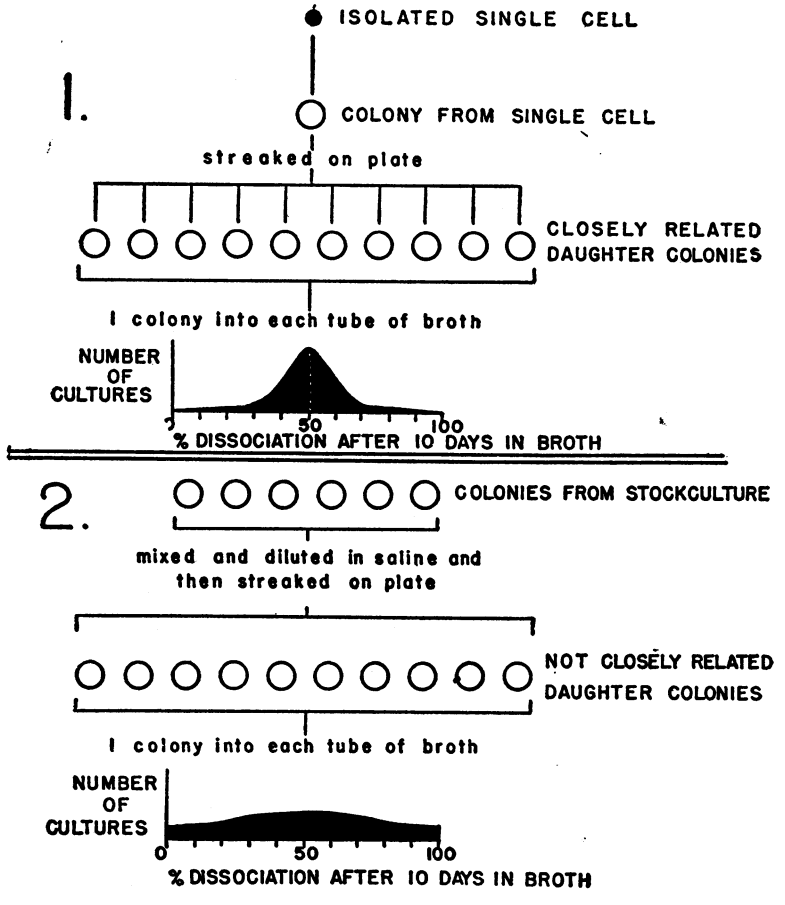

FIG. 3. DIAGRAMS ILLUSTRATING THE METHOD USED IN DEMONSTRATING THE EXISTENCE OF INHERENT FACTORS CONTROLLING DISSOCIATION PERCENTAGES

For further details see text

Since the individual colonies originated from a single cell, the dissociation indices observed after growth in each of the broth cultures were expected to be fairly close to a mean if the inherent dissociation potential is greater than the variability (mutability) of an individual cell. This is illustrated in diagram 1 of figure 3.

At the same time a similar test was made on colonies which, although from the same culture, originated from various single cells. As illustrated in diagram

2 of figure 3, a considerable number of colonies were taken from the same stock culture from which the single cell used in the first part of this experiment originated. The colonies were suspended in saline and mixed, and the diluted suspension was then streaked on a 2-1 agar plate. Again, isolated colonies were picked and individually suspended in a tube of broth. The rest of the experiment was exactly like that in the first part: after 10 days samples of the

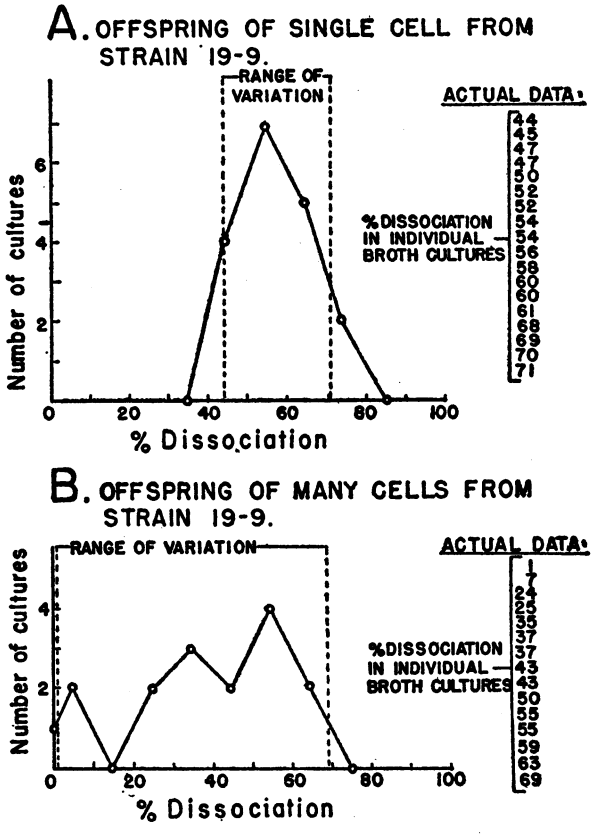

FIG. 4. A DEMONSTRATION OF THE EXISTENCE OF INHERENT FACTORS CONTROLLING DISSOCIATION PERCENTAGES

Actual results obtained in experiments conducted according to the method illustrated in figure 3.

broth cultures were streaked on plates, and the percentage of dissociated colonies was recorded 4 days later. If differences in inherent factors controlling dissociation percentages exist between cells of different origin and if such potentials are greater than the variability (mutability) of each cell, then the dissociation indices observed in this second experiment would be expected to be more widely scattered around a mean than those observed in the first experiment in which colonies originated from one single cell. This is graphically illustrated in diagram 2 of figure 3.

The actual results followed the expectations just expressed, supporting the idea that inherent factors control dissociation percentages. Figure 4A illustrates the results of an experiment which was started from a single cell according to diagram 1 of figure 3. The dissociation indices of 18 broth cultures, started from closely related colonies, show a distinct mean around 55 per cent, with relatively little variation extending to 44 per cent and 71 per cent. Considerable variation from a mean could be observed in the control experiment, which was

TABLE 3

Examples of dissociation indices in various clones (B-F)

	A	B	C	D	E	F
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	1	44	74	28	7	0.0
	7	45	76	32	7	0.0
	24	47	78	36	7	0.0
	25	47	80	36	7	0.3
	35	50	81	41	9	3
	37	52	82	42	9	3
	37	52	87	43	9	4
	43	54	88	44	13	5
	44	54	91		14	13
	50	56	95		15	
	55	56	95			
	55	60				
	59	60				
	63	61				
	69	68				
Dissociation constant*.	40± 5.09	54± 1.74	84± 2.23	38± 1.99	10± 0.95	3± 1.27
Number of colonies counted...	1,640	1,586	1,777	1,220	1,687	1,929

A. Dissociation indices of individual colonies originating from many single cells isolated from strain 19-9.

B. Dissociation indices of individual colonies originating from one single cell isolated from stain 19-9.

C–F. Examples of dissociation indices of colonies from strains started from single cells.

* Dissociation Constant = most representative dissociation index of a population = p of Hendricks, *Poul. Science*, 14: 365.

conducted according to diagram 2 of figure 3 and the results of which are presented in figure 4B. The graph illustrates clearly the irregular dispersion of dissociation indices in this less related material, the percentages of dissociation varying from 1 per cent to 69 per cent.

Subsequently, progenies from many other single cells, isolated from various strains, were tested, and the similarity of dissociation indices within clones was confirmed. A few representative samples are given in table 3 together with the

results of a statistical analysis which proved that differences in dissociation indices between clones are statistically highly significant. The clones with different dissociation indices were obtained by systematic selection. This means that single cells from existent strains were tested for dissociation indices, and the heterogeneity or homogeneity of various strains were thus established (examples of four B.A.I. strains are presented in table 4). If a strain showed a fairly high percentage of low dissociating cells, attempts were made to establish low dissociating clones from that strain (for example, see clone D of table 3, which was obtained from strain 19-12 of table 4). Correspondingly, high dissociating clones were obtained from strains for which preliminary tests had

TABLE 4

Examples of dissociation indices of colonies from four B.A.I. strains

STRAIN	TOTAL NUMBER OF COLONIES COUNTED	DISSOCIATION INDICES OF COLONIES	MEAN
		per cent	*per cent*
19-9	1,640	See B in fig. 4	54
19-11	952	21 29 33 46 78	41
19-12	842	22 40 52 81 96	58
19-13	990	70 77 79 95 96	83

TABLE 5

Dissociation indices of colonies from a heterogeneous strain illustrated by three broth cultures from each colony

		PARENT COLONY									
		A	B	C	D	E	F	G	H	I	K
Dissociation indices (%) in 3 broth cultures from each colony	(1)	5	5	5	28	24	37	33	36	52	4
	(2)	3	8	11	19	40	20	32	49	45	4
	(3)	2	8	6	25	24	35	56	49	68	62 (!)
Total number of colonies counted		544	509	531	517	435	471	430	465	459	462

indicated the presence of high dissociating cells. A survey of available strains proved that many of them are highly heterogeneous in regard to the dissociation index, whereas only a few are fairly homogeneous. Selected clones have so far shown great stability in regard to retention of their original dissociation index if preserved on agar slants at low temperatures.

Additional proof for the genetic control of dissociation percentages was obtained by the following test: Single colonies, picked from a plate made from a heterogeneous stock culture, were individually suspended in 1 ml of saline, and 3 broth cultures were then inoculated with equal suspensions (0.3 ml) of the same colony. The results, compiled in table 5, show that generally the dissociation indices of cultures from the same colony are very much alike, but

dissociation indices differ considerably between colonies. The occasional lack of agreement between cultures made from one colony of this heterogeneous population is to be expected, since colonies do not always arise from one cell only. Furthermore, in this and all other experiments on dissociation percentages in clones, a small percentage of spontaneous changes in inherent factors controlling dissociation percentages has to be taken into consideration. Future experiments are expected to yield information on the actual frequency of such changes.

Environmental Factors, Other than pH, Affecting Dissociation Percentages

After clones with known dissociation indices under standard conditions had been established, it became possible to test the modifying effects of a number of environmental factors upon genetically controlled dissociation percentages.

Daily transfer. An S clone which showed 45 per cent dissociation after remaining for 8 days in the same tube of broth (buffered pH 6.8) was used for a test in which 0.1 ml of a saline suspension of this strain was inoculated: (a) into a number of broth cultures which were not disturbed for 8 days, and (b) into a number of identical broth cultures of the same batch of broth, which did not remain undisturbed for 8 days, because 0.5 ml of 1-day-old broth cultures were daily transferred into fresh tubes of broth.

When plates were made on the eighth day from the 8-day-old undisturbed cultures of (a), 45 per cent dissociation was recorded; whereas plates made from the seventh transfer of (b), i.e., organisms which had grown for 8 days in daily renewed broth, showed less than 1 per cent dissociation. It was thought that this difference in dissociation percentages between growth in aging broth and growth in daily renewed broth might be due to one or more of a combination of three factors: (1) Actively growing (multiplying) cells may produce a metabolic product which enhances dissociation, and the effect of such metabolites would be lessened in case of daily transfers. (2) The growth of organisms may produce a deficiency in the broth which enhances dissociation. Again daily transfers would almost nullify the effect of such a deficiency. (3) Since, in comparison with aging broth cultures, daily transferred cultures have a smaller size of population per ml of broth, a lack of population pressure may be responsible for the failure of dissociated types to establish themselves within the daily transferred population. This infers that population pressure has a major role in the establishment of dissociated types and suggests that differences in population pressure may affect the propagation and survival of S types and dissociated types differentially. In daily renewed broth dissociated types may then have less of a chance to establish themselves because of the lack, or low intensity, of population pressure; but in aging cultures, in which the struggle for survival of different types can be assumed to be much more intense, a high degree of population pressure exists, favoring the establishment of dissociated types.

It was possible to eliminate the first two possibilities, (1) and (2), as responsible factors through the following test: S type organisms were suspended in filtrates

of broth in which other S type organisms of *Brucella* had grown and dissociated previously for 2 weeks. Daily transfers to tubes containing 5 ml of filtrate only and to tubes containing 3 ml of filtrate plus 2 ml of fresh broth were then made. In another set the organisms remained for 10 days without transfer in tubes with filtrate only or in tubes with filtrate plus fresh broth. Controls consisted of tubes with fresh broth only and tubes with 3 ml fresh broth plus 2 ml saline. Daily inspection of the percentage of dissociation did not reveal any differences between controls and tubes containing filtrate. After the possibility had thus been eliminated that metabolite or deficiency factors caused the striking differences in dissociation percentages between growth in aging broth and daily renewed broth, the role of population pressure in dissociation came into the foreground of attention.

Different batches of broth. The next suggestive evidence regarding an effect of population size on dissociation percentages was obtained when it was observed that certain batches of broth yielded different dissociation indices when cells from identical clones were used (see example in table 6). Although the proportion of ingredients was the same in all batches of broth, the peptone and beef

TABLE 6

Effect of different batches of broth upon dissociation index (D. I.) and growth

(Averages for 21 cultures)

BROTH BATCH	D. I. OF NO. 1287	D. I. OF NO. 1863	D. I. OF NO. 1983	TOTAL COUNT PER ML AFTER 16 DAYS
	per cent	*per cent*	*per cent*	*billions*
IX	0	1	0	1.375
X	13	7	5	2.475

extract used did not originate from the same lot. Differences in nutrient material supporting growth were, therefore, suspected, and an inspection at different times of the total number of bacteria in two such batches of broth, originally inoculated with an equal number of cells, revealed such growth differences. Table 6 illustrates the higher dissociation percentages observed in broth which supported better growth.

Daily plating versus one plating, and the effect of temperature changes. Another striking effect of modified growth rates upon dissociation percentages of cells from the same clone was observed in a large number of experiments, which were conducted as follows: Tubes with broth (from the same batch of broth) were inoculated with equal amounts of a suspension made from cells belonging to one clone. One set of tubes was disturbed daily when samples were removed for streaking on plates; another set of tubes was left undisturbed for 10 days. Table 7 shows a representative example of the results obtained.

The dissociation index in a daily disturbed population is much lower than the dissociation index of the genetically identical population left undisturbed for 10 days. Counts revealed that the total number of cells after daily disturbance is smaller than the number of cells in equally old but previously undisturbed

cultures. Again, less growth produced a lower dissociation index. The growth differences between "disturbed" and "undisturbed" cultures are probably caused

TABLE 7

Dissociation percentages and total count in buffered and unbuffered broth cultures from one clone after frequent plating vs. one plating on 10th day only

(Averages for 32 cultures)

Broth	DISSOCIATION PERCENTAGE ON VARIOUS DAYS AFTER START OF BROTH CULTURES					TOTAL COUNT PER ML ON 10TH DAY
	2	4	6	8	10	
					per cent	
Buffered	None	None	None	None	0.1	1.2 billions
Buffered (Plated 10th day only)					11	1.75 billions
Unbuffered	None	None	None	None	None	550 millions
Unbuffered (Plated 10th day only)					0.01	700 millions

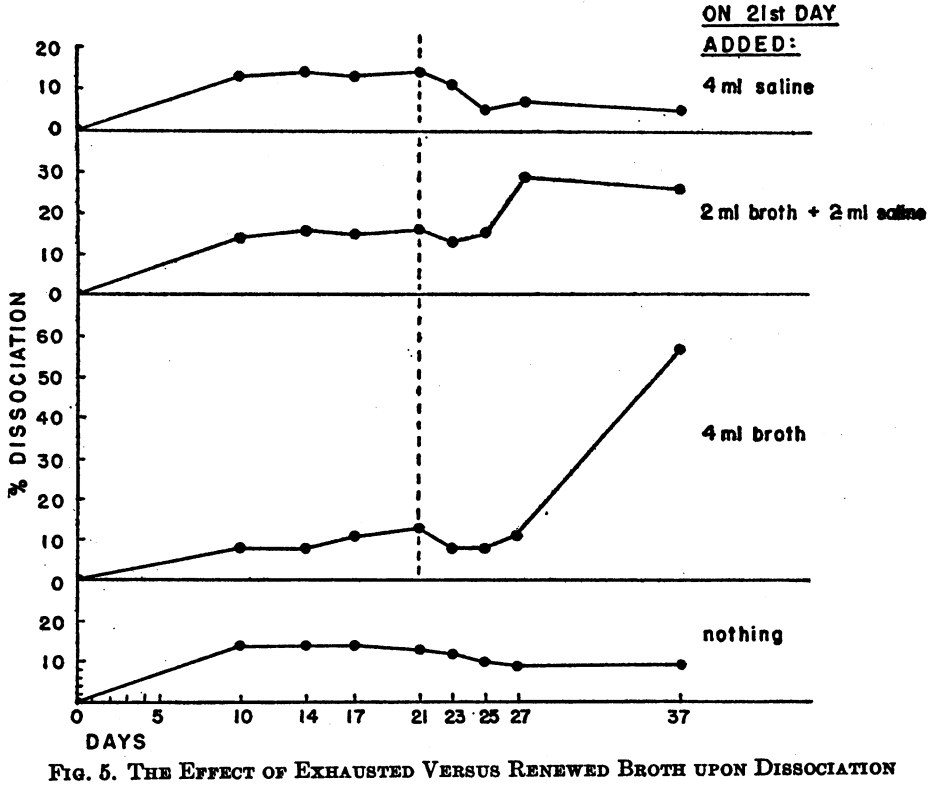

FIG. 5. THE EFFECT OF EXHAUSTED VERSUS RENEWED BROTH UPON DISSOCIATION PERCENTAGES

by temperature changes to which the "disturbed" cultures were subjected when they were daily removed from the incubator for streaking. It was subse-

quently established that temperature differences have a pronounced effect on growth rate, viability, and dissociation.

Exhausted versus renewed broth. The dependency of dissociation upon growth was next proved by adding fresh broth to aged broth cultures which had ceased to support growth. It had previously been observed that after about 10 days of growth in broth dissociation percentages reached an equilibrium (see figure 1), and counts of the total number of cells later showed that growth actually ceases at this point. Therefore, a number of broth cultures were now started with identical inocula; dissociation percentages were then checked on the 10th, the 14th, the 17th, and the 21st days. To four of the 21-day-old broth cultures 4 ml of fresh broth were added; to two others 2 ml of fresh broth and 2 ml of saline were added; to four others 4 ml of saline were added; and two cultures were left undisturbed. Dissociation percentages were then checked 2, 4, 6, and 16 days later. Representative results are presented in figure 5. It can be observed that dissociation percentages, after having reached an equilibrium in exhausted broth, rise significantly if fresh broth is added, i.e., if new growth and subsequent competition is initiated.

TABLE 8

The effect of reduced O/R potential of broth upon dissociation index (D. I.), growth, and viability

	D. I. OF NO. 1287 (AVERAGE OF 16 CULTURES)	TOTAL COUNT PER ML ON 14TH DAY	VIABLE COUNT PER ML ON 14TH DAY
	per cent	*millions*	*millions*
Normal Broth	13	980	620
O/R Broth	less than 1	700	500

Reduced O/R potential. The most striking demonstration of the effect of growth rates upon the establishment of dissociated types within a population was obtained when broth with a lowered oxidation-reduction potential was used. Such broth was prepared by the addition of 0.1 per cent agar and 0.1 per cent sodium thioglycolate to standard broth (Reed and Orr, 1943). Eight tubes of standard broth and eight tubes of "O/R broth" were each then inoculated with 0.2 ml of the same suspension of bacteria. Plates made from these cultures indicated a considerable percentage of dissociated cells in samples from the standard broth, but less than 0.1 per cent of dissociated cells were found in cultures made with "O/R broth" (table 8). Counts made on the fourteenth day of the total number of cells and the number of viable cells revealed a far smaller number of both per ml of "O/R broth" than per ml of standard broth (table 8). Thus it was found again that environmental conditions which alter growth rates and affect viability cause a striking modification of genetically controlled dissociation percentages.

Growth Rates and Dissociation Percentages

The results described above provided a great deal of indirect evidence on the major role of growth rates and population pressure in the determination of

dissociation percentages. The necessary direct evidence was finally supplied by actual counts of bacteria.

Before the results of such counts are reported, some general principles of growth in bacterial populations may be stated. Jordan and Jacobs (1944) reported on observations with *Bacterium coli* which showed that as long as food is supplied the total number of organisms increases steadily, but the number of viable cells soon reaches an equilibrium. In the present work with *Brucella abortus* these observations were confirmed (see figure 6). Furthermore, it was

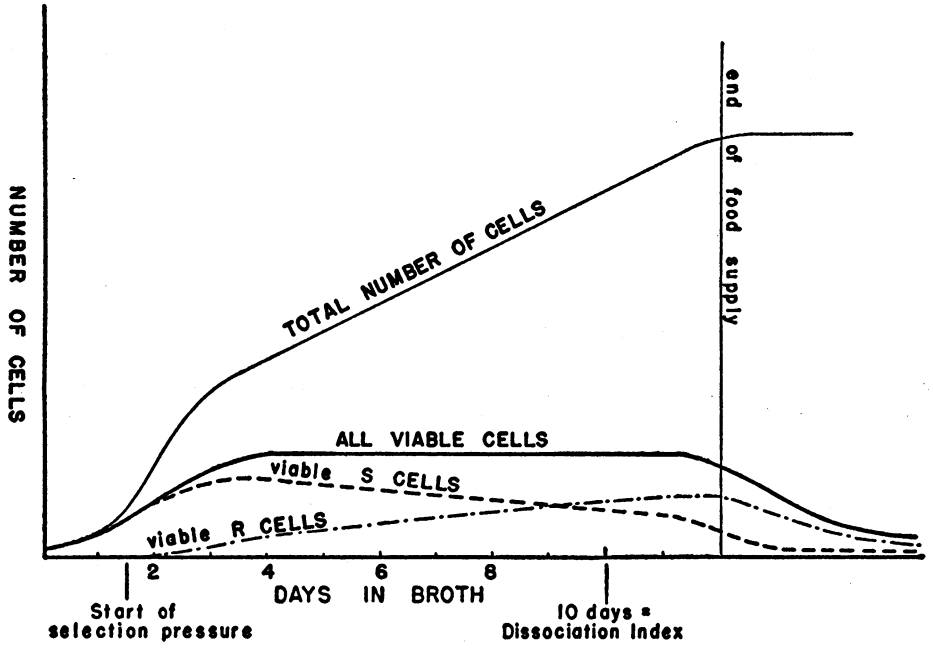

FIG. 6. A GRAPHICAL REPRESENTATION OF THE TOTAL NUMBER OF CELLS AND OF THE NUMBER OF VIABLE CELLS DURING GROWTH IN BACTERIAL POPULATIONS AND AFTER CESSATION OF GROWTH DUE TO EXHAUSTION OF FOOD SUPPLY

The curves of "total numbers of cells" and "all viable cells" are based on experimental results by Jordan and Jacobs (1944) with *Bacterium coli*, which have been substantiated in the studies with *Brucella abortus* reported here. For an explanation of the curves of "viable S cells" and "viable R cells" see discussion.

determined that a maximum exists for the number of viable cells which can be found per ml of broth (approximately 500 millions per ml with possible slight variations between clones). Regardless of the size of the inoculum this maximum is always reached within the first 4 days after the start of growth in broth and is retained as long as growth persists. If a culture is started with more than 500 million per ml of viable cells, the number of viable cells will decrease to the "maximum level" of around 500 millions per ml within 24 hours and remain at that level as long as growth persists (figure 7).

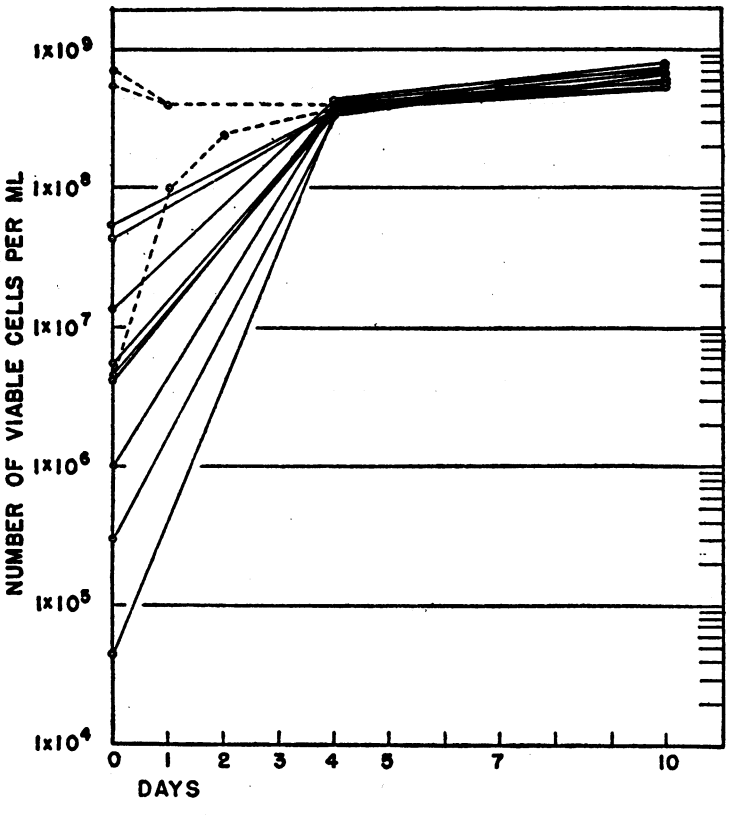

FIG. 7. NUMBER OF VIABLE CELLS PER ML IN BROTH CULTURES STARTED WITH VARYING AMOUNTS OF INOCULA, ILLUSTRATING THE LIMITS FOR VIABLE CELLS PER ML OF BROTH REGARDLESS OF THE AMOUNT OF THE INOCULUM

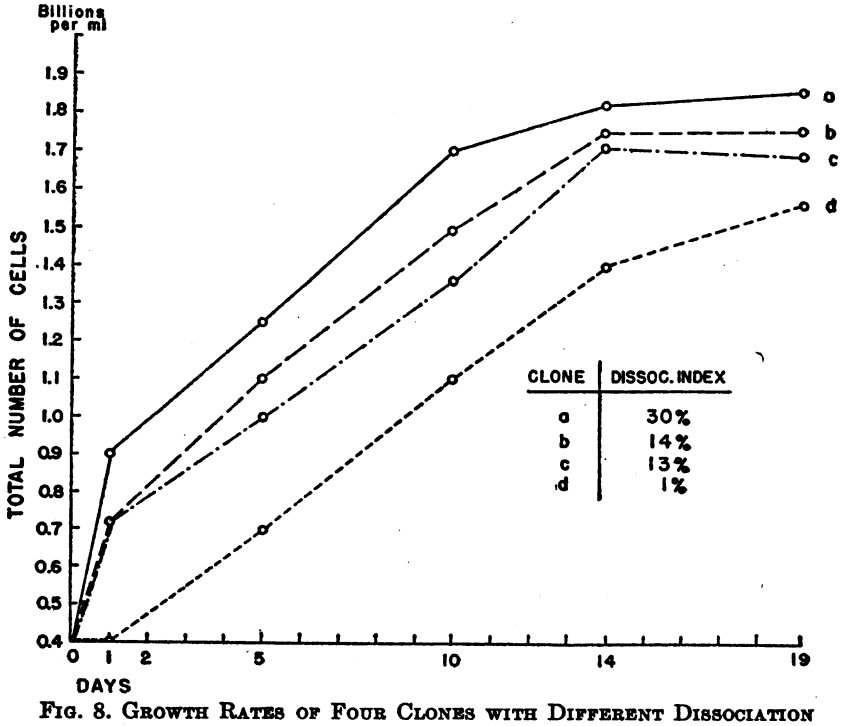

FIG. 8. GROWTH RATES OF FOUR CLONES WITH DIFFERENT DISSOCIATION INDICES

The total number of cells per ml have been adjusted to compensate for evaporation of the broth.

An effect of the size of the inoculum upon dissociation percentages has been observed and can be explained in terms of differential initial population pressure due to the limits for number of viable cells per ml. In the majority of tests, broth cultures with larger inocula (and thus presumably greater initial competition) showed higher dissociation percentages than cultures of the same clone inoculated with a smaller number of organisms. A representative example is given in table 10.

The total number of cells and the number of viable cells in growing populations of several clones were determined in broth cultures which had been started in duplicate with equally large inocula. The results which are presented in figure

TABLE 9

Average of total number of cells and number of viable cells in smooth and rough cultures, respectively, after 7 and 10 days of growth in broth

		DAYS AFTER START OF CULTURES	
		7	10
Smooth	Total count per ml	0.85×10^9	2.10×10^9
	Viable count per ml	0.49×10^9	0.55×10^9
Rough	Total count per ml	0.54×10^9	1.00×10^9
	Viable count per ml	0.54×10^9	0.54×10^9

TABLE 10

The effect of the size of the inoculum upon dissociation percentages of one clone

NO. OF CULTURE	INITIAL NUMBER OF VIABLE CELLS PER ML	DISSOC. PERCENTAGES ON 9TH DAY
2528	280 millions	11
2529	280 millions	7
2530	280 millions	10
2531	280 millions	12
2532	280 thousands	3
2533	280 thousands	4
2534	280 thousands	4
2535	280 thousands	4

8 demonstrate inherent differences in growth rates and differences in duration of the lag phase between clones. These differences are clearly associated with the dissociation indices of the clones tested: the clone with the highest dissociation index has the fastest growth rate; clones with low dissociation indices exhibit slower growth rates and longer duration of the lag phase.

Differential Viability of Variants

Differences in viability, i.e., the ratio of the total number of cells to the number of viable cells, between smooth populations from different clones during growth in broth have so far not been thoroughly analyzed. However, significant differ-

ences in viability between smooth clones under special environmental conditions, namely, suspension in 1 per cent urea solutions, have been observed and will be reported separately. Recently, significant differences in viability during growth in broth have been found between cultures started from smooth cells and cultures started from rough cells. Both S and R had been isolated from one 10-day-old broth culture which had been started with S cells only. The results of one such test, compiled in table 9, indicate higher viability but slower growth rate in the rough culture as compared with the smooth culture.[3]

DISCUSSION

The foregoing experiments have revealed that inherent factors control dissociation percentages and that it is, therefore, possible to select clones with different dissociation indices. The experiments also revealed that these genetically controlled dissociation indices can be modified by changes in the environment, particularly changes which affect the growth rate and viability. Under such altered environmental conditions the absolute degree of dissociation is changed, but the relative differences between two clones, such as a high dissociating one and a low dissociating one, are retained; i.e., environmental influences which lower the dissociation index will decrease the dissociation index of a high and a low dissociating clone proportionally. Finally, a demonstration of growth rate differences between clones with different dissociation indices directly confirmed the important role of inherent growth rates in the control of dissociation indices.

When inherent differences in dissociation percentages between clones were first observed, it was thought that these might be due to a different rate of appearance of variants in different clones. However, the subsequent studies on environmental effects upon the dissociation index in selected strains suggested that the percentage of dissociation, as such, is not an inherent characteristic, but rather a secondary indicator of primary inherent differences, such as differences in growth rate and viability between clones. In order to substantiate this suggestion, it is now necessary to demonstrate that differences in growth rate and viability alone can lead to differences in dissociation percentages, even at an equal rate of appearance of variants. The data here collected permit such a demonstration, provided the dissociation phenomenon is interpreted as a process of natural change (mutation?)[4] and selection, under the control of inherent and environmental factors. During periods of active multiplication a small percentage of variants arises continuously. The chances of these variants to establish themselves within a population (i.e., dissociation percentages) depend on their growth rates and viability within a given environment plus the degree

[3] A mathematical calculation based on the data of table 9 revealed that, despite its slower growth rate, the higher viability of the R type here tested is sufficient to give the R a higher selection value than the S if they are competing within one population.

[4] Although the work with *Brucella abortus* has not supplied any direct evidence that the actual change from one type to another is a mutational step, recent work by Humphries, Demerec, Luria and Delbrück, and others makes it highly probable that such is the case.

of population pressure existent in the population in which they arise. This population pressure, in turn, is determined by the growth rate and viability of the original members of the population.

This concept, which emphasizes the role of population dynamics and natural selection in dissociation, will now be amplified. As expressed above it necessitates the existence of population pressure for the establishment of variant types within a population. The work of Jordan and Jacobs on the growth of bacteria in liquid media and the work reported here with *Brucella abortus* have shown that, after an initial growth phase, crowding and competition between types will result. Since limits exist for the size of the viable population which can successfully maintain itself within a given amount of liquid, selection will take place. The point at which population pressure begins to act as selection pressure, producing conditions which permit the establishment of variant types with positive selection value, may vary and is determined by growth rates and viability. In figure 9 (A, B, and C) it has been attempted to illustrate the effects of differences in growth rates and viabilities upon variations in the time of the beginning of selection pressure, and it is shown how this will subsequently lead to different dissociation indices. It may be seen that if a strain A has an inherently fast growth rate, the point at which population pressure starts will be earlier in this strain than in a strain B with slow growth rate. Consequently the dissociation index (after 10 days of growth) will be higher in strain A, although the percentage of variants arising may be the same. The difference in growth rate, controlled by inherent factors in strains A and B, can also be produced by environmental factors, like pH, and different dissociation indices for one strain may thus result under different environmental conditions. Figure 9 C illustrates the possibilities for a shift in the start of selection pressure through higher viability of the original type, without change in growth rate, and the subsequent effect on dissociation indices.[5] Finally, D in figure 9 illustrates the effect of changes in the viability or the growth rate of the arising variant upon the dissociation index. The same graph, D, would also apply to changes in dissociation indices through changes in the rate of appearance of variants (differential mutation rates); this possible *additional* control of dissociation percentages through differences in mutation rates cannot be eliminated.

It has thus been shown how inherent factors which determine growth rates and viabilities can cause the experimentally demonstrated differences in dissociation indices of different homogeneous populations, even if variants appear at a constant rate. Two additional facts may be cited in further support of this interpretation. First, the implied necessity of population pressure for the establishment of variant types within a population is substantiated by the conditions under which the appearance of variant types are most commonly observed.

[5] Experimental evidence for this possibility has just been obtained: the growth rate of one clone used in a temperature experiment was similar at 34 C and 38 C; despite equal inocula the number of viable cells, however, differed greatly, i.e., 204 millions per ml at 38 C compared to 445 millions per ml at 34 C on the sixth day. The dissociation index at 34 C was 3 per cent, at 38 C 63 per cent.

Little dissociation has been observed when growth takes place on a solid medium. Here the chances for multiplication are physically limited, and, therefore, the population pressure is low. In liquid media, however, where dissociation is usually observed, far fewer limits exist for multiplication. Therefore, crowding and competition between types will result. This population pressure will then permit the establishment of variant types with positive selection value. Second,

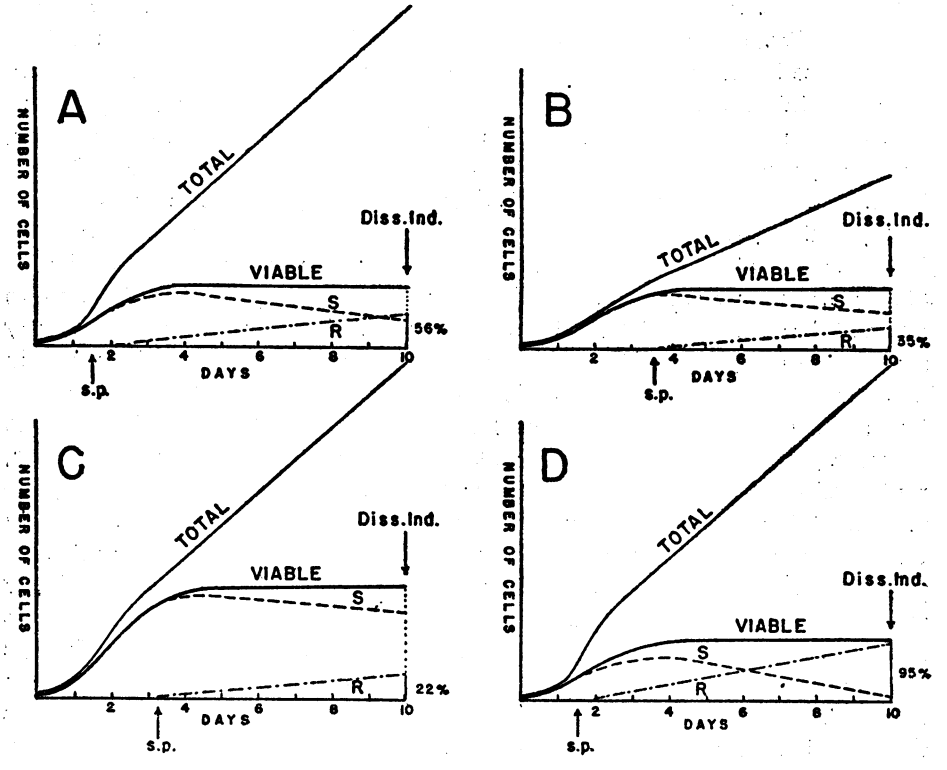

FIG. 9. DIAGRAMS ILLUSTRATING THE ESTABLISHMENT OF VARIANTS WITHIN A POPULATION THROUGH SPONTANEOUS OCCURRENCE (MUTATION) AND SUBSEQUENT SELECTION AND THE EFFECTS OF VIABILITY AND GROWTH RATE UPON THE DISSOCIAION INDEX

A. basic diagram; B. slower growth rate of original type; C. higher viability of original type; D. faster growth rate, or higher viability of variant, or higher rate of appearance (higher mutation rate). For further details see discussion. s. p. = start of selection pressure; Diss. Ind. = Dissociation Index;------viable S cells;—·—·—viable R cells.

according to the graphs in figure 9, some time is expected to elapse before sufficient population pressure is established in cultures started with a small inoculum. Consequently, not many dissociated types are expected to be found during the early period of population growth. Experimental data substantiate this expectation, since it has been shown that dissociation can first be observed a few days after the start of a broth culture (figure 1).

It should be mentioned that a relationship between morphologic variations and growth rates in various species of bacteria has previously been described by Henrici (1928). Henrici, however, interpreted his data in terms of regular and orderly changes which a bacterium undergoes during its stages of growth (cytomorphosis), claiming that "each character reaches its maximum development in some particular phase or at some particular point of inflection of the growth curve." Without going into a detailed criticism of Henrici's "cytomorphic" interpretation, it may be stated that his data fit equally well into the concept of dissociation here advanced, especially if it is kept in mind that Henrici failed to use clones in his studies.

The interpretation of dissociation in terms of the spontaneous appearance of variants, and their subsequent establishment under the control of inherent and environmental factors governing population dynamics, is meant to apply only to the occurrence of dissociation under natural conditions of population growth. It is likely that other mechanisms may cause dissociation by a more direct action on the cell, such as X-rays (Gray and Tatum, 1944), ultraviolet rays (Haberman, 1941; Hollaender and Emmons, 1941; Braun, 1943), possibly antibodies (Dawson and Sia, 1931; Emerson, 1944), and a specific transforming substance, a form of desoxyribonucleic acid, recently isolated by Avery *et al.* (1944). (In the case of antibodies, however, the effect may not be a direct one, but rather come through the creation of a specifically selective environment due to the presence of "suppressive" antibodies.)

During the work with clones of *Brucella abortus* inherent factors other than those controlling dissociation have been recognized. One of them is the inherent control of ability to withstand toxic effects, already mentioned. Another inherent characteristic seems to be the "pattern of dissociation," i.e., whether primarily from S to R, or from S to Br. Also, some clones appear to give rise to R types which can revert to a true S type; others give rise to R types which yield the so-called S^R type (Henry, 1933). This genetic control of the "pattern of dissociation" explains why some workers observed an S^R type (Henry, 1933) and others did not (Huddleson, 1943).

It can be hoped that recognition of the role of inherent and certain environmental factors in dissociation, as well as in the control of other characteristics of bacteria, will eventually lead to an improvement of vaccines through systematic selection of clones with desired characteristics for the manufacture of vaccines. Work in this direction is now under way.

SUMMARY

With the help of single cell isolation the existence of inherent differences between clones of *Brucella abortus*, strain 19, in regard to dissociation percentages has been demonstrated under standardized environmental conditions. Clones with statistically significant differences in dissociation indices have been systematically selected and have remained stable if stored at low temperatures.

These inherent dissociation percentages can be modified by environmental changes, particularly, changes which affect growth rates and viability. Thus,

changes in pH, daily transfers, changes in temperature, differences in available nutrients, or reduced oxidation-reduction potentials alter the dissociation index of any given clone. Actual counts of bacteria revealed that environmental conditions which decrease the growth rate lower dissociation percentages. It was found, finally, that, under standard environmental conditions, clones with different dissociation indices have inherently different growth rates.

The studies on environmental effects suggested that dissociation percentages, as such, are not an inherent characteristic, but rather secondary indicators of primary inherent differences in growth rate and viability between clones. An interpretation of the experimental results is offered which demonstrates that inherent or environmentally induced differences in growth rate and in viability can cause differences in dissociation percentages, even at an equal rate of appearance of new variants. This interpretation is based on observed phenomena of population dynamics and the idea (substantiated by the work of others) that a small number of variants (mutants) arises constantly during periods of active multiplication. Counts of the number of total and viable bacteria, respectively, had shown that during population growth the total number of cells increases steadily, whereas the number of viable cells, regardless of the size of the inoculum, soon reaches a maximum which is retained as long as growth persists. At the point of population growth at which the total number of cells becomes steadily greater than the viable number of cells, population pressure starts. This population pressure can act as selection pressure and will permit the establishment of spontaneously arising variants (with positive selection value) within a population. The growth rate and the viability of the original members of a population determine the point at which population pressure starts, and the growth rate and the viability of a variant determine its chances to establish itself within a population after population pressure has started. Therefore, any changes in growth rate or in viability can produce differences in dissociation indices.

Bacterial dissociation is thus interpreted in terms of the spontaneous appearance of variants (mutants) and their subsequent establishment under the control of the inherent and environmental factors which govern population dynamics.

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