Modification of Bactericidal Effects of Human Sera.* (25291)

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We employed antigenically similar strains of *Shigella dysenteriae* with inherent differences in their susceptibility to bactericidal effects of so-called normal human serum(1) to study modifying effects of carbohydrates and amino acids on serum susceptibility of bacteria *in vitro*. Changes in bacterial sensitivity to serum following changes in conditions affecting bacterial metabolism have been noted previously(2,3). In our studies we became aware of such modifications when, in studies on serum spheroplasts(4), pregrowth of bacteria in different broth media resulted in significant alteration of their subsequent susceptibility to antibody- and complement-containing human serum. This altered susceptibility represented merely a phenotypic modification without genetic changes, and altered sensitivity was also obtained when the bactericidal system was directly supplemented with different concentrations of various growth media. The latter condition, therefore, was utilized as a routine test system for measuring modifying effects of non-bactericidal and non-bacteriostatic concentrations of various supplements on serum susceptibility of *Sh. dysenteriae* and *Escherichia coli*.

Methods. Previously described(1,4) serum-resistant (S_1) and serum-sensitive (S_3) strains of *Sh. dysenteriae,* and a serum-resistant (SPI) and a serum-sensitive strain of *Escherichia coli,* were employed. Bactericidal serum effects were tested by determination of survivors following exposure of approximately 3 to 4,000 bacteria/ml to appropriately diluted serum in ABA buffer ± supplement for 60 minutes at 37°C(1). All tests were made at least in triplicate and plated in duplicate; as a rule standard errors approximated 5 to 10% of survival values(1). Preliminary tests employed determinations of the 50% endpoint in serial dilution tests, conducted in cup-like depressions of plastic trays (Disposo trays, Linbro Chemical Corp., New Haven, Conn.) where bactericidal reactions were terminated by addition of 2 ml of melted agar to 0.5 ml of a 60-minutes-old bacteria-serum suspension(5). All supplements were dissolved in saline, pH adjusted to 6.8, and added to equal volume of ABA buffer + serum. Serum from one human donor was used throughout; this serum apparently contained low levels of specific antibodies, since absorption with homologous organisms significantly reduced its bactericidal activity(4).

Results. Effects of addition of broth on serum susceptibility of 2 strains of *Shigella* are illustrated in Table I; high concentrations of broth protected bacteria from bactericidal serum effects, whereas low concentrations (<1:1000) enhanced their susceptibility. Supplementation with nutrient broth (Difco) and penassay broth (Difco) gave comparable results.

To test involvement of specific broth constituents in these modifications, the influence of glucose and certain other carbohydrates, added directly to the bactericidal serum system, was tested. Table II shows that 1 mg of glucose/ml significantly enhanced bacterial sensitivity, whereas other carbohydrates, in-

TABLE I. Effects of Broth on Serum Susceptibility of *Shigella dysenteriae,* Strains S_1 and S_3.

Broth added	Dilution	% survivors after 60′ exposure S_1	S_3
Tryptose	1:1	88	102
	1:10	36	32
	1:100	9	19
	1:1000	40	37
Casein hydrol.	1:1	96	108
	1:10	40	29
	1:100	12	9
	1:1000	47	36
Brain-heart	1:1	101	82
	1:10	53	35
	1:100	9	10
	1:1000	30	36
None (serum control)		42	31
	Serum dil.:	1:2.5	1:10
	Inoc. size:	3070/ml	3550/ml

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TABLE II. Effects of Carbohydrates on Serum Susceptibility of *Shigella dysenteriae*, Strains S_1 and S_3.

Sugar added	Conc., mg/ml	% survivors after 60′ exposure S_1	S_3
Glucose	10	39	28
	1	7	10
	.1	26	36
Galactose	10	42	40
	1	22	25
	.1	30	38
Lactose	10	36	40
	1	40	43
	.1	31	53
None (serum control)		34	45
	Serum dil.:	1:2.5	1:10
	Inoc. size:	3720/ml	3550/ml

cluding galactose, lactose, and also ribose, sucrose, and soluble starch, had no significant effects. Since glucose is the most readily utilized carbohydrate for organisms used, these observations were the first to suggest possible relationships between metabolism and bacterial sensitivity to serum.

This relationship was further indicated by the finding that presence of amino acids known to play a prominent role in metabolism of these bacteria similarly enhanced their sensitivity to bactericidal serum effects. Following determination of the range of nontoxic concentrations of 21 different amino acids, appropriate amounts of dl-forms of these acids were added to the bactericidal serum systems. Table III illustrates the different types of modified serum effects obtained with strain S_1 in the presence of certain amino acids; comparable data were obtained with strain S_3 and *E. coli* strains.

Since the most striking enhancement of bactericidal effects occurred in the presence of glucose and of those amino acids known to stimulate metabolic activity of bacteria here employed, it was believed that bacterial lysis resulting after injury to bacterial wall components may be dependent upon the extent of metabolism of injured cells. In other words, the previously demonstrated interference of serum factors with the integrity of bacterial wall components(4) may not suffice to cause bacterial lysis. The latter may occur only if wall-damaged bacteria "grow out of their damaged walls." If these considerations were correct, interference with metabolism would be expected to prevent bacterial lysis and killing even in presence of damaged walls. This was the case. As shown in Table IV, addition of 2 amino acid analogues, 5-methyl tryptophan, and fluorophenylalanine, interfered with bacterial killing in the presence of usually bactericidal concentrations of serum. Similarly, in studies with *E. coli* strains, it was demonstrated that presence of chloramphenicol (Table V), or of the just cited analogues, interfered with serum lysis of bacteria. Inhibitory effects of analogues or of chloramphenicol were absent, or significantly reduced, when bacteria were exposed to these compounds prior to treatment with unsupplemented serum. These data thus strongly supported the existence of a fundamental relationship between extent of bacterial susceptibility to serum and extent of bacterial metabolism subsequent to, or at time of, wall damage.

Two somewhat different types of protective effects of high concentrations of certain basic amino acids (Table III) were observed; both of these may be interpreted conditionally as

TABLE III. Effects of Amino Acids on Serum Susceptibility of *Shigella dysenteriae*, Strain S_1.

Amino acids added	% survivors after 60′ exposure in presence of: 1 mg/ml	10 mg/ml
None (serum control)	38	38
Enhancing		
dl-alanine	10	17
-aspartic acid	11	6
-glutamic "	10	8
-asparagine	11	17
Glycine	23	17
DAP	15	33
Protecting		
dl-cysteine	34	72
-ornithine	44	78
-arginine	53	86
Concentration-dependent		
dl-lysine	17	84
-histidine	13	57
Ineffective		
dl-serine	dl-citrulline	
-threonine	-phenylalanine	
-valine	-tyrosine	
-methionine	-proline	
-leucine	-tryptophane	

Serum dil.: 1:2.5. Inoc. size: 3260/ml.

TABLE IV. Influence of 2 Amino Acid Analogues on Susceptibility of *Shigella dysenteriae*, Strains S_1 and S_3, to Bactericidal Serum Effects.

Compound added	Conc., mg/ml	% survivors after 60′ of exposure: S_1 Inhibitor control	S_1 Inhibitor + serum	S_3 Inhibitor control	S_3 Inhibitor + serum
5-methyl dl-tryptophan	1	94	61	87	77
	5	110	78	97	90
dl-p-fluorophenylalanine	1	91	57	103	62
	5	100	70	85	84
None (serum control)	0	92	32	97	40
	Serum dil.:	1:2.5		1:10	
	Inoc. size:	3420/ml		3480/ml	

involving an interference with attachment of serum bactericidins to sites on the bacterial surface. The first type of effect (upper part of Table VI) was dissociable; this means that following 2 hours incubation of bacteria with so-called basic amino acids in buffer, the protective effect was removed by simple washing with saline prior to serum treatment. In contrast, if nutrients, such as tryptose broth, were present during pretreatment with basic amino acids (lower part of Table VI), the protective effect was *not* dissociable by washing.

TABLE V. The Effect of Chloramphenicol on Serum Susceptibility of *Escherichia coli* Strains.

Chloramphenicol, μg/ml	Coli SP1 % survivors after 60′ exposure to: S-F*	S-C*	Coli S S-F	S-C
10	92	46	95	59
5	99	82	101	84
1	103	72	116	68
.5	95	47	96	43
.1	84	37	92	39
.05	103	34	94	52
.01	92	33	104	37
—	103	34	99	39
Serum dil.:	1:1.5		1:10	
Inoc. size:	2510/ml		3110/ml	

* S-F, serum-free suspensions; S-C, serum-containing suspensions.

Absence of any irreversible *direct* effects of basic amino acids on bactericidal serum itself was indicated by finding that sera first exposed to amino acids for various periods of time and then dialyzed against saline did not show any altered bactericidal effects.

The protective effects of basic amino acids occurred only in pH ranges of 6.8 and above, and disappeared in more acid pH ranges. A comparison of related serum-resistant and serum-sensitive strains revealed that serum-sensitive strains had a requirement for higher concentrations of basic amino acids, to achieve the same degree of protection obtainable by lower concentrations with serum-resistant strains.

When mixtures containing both enhancing and protective amino acids were added to serum, the protective effects always counteracted any enhancing effects (Table VII). Further, combinations of different enhancing amino acids were more effective in increasing bacterial susceptibility to serum than comparable amounts of single amino acids (Table VII). Finally, non-bactericidal concentrations of dipeptides, added to bactericidal serum systems, produced greater enhancement of bactericidal effects than the same amount of their constituent amino acids (Table VIII). This last effect might be attributable to the cells' greater capability to take up dipeptides in comparison to amino acids(7).

Discussion. The foregoing data support the

TABLE VI. Effects of Pre-exposure to Basic Amino Acids, plus or minus Broth, upon Subsequent Serum Susceptibility of *Shigella dysenteriae*, S_1 or S_3 Organisms.

Pretreatment with:	% survivors after 60′ exposure S_1	S_3
dl-lysine (10 mg/ml)	46	33
-arginine "	40	33
-lysine in tryptose broth	91	88
-arginine *idem*	82	75
Serum control	32	29
Serum dil.:	1:2.5	1:10
Inoc. size:	3410/ml	3010/ml

conclusion that the extent of bacterial lysis by serum is greatly dependent upon metabolic activity of bacteria at time of, or very shortly after, serum damage to wall components. This relationship appears similar to the well-established dependence of penicillin lysis upon metabolic activity(6), and may possibly be a reflection of the need for growth and/or cell division to render partially damaged walls lethal for the affected bacterium. However, involvement of more complex interactions between metabolism and serum lysis cannot be ruled out. The present finding that inhibition of protein synthesis by chloramphenicol interferes with bactericidal serum effects, at first appears to be in contrast to prior data (3) on the additive killing effects of serum and chloramphenicol observed with *Salmonella*. However, earlier data were obtained not only with entirely different bacteria but apparently involved the use of bactericidal antibiotic concentrations, whereas only bacteriostatic concentrations have been employed here. One possible explanation for the observed protective effects of basic amino acids could be based on the assumption that these compounds may be able to combine with, and to protect, bacterial surface sites which act as receptors for bactericidins. The proper conditions for such combinations may exist only in environments with approximately neutral or slightly basic pH. Whereas such combinations appear easily dissociable after exposure in a nutrient-free environment, their irreversibility after exposure in the presence of nutrients might reflect a need for growth or active metabolism to permit firm attachment or partial penetration of basic amino acids into the bacterial wall. Studies are under way to elucidate the nature of these effects and to test the capability of specific carbohydrates and amino acids to modify bactericidal serum effects *in vivo*.

TABLE VII. Effects of Addition of Single and Mixed Amino Acids on Serum Susceptibility of *Shigella dysenteriae*, S_1 and S_3.

Amino acids added	Conc., mg/ml	% survivors after 60′ exposure S_1	S_3
Single additions			
dl-glutamic acid	1	39	36
-aspartic "	1	44	41
-alanine	1	53	46
-lysine	10	89	87
Mixed additions			
dl-glutamic acid	.33	13	23
-aspartic "	"		
-alanine	"		
-glutamic acid	.5	83	85
-aspartic "	.5		
-lysine	10		
None (serum control)		70	58
	Serum dil.:	1:5	1:20
	Inoc. size:	3140/ml	2650/ml

TABLE VIII. Effects of Dipeptides on Serum Susceptibility of *Shigella dysenteriae*, S_1 and S_3.

Compound added	Conc., mg/ml	% survivors after 60′ exposure S_1	S_3
l-lysyl-glycine	1	12	16
-lysine + glycine	.5 of each	37	35
Glycyl-dl-phenyl-alanine	1	14	19
Glycine + dl-phenyl-alanine	.5 of each	33	37
Glycyl-l-glutamic acid	1	13	18
Glycine + l-glutamic acid	.5 of each	30	34
dl-alanyl-dl-alanine	1	17	22
-alanine	1	29	33
None (serum control)		41	47
	Serum dil.:	1:3	1:15
	Inoc. size:	3710/ml	2920/ml

Summary. Studies with *Shigella dysenteriae* and *Escherichia coli* strains revealed enhancement of bacterial susceptibility to normal human serum *in vitro* in the presence of low concentrations (0.1%) of broth, of glucose, or of certain amino acids known to play a prominent role in bacteria's metabolism. Dipeptides containing these amino acids were more effective than single amino acids. In contrast, metabolic inhibitors, including 5-methyltryptophan, fluorophenylalanine, and non-bactericidal concentrations of chloramphenicol, decreased bacterial susceptibility to serum. Such decrease also was obtained in the presence of, or following exposure to, relatively high concentrations (0.5%) of so-called basic amino acids. The latter effects were reversible by washing when bacteria had been exposed in the absence of nutrients, but they were irreversible following exposure in the presence of nutrients.

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Influence of Nitrogen Source on Growth of *Streptococcus Bovis.** (25292)

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Niven *et al.*(1) showed that a number of strains of *Streptococcus bovis* have no absolute requirement for any single amino acid, and that most strains studied could grow in a medium containing arginine as the only source of amino nitrogen. Our previous work has shown growth dependence of 2 strains of *S. bovis* on CO_2 when arginine served as the nitrogen source(2), and demonstrated certain interactions between CO_2 and various individual amino acids during growth in this medium(3). Ability of the organism to grow well in media containing arginine as sole source of amino nitrogen suggested that other compounds might similarly serve in this capacity. This report describes ability of various individual amino acids and other nitrogenous compounds to support growth of 3 strains of *S. bovis* in the presence of added CO_2.

Materials and methods. The cultures were *Streptococcus bovis* ATCC 9809 and 2 cultures from Cornell University collection designated as P-10 and P-2. Maintenance of cultures and preparation of inocula have been described(2). *Basal medium* consisted of glucose, K_2HPO_4, sodium thioglycollate, adenine, guanine, uracil, a vitamin mixture, and salts mixture in the same proportions described by Prescott and Stutts(2). Various nitrogen sources were added for each experiment, usually at levels of 0.05 or 0.1 m mole/6 ml culture, although graded levels of these compounds were frequently tested. Also, some experiments compared responses to different amino acids supplied at the same level of total nitrogen. Filter sterilized $NaHCO_3$ was added to autoclaved medium at 0.05 or 0.2 m mole/6 ml culture to supply the CO_2 requirement (2,3). *Procedures for growth tests* were identical to those used in earlier experiments(2). Growth was determined turbidimetrically, either at a fixed time, or more commonly, at different times during the growth phase. Incubation was usually terminated at 72 hours or less, and was never carried beyond 96 hours.

Results. Ability of compounds to serve as sole nitrogen source.† Thirty-seven amino acids and related organic nitrogenous compounds were tested for ability to serve as sole source of amino nitrogen for 3 strains of *S. bovis*. A list of compounds tested and their effects on 2 strains are shown in Table I. These data represent average responses, and evaluations shown are based on the following factors: (a) total growth, (b) rate of growth, and (c) consistency of growth response from one experiment to another. Results obtained with strain P-2 are not shown, since this culture did not show consistent heavy growth with any of the individual compounds listed in Table I, although it grew well with a complete amino acid mixture, as did the other 2 strains.

Relatively few amino acids tested supported

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† Exclusive of vitamins, purines, and pyrimidines. Purines and pyrimidines are not essential for growth under conditions employed(1), but were routinely included in the basal medium to insure more rapid and regular growth.