Toxicity of Autoclaved Cystine for *Lactobacillus bifidus.** (19996)

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In connection with studies on the ability of different sugars to support the growth of *Lactobacillus bifidus* and its unbranched mutants(1), it was observed that if any of the sugars was sterilized by filtration and added to autoclaved, sugar-free medium, there was usually a negligible amount of growth. Orla-Jensen(2) found that for growth of lactobacilli sugar must be autoclaved with the medium or with the nitrogen source (yeast extract). Sugar autoclaved separately and added to autoclaved medium did not support growth. The effect was attributed to the action of decomposition products of sugar such as methylglyoxal. Methyglyoxal itself was tested and found to be effective, but it also had to be autoclaved with the yeast extract. Smiley, Niven, and Sherman(3) working with *Streptococcus salivarius,* and later Rabinowitz and Snell(4) and Snell, Kitay and Hoff-Jorgensen(5), using *L. bulgaricus* observed a somewhat different reaction. They found that growth of the organisms was supported when the sugar was autoclaved separately under alkaline conditions and added to autoclaved medium. It was suggested that in the presence of such decomposition products of sugar as pyruvic acid, a favorable oxidation-reduction potential was developed in the medium. Pyruvic acid could, in fact, replace the autoclaved sugar. The present investigation describes studies carried out to determine what factors were involved in the requirement of *L. bifidus* for autoclaved sugar. The first experiments were directed toward identification of a possible growth stimulant, either a degradation product of sugar or some substance formed by reaction of sugar with amino acids, such as the compounds described by Maillard(6) and others. It was found that sugar autoclaved with cystine supported growth in medium autoclaved without sugar. Subsequent tests indicated, however, that this effect did not depend on the formation of a growth stimulant during autoclaving since the same result could be obtained by using sugar and cystine sterilized by seitz-filtration, or indeed by sterilizing the whole medium by seitz-filtration instead of by autoclaving. The medium contained a high level of cystine, 2 mg per tube, since it has been shown that there may be extensive destruction of sugar during autoclaving(7,8). It was established that failure of *L. bifidus* to grow in medium autoclaved in the absence of sugar was a result of the formation in medium so treated of some toxic decomposition product of cystine. Sugar autoclaved with the medium prevented the formation of such products by its reducing power. The toxicity could be overcome by adding to the autoclaved medium certain reducing substances, in particular those containing sulfhydril groups.

Experimental. Studies were made on *L. bifidus* and the unbranched mutant, Strain A, which have been described(9). Since the results with the two types of organism were identical, data will be presented only on the bifid strain. The medium was the modification of the Teply-Elvehjem medium used by Norris, *et al.*(9). Double strength medium (without sugar) was prepared in advance and sterilized in 2 liter flasks by autoclaving for 10 minutes at 15 lb pressure. At the time of the experiment, 5 ml of basal medium was transferred to each culture tube, any desired supplements were added, and the tubes were autoclaved for 10 minutes. Ascorbic acid (1 ml of a 1% solution) was always seitz-filtered and added aseptically to the autoclaved medium. Where noted, sugar and other supplements were either autoclaved separately from the medium or seitz-filtered and added aseptically. Solutions were prepared so that the amount of supplement for each test was contained in 1 to 3 ml. In all cases the

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TABLE I. Production and Prevention of Cystine Toxicity in Autoclaved Medium.

Treatment of sugar-free medium	Treatment of lactose	Supplements, mg		Acid production, ml .1 N in 40 hr
		Exp. 1—Regular medium		
Autoclaved	Autoclaved with medium			*21.4 ± .4
"	" separately, pH 10			.1
"	Seitz-filtered			.2
"	"	Pyruvic acid, 10		.5
"	"	Cystine, 6		22.4
"	"	Cysteine • HCl, 6		22.7
"	"	Thiomalic acid		17
"	"	Thioglycollic acid	Seitz-filtered	15.5
"	"	Ascorbic acid,‡ 15		0
"	"	" " ‡ 40		0
"	"	Reductone, 50		12.9
"	"	Ascorbic acid,‡ 40 + reductone, 50		15
Seitz-filtered	"			21.7
		Exp. 2—Medium with no added cystine		
Autoclaved	Autoclaved with medium			8 †
"	Seitz-filtered			14.2†
"	Autoclaved with medium	Cystine, 2	Autoclaved with med.	22
"	Seitz-filtered	" 2	" " "	7.7
"	Autoclaved with medium	Cysteine • HCl, 2	" " "	21.7
"	Seitz-filtered	" " 2	" " "	23.4
"	"	Cystine, 2	separately, pH 7	.4
"	"	" 2	" pH 1.5	.4
"	"	Cysteine • HCl, 2	" pH 1.5	21.6
"	"	" " 2	Seitz-filtered	23.8
		Exp. 3—Medium with no ascorbic acid		
Autoclaved	Seitz-filtered	Cystine, 6	Seitz-filtered	11.6
"	"	Cysteine • HCl, 6	"	20.5

* Data in Exp. 1 have been taken from four assays. Since the control titration was almost the same in the 4 tests, individual experiments have not been tabulated separately.

† The difference between these samples was even more marked at 16 hr when the titrations were 1.8 ml and 7 ml.

‡ In addition to the 10 mg of ascorbic acid routinely present in the medium.

final volume in each tube was 10 ml. The concentration of sugar used was 350 mg per tube. In the experiments reported lactose was used. Similar results were obtained when glucose or galactose was substituted for lactose.

If for *L. bifidus* as for *L. salivarius* and *L. bulgaricus* (3-5) the favorable effect of autoclaving sugar depended on the formation of some breakdown product, addition of autoclaved sugar to medium sterilized without sugar should stimulate growth. However, sugar autoclaved separately from the medium under alkaline conditions allowed only slight growth. Neither did addition of pyruvic acid (10 mg per tube) stimulate growth (Table I, Exp. 1). To test the possibility of the formation of a growth stimulant by a Maillard reaction, lactose was autoclaved with a mixture of alanine, cystine, and tryptophane. These 3 amino acids were always present in the medium as supplements to the casein digest used as the source of nitrogen; the amount added with the lactose doubled the usual concentration. When the autoclaved lactose-amino acid mixture was added to medium autoclaved without sugar, growth was as good as when the lactose was autoclaved with the medium. When each amino acid was tested individually by autoclaving it with lactose, it was found that only the combination of cystine and lactose supported growth.

These findings appeared to be in accord with the assumption that interaction of lactose with cystine during autoclaving might lead to formation of a specific growth stimulant. However, this hypothesis became untenable when it was found that the mixture of cystine and

lactose was as effective when it was sterilized by seitz-filtration as when it was autoclaved (Table I, Exp. 1). That the value of autoclaving cystine with sugar did not depend on the formation of a growth stimulant was verified by sterilizing the whole medium by filtration. Unautoclaved medium promoted growth as effectively as did autoclaved.

In another series of experiments (Table I, Exp. 2) the basal medium contained no supplement of cystine, so that the only source of this amino acid was the casein digest which furnished about 0.2 mg per tube. This low-cystine medium also was autoclaved with and without lactose. When the lactose was added before autoclaving growth of the organism was very poor. However, when the lactose was added aseptically to medium which had been autoclaved, acid production approached maximum rate and amount. These results were the opposite of those obtained with medium of high cystine content: in high-cystine medium autoclaving with sugar was essential, growth in medium to which the sugar was added after autoclaving being small or completely inhibited, whereas in low-cystine medium autoclaving in the absence of sugar allowed good growth while addition of sugar before autoclaving reduced acid production to about one-third the usual level.

It has been reported on the basis of both chemical(10) and microbiological(7,8) studies that the presence of reducing sugar increases the rate of destruction of cystine and cysteine during autoclaving. It is probable that in the low-cystine medium in the presence of sugar so much cystine was destroyed during autoclaving that full growth of the organism was impossible, while in the absence of sugar or in medium supplemented with cystine, the cystine level was not reduced below the amount required for good growth. That there was no growth in high-cystine medium autoclaved in the absence of sugar in contrast to the excellent growth in low-cystine medium might be explained by the formation of some inhibitory substance from cystine, which was present in effective amount only when the level of cystine in the medium was high. Evidence for the toxic origin of growth inhibition in the high-cystine medium autoclaved without sugar was furnished by experiments in which cystine in aqueous solution (2 mg in 3 ml water), autoclaved for one-half hour, was found to inhibit the growth of *L. bifidus* when it was added to autoclaved complete medium at the usual level of 2 mg per tube. Cysteine, on the other hand, was not toxic whether autoclaved with the medium or separately (Table I, Exp. 2).

It has been mentioned that cystine or cysteine added aseptically with lactose to high-cystine medium autoclaved without sugar were effective in overcoming the inhibitory effect of the autoclaved cystine. Cystine was as effective as cysteine only when the medium contained ascorbic acid. In medium from which the ascorbic acid was omitted the rate of acid production was much slower with cystine than with cysteine (Table I, Exp. 3). Thioglycollic and thiomalic acids could be substituted for cystine or cysteine, but were somewhat less effective. Ascorbic acid up to a level of 50 mg was ineffective in counteracting the effect of autoclaved cystine. In contrast, reductone† at the 50 mg level allowed considerable growth, although it was much less effective than the sulfhydril compounds.

The protective effect of autoclaving cystine in the presence of lactose was probably due to the reducing properties of lactose. A non-reducing sugar would not be expected to prevent the development of toxicity in autoclaved cystine. This would explain the fact that under the usual experimental conditions sucrose did not support the growth of *L. bifidus*(1). There was growth on sucrose when it was used in unautoclaved medium or in medium supplemented, after autoclaving, with unautoclaved cysteine, although even under these conditions neither the rate nor the total amount of growth was as great as with lactose.

Discussion. The toxic substance formed from cystine was presumably an oxidation product since autoclaving the medium in the presence of reducing sugar prevented its appearance and the toxicity was reversed by the addition of cysteine or related sulfhydril compounds or of reductone (ene-diol form of

† Kindly furnished by Professor R. Kuhn, Heidelberg.

hydroxymethylglyoxal). It is of interest that reductone was active whereas ascorbic acid was ineffective since both owe their reducing activity to an ene-diol structure.

Schuhardt *et al.*(11) and Rode *et al.*(12) found that cystine when autoclaved in neutral solution (but not when autoclaved at pH 1.5) was toxic for brucellae, and explained the toxicity of some lots of tryptose by the formation of oxidation products of cystine on standing. Under the conditions of our experiments cystine was toxic after autoclaving in acid as well as in neutral solution. Recently Schuhardt *et al.*(13,14) have identified the substance toxic to brucellae as elemental sulfur. Following this report the toxicity of colloidal sulfur‡ for *L. bifidus* was tested. Growth of the organism was inhibited by sulfur added to the autoclaved medium but the amount required for complete inhibition was large, about 10 γ per ml, whereas the growth of brucellae had been inhibited by 0.1 to 0.2 γ of sulfur per ml. The toxic effect of the sulfur could be reversed by autoclaving it with the complete sugar-containing medium or by addition of cysteine with the sulfur.

These experiments emphasize the importance and variety of interactions among the components of the medium, particularly under the severe conditions imposed by autoclaving. With a high level of cystine in the medium autoclaving with sugar was beneficial, with a low level of cystine, detrimental. Low concentrations of sugar were not tested but would surely modify the picture. An apparent complete failure of sucrose to support growth in the usual medium was a reflection of the fact that it is not a reducing sugar.

Summary. Autoclaved cystine is toxic to *L. bifidus*. Autoclaving in the presence of reducing sugar prevents development of the toxic product. The toxicity may be counteracted by addition, after autoclaving, of cysteine, thioglycollic or thiomalic acids, reductone, or cystine itself with ascorbic acid, but not by addition of ascorbic acid alone.

‡ The method by which the colloidal sulfur used in his experiments was prepared was kindly furnished to the authors by Dr. V. T. Schuhardt.

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Mono- and Disaccharides in Growth of *Lactobacillus bifidus* and Its Mutants. (19997)

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Wright(1) described a strain of *Streptococcus thermophilus* which grew more readily with lactose or sucrose than with any other sugar. Since that time, several workers have discussed organisms which utilize disaccharides more readily than their constituent