tein. The relationship of this complex to the collagen fiber is still to be elucidated.

Collagen breakdown occurs during the degradation of articular cartilage by papain although native collagen is not susceptible to digestion by papain at the temperature and pH employed in this experiment(16). Denatured collagen, however, is readily digested by papain. The fact that hydroxyproline appears in the filtrate only after chondromucoprotein breakdown is much advanced, suggests that denaturation of collagen occurs as chondromucoprotein is lost from the cartilage. It is also possible that collagen breakdown is catalyzed in some way by the products of cartilage breakdown.

Summary. The digestion *in vitro* of diced fragments of bovine cartilage by a 0.1% solution of papain was investigated over a 2-hour period. At half-hour intervals, measurements were made of the gravimetric loss of cartilage, and of the amount of cartilage nitrogen, hexosamine, and hydroxyproline present in the supernatant solution. From these determinations, the quantities of chondroitin sulfate, collagen and non-collagen protein released from the cartilage were calculated and their total compared with the weight loss of cartilage. During the first hour, the cartilage lost 88% of the chondroitin sulfate and 63% of the non-collagen protein. After 2 hours, more than 90% of each of these components were found in the supernatant solution. Collagen breakdown proceeded at the rate of about 9% per half-hour. Gravimetric loss measured 56% of the dry weight of the cartilage at the end of 2 hours.

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Iron Reversal of Serum Inhibited Respiration by *Bacillus subtilis.** (31906)

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Bactericidal effects of serum against *Bacillus subtilis* have been studied manometrically during growth of the microorganism in a nutrient broth(1). In this growth system it was found that serum antirespiratory activity was proportional to serum bactericidal activity, that the antirespiratory agent was a serum globulin, and that ferric iron in excess of the amount needed to saturate transferrin re-

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versed serum inhibited growth. Both human and rabbit serum inhibited oxygen consumption in this growth system, and human 7S-gamma globulin displayed the greatest specific antirespiratory activity observed.

The present report describes the ability of serum to inhibit oxygen consumption by washed cellular suspensions of *B. subtilis* in the presence of glucose. Such studies allow the antibacterial effects of serum in a respiratory system to be compared with those previously studied in the growth system. A variety of plasma fractions is employed in place of whole serum, to identify the inhibitor. Moreover, studies employing iron to reverse the inhibition of oxygen consumption are reported, and evidence pointing to the existence of serum inhibitors of respiration different from those of growth is presented.

Materials and methods. The same laboratory strain of *B. subtilis* employed previously was used for most experiments. *B. subtilis* ATCC #6633 was used for some studies. Similar results were obtained with cellular suspensions of either strain. Cells were routinely harvested after centrifugation from 18-hour cultures incubated at 37°C on a rotary platform shaker in 50 ml of Trypticase Soy Broth (BBL), washed twice with distilled water, and resuspended with Sorensen's phosphate buffer (0.1 *M*; pH 7). The turbidity of the cellular suspension to be added to Warburg flasks was adjusted so that a 10-fold dilution had an optical density of 0.20 ± 0.02 at 540 mμ employing a Coleman Jr. spectrophotometer. A standard curve for optical density related to dry weight of cells washed with distilled water indicated that the standardized suspensions weighed 2.0 mg per ml, and contained between 10^8 to 10^9 cells per ml.

The manometric method used to determine ability of serum to inhibit the growth of *B. subtilis* has been described(1,7). Standard manometric procedures were used for oxygen consumption studies in the presence of glucose. All experiments and assays, along with suitable controls, were done in triplicate, and numerical results were averaged. The center well of Warburg flasks contained 0.2 ml of 10% KOH and a coiled filter paper to trap CO_2. All studies were done at 37.5°C.

Blood was collected from adult male New Zealand rabbits by intracardiac puncture. The serum was pooled, divided into small working portions, and frozen. Pooled human serum (Microbiological Associates, Inc., Bethesda, Md.), human 7S-gamma globulin (Mann Research Laboratories, New York), and human and animal plasma fractions (Hyland Laboratories, Los Angeles, Calif.) were purchased. All fractions were made up in saline solution at a concentration of 40 mg/ml (W/V). Ferrous and ferric salts, used to reverse respiratory inhibition, were reagent grade with the exception of ferric ammonium citrate, which was prepared from ferric chloride(5).

For some experiments measured volumes of serum were dialyzed against glass distilled water in the cold. The sack of dialyzed serum was immersed in Aquacide (Calbiochem, Los Angeles, Calif.) overnight in the cold to concentrate the serum, and the contents were reconstituted to original volume with saline solution.

Results. Inhibition by whole serum. Oxygen consumption by cellular suspensions in the presence of glucose was linear with respect to time, and was inhibited by serum. During a typical experiment, the mean oxygen consumption in 3 hours in the presence of glucose and pooled rabbit serum was 30 μl (range 29 to 32 μl), and in the presence of glucose alone was 352 μl (range 328 to 374 μl). When rabbit serum was serially diluted, oxygen consumption increased proportionally and the respiratory rate increased 2- to 3-fold with each dilution (Fig. 1). This result was entirely different from that obtained in the previously studied growth system where the inhibitory effect of serum was completely removed when serum was diluted in half, and where there was a 2-hour lag period before oxygen consumption.

Both rabbit and human serum inhibited oxygen consumption in the presence of glucose. However, the inhibition by each was markedly enhanced after the serum had been dialyzed against distilled water (Table I). The controls indicated that in the absence of added glucose each serum contained substrates allowing considerable oxygen uptake,

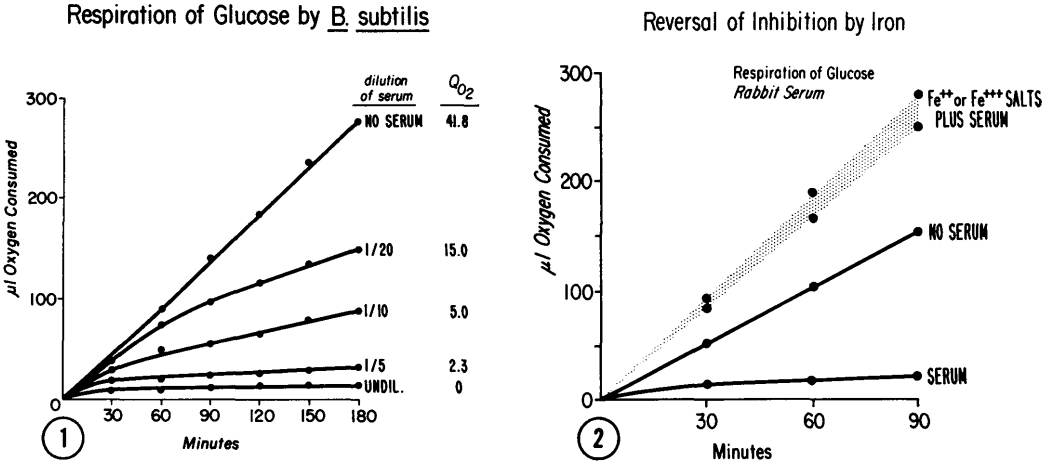

Fig. 1. Respiration by suspensions of *B. subtilis* in the presence of serially diluted rabbit serum. One half ml of 0.1 *M* glucose and 0.5 ml of water or serum in the side arms, and 1 ml of cellular suspension (2.2 mg dry wt) and 1 ml buffer in the main compartment of Warburg flasks Q_{O_2}, equals the rate of oxgen uptake in µl consumed per mg of dry weight per hour between the second and third hour.

Fig. 2. Inhibition of respiration by rabbit serum, and reversal of inhibition by iron salts. One half ml of rabbit serum, and 0.5 ml of iron salt (75 µg Fe) in side arms, and 1 ml of washed suspension of *B. subtilis* and 1 ml of 0.02 *M* glucose in the main compartment of Warburg flasks.

but did not support respiration after dialysis.

Inhibition by plasma protein fractions. A wide assortment of human and animal plasma protein fractions was assayed for ability to inhibit respiration by washed cells in the presence of glucose (Table II). Oxygen consumption was inhibited most strongly by fractions consisting of mixed beta and gamma globulins (Fractions II + III-W), of pure beta globulin (Fraction IV-7), and of an

TABLE I. Enhanced Inhibition of Oxygen Consumption by Washed Cells of *Bacillus subtilis* After Dialysis of Serum.

Substrate	Q_{O_2}* Rabbit serum	Q_{O_2}* Human serum
Glucose (no serum)	100	100
Glucose plus serum	45	77
" " dialyzed serum	15	21
Controls (no glucose):		
Serum	55	43
Dialyzed serum	5	0
Endogenous (cells alone; no serum or glucose)	8	—

* Microliters of oxygen consumed per mg dried cells between the second and third hour; relative to Q_{O_2} for glucose set at 100.

Each Warburg flask contained 0.5 ml of 0.1 *M* glucose and 0.5 ml of serum in the side arms, and 1.0 ml of cellular suspension along with 1.0 ml of phosphate buffer in the main compartment.

TABLE II. Survey of Inhibitory Effect of Protein Fractions on Oxygen Consumption by Washed Cells of *Bacillus subtilis* in Presence of Glucose.

Fraction	Q_{O_2}*
Plasma proteins:	
Human I (fibrinogen)	173
" II + III (β/γ globulin)	38
" II + III-W (β/γ globulin)	1
" II (γ globulin)	95
" III (β globulin)	63
" III-o (β lipoprotein)	102
" IV-1 (α globulin)	97
Horse IV-1 "	137
Sheep IV-1 "	129
Human IV-1/IV-4 (α/β globulin)	166
" IV-5, 6 (α globulin)	159
" IV-7 (β globulin)	6
" IV-8 (impure albumin)	148
" V (albumin)	119
" VI (albumin/glycoprotein)	0
Serum proteins:	
Human 7S-γ globulin	119
" albumin	127
Rabbit "	142
Bovine "	128

* Microliters of oxygen consumed per mg dried cells between the second and third hour; relative to Q_{O_2} for controls without protein fractions set at 100.

Each Warburg flask contained 0.5 ml of 0.02 *M* glucose and 0.5 ml of protein solution (40 mg/ml of saline) in the side arms, and 1.0 ml of cellular suspension and 1.0 ml of buffer in the main compartment.

albumin-glycoprotein mixture (Fraction VI). Immunoelectrophoretic analysis of these fractions indicated that beta $_{2A}$ globulin was the only protein common to each of these fractions. Since albumins, gamma globulin, and alpha globulin did not inhibit, a role for these individual components of the mixed fractions appeared unlikely.

The 7S-gamma globulin was a highly purified preparation. Immunoelectrophoretic analysis showed it to be free of contaminating protein. This preparation did not inhibit oxygen consumption by washed cells in the presence of glucose, but was previously found to be a potent inhibitor of oxygen consumption in the growth system(1).

Reversal of serum inhibition. The ability of iron to reverse serum inhibited oxygen consumption by cellular suspensions was investigated in the presence of each of the following salts (75 μg Fe added to each Warburg flask): ferric ammonium citrate, ferric ammonium sulfate, ferric nitrate, ferrous ammonium sulfate, ferrous sulfate, and ferrous chloride. All reversed serum inhibition and restored oxygen consumption. Indeed, cellular suspensions in the presence of rabbit serum and added iron consumed more oxygen than did controls in the absence of serum and iron (Fig. 2). Added iron reversed serum inhibition and stimulated oxygen consumption to the same extent regardless of the use of either ferric or ferrous salts, or the presence of an ammonium anion. Inasmuch as ferrous chloride was readily soluble in water and its anion offered no source of carbon or nitrogen, serial dilutions of ferrous chloride were used to titrate the reversal of serum inhibition. Addition of approximately 18 μg of iron was needed to reverse serum inhibition and enhance oxygen consumption to the level obtained in the absence of serum. When 75 μg of iron were added to blank reaction vessels containing serum, glucose, and buffer, but without added cells, no oxygen consumption occurred. Thus, oxygen uptake in the presence of iron at the high, unphysiological levels employed was not an artifact of the manometric procedure attributable to gaseous exchange as a result of oxidation-reduction reactions.

The inhibitory effect of dialyzed human serum on oxygen consumption by washed cells was not reversed by added iron, nor was that of any of the inhibitory protein fractions studied above.

We previously reported that ferric ammonium citrate reversed rabbit serum inhibited growth in Trypticase Soy Broth. This phenomenon was reinvestigated in the same growth system. Addition of 30 μg iron as ferric ammonium citrate reversed serum inhibited growth and restored oxygen consumption, but the same concentration of iron as ferric ammonium sulfate, ferric nitrate, or ferrous chloride was without effect on rabbit serum inhibited growth.

Discussion. The results of the present study with *B. subtilis* point to the existence of serum inhibitors of respiration different from those of growth. When measured manometrically, growth in Trypticase Soy Broth and respiration in glucose responded differently to serial dilution of rabbit serum; inhibition of growth was essentially removed once serum was diluted in half(1), but inhibition of oxygen consumption in the presence of glucose diminished as the serum was diluted. Beta $_{2A}$ globulin inhibited respiration. Highly purified 7S-gamma globulin was a potent inhibitor of growth, but did not inhibit respiration. Both ferrous and ferric salts reversed rabbit serum inhibited respiration to the same extent, but only ferric ammonium citrate reversed serum inhibited growth. Dialysis of both rabbit and human serum enhanced its inhibitory effect on glucose respiration.

The enhanced inhibition of oxygen consumption by washed cells after serum has been dialyzed emphasizes the necessity of iron for respiration of *B. subtilis*. Aerobes require iron for metabolic reactions utilizing cytochromes and iron-containing cofactors and enzymes. Transferrin (iron-binding β_1-globulin) is known to make serum nutritionally deficient for aerobes possessing high metabolic requirements for iron(6). Although transferrin was devoid of bactericidal activity measured manometrically(1), it might conceivably be one component of serum contributing to inhibited oxygen consumption by *B. subtilis* in the presence of glucose. Thus in the ab-

sence of added iron, serum would bind all available iron in the system, and no respiration in the presence of glucose would be possible. When iron was added, however, the iron-binding capacity of the serum would be saturated, and there would then be excess iron available to satisfy metabolic requirements. Since 1 ml of rabbit serum binds approximately 3 μg of iron(1), the 18 μg of added iron needed to cancel out the ability of 0.5 ml of serum to inhibit oxygen consumption was more than twelve times the quantity needed to saturate transferrin. Accordingly, transferrin would not appear to be of singular importance in serum inhibited oxygen consumption in the presence of glucose.

The reversal of rabbit serum inhibited oxygen consumption by iron is the only property shared by both the manometrically studied growth and respiratory systems. However, only ferric ammonium citrate reversed serum inhibited growth. No reversal of growth has been obtained with the corresponding salts of aluminum, magnesium, manganese, copper, cobalt, or zinc, and the iron has been attributed to act on the serum rather than on the bacteria(1). As with the respiratory system, the amount of iron needed to reverse serum inhibited growth was greatly in excess of that needed to saturate transferrin. We previously interpreted the unique reversal of serum inhibited growth by ferric ammonium citrate as possibly being due to the catalytic reduction of S-S bridges in serum proteins by Fe^{+++}(2; *cf* p 123). However, the ineffectiveness of ferric ammonium sulfate and ferric nitrate in reversing serum inhibited growth weakens this hypothesis. Alternatively, the bactericidal activity of serum against *B. subtilis* and *Staphylococcus albus* has been found to be destroyed by added sodium citrate(3,4). Myrvik(4) has suggested that the mechanism of citrate inactivation involved factors other than calcium binding, and has shown that serum bactericidins against Gram-positive bacteria are inactive in ionic strengths of 0.1 or more. Further studies of the influence of citrate on our serum antirespiratory activities are needed.

Yotis(8) has studied the mode of action of a purified globulin fraction from human serum which markedly inhibited the oxidation of glucose by *B. subtilis* and staphylococci, and has postulated that bacterial susceptibility to the serum factor depends upon the attachment of certain minimal amounts of the factor to cells. Absorption of the serum factor was nonspecifically inhibited by the concentration of various salts as well as by other experimental conditions employed. The globulin fraction exerted a lethal and partially lytic effect on staphylococci, and did not appear to function as a metabolic inhibitor (9). Future studies may show a similar mechanism to account for our results.

It now appears that beta $_{2A}$ globulin is one antibacterial agent in the respiratory system and that 7S-gamma globulin is the major agent in the growth system. However, the inability of added iron to reverse the antirespiratory activity of the several inhibitory protein fractions and of dialyzed serum indicates a role for at least one additional serum inhibitor of respiration which is as yet unknown.

Summary. Oxygen consumption by washed cells of *Bacillus subtilis* respiring in the presence of glucose and by cells growing in Trypticase Soy Broth was inhibited by rabbit and human serum. The inhibition of respiration was markedly enhanced after serum was dialyzed. Both ferrous and ferric acid salts reversed serum inhibited respiration. Only ferric ammonium citrate reversed serum inhibited growth. Although 7S-gamma globulin was previously shown to be a potent inhibitor of growth, it did not inhibit respiration. Beta $_{2A}$ globulin was the only protein common to the 3 inhibitory plasma protein fractions, and appeared to be one inhibitor of respiration. However, the inability of added iron to reverse the antirespiratory activity of these inhibitory protein fractions and of dialyzed serum indicates a role for at least one additional serum inhibitor of respiration which is as yet unknown.

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Comparison of Ratio and Covariance Analysis of TSH Assays. (31907)

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Sakiz and Guillemin(1) examined the calculation of the results of the McKenzie(2) assay for TSH activity. They recommended: a) transformation of both the initial and the response radioactivity counts to logarithms; b) analysis of the log response counts using the log initial count as a covariate. They demonstrated that the transformation removes the heterogeneity of variance inherent in the original (untransformed) counts. Levy *et al* (3) analyzed the logarithm of the ratio of the response count to the initial count, remarking that they used this procedure because in prior work they had found only minor gains in precision by using covariance analysis. It is the purpose of this paper to examine the consequences of using as the response metameter the logarithm of the ratio of the response count to the initial count as compared with the technique of covariance analysis.

The mathematical model underlying the covariance analysis may be written

$$y = a + b \log \text{dose} + c\,z + e$$

where y is the logarithm of the response count,
z is the logarithm of the initial count
a,b,c are (unknown) constants and
e is a normally distributed random variable with mean zero and variance s^2

The mathematical model for the "ratio analysis" is similar to the above, with the a priori assumption that $c = 1$. We may then write

$$y' = a' + b' \log \text{dose} + e'$$

where $y' = \log$ (response count/initial count) $= y - z$
a', b' are constants, and e' is a random variable.

From a strictly statistical point of view, the question at issue is the appropriateness of either of these models to the biological system; the only 'proper' analysis is the one based on the 'right' model. However, the experimenter's interest is in the usefulness of the results, so rather than investigate the appropriateness of the models, we ask, instead, what (if any) difference does it make which analysis is performed?

Since the error of the estimated relative potency is affected by the experimental design used (*e.g.*, number of levels and actual levels of dosage for standard and unknown, number of animals allocated to the different dosage levels of standard and unknown, etc.), as well as the form of model assumed, we avoid confusion due to effects of design by considering responses to known dosages of standard TSH. The data examined were 6 assays using mice (Veterans Research Hospital, Chicago) and 9 assays using chicks (VA Hospital, Indianapolis). These dose-response curves were calculated according to both models with results as tabulated.

Using the Wilcoxon signed rank test for the hypothesis that the parameter estimates from the two methods of calculation do not differ (for the 13 assays giving legitimate estimates of all parameters) we have the following p-values:

slopes (on log dose),	$p > .60$
standard deviation,	$p > .31$
lambda,	$p > .86$