what analogous to the findings of Witebsky (3), Ervin and Young(4) and Wiener(5) in man where incompatible transfusions or heterospecific pregnancies involving the A-B-O blood groups occasionally result in the formation of "blocking" or incomplete antibodies against blood groups A or B. The "blocking" antibody in turn is of importance in the pathogenesis of erythroblastosis fetalis and in transfusion reactions.

Whether selective fetal death in the pig is ever the result of transplacental isoimmunization and the formation of "blocking" antibodies, is unanswerable at the present time. The studies of Corner(6), however, may be pertinent in this regard, and his concept of embryonic morbidity in mammals as a sequel to internal defects of the germ cells may profitably be re-investigated in the present light of demonstrable blood group incompatibilities in many mammals, including the pig.

Hemolysis in the transfused pigs was apparently not great as judged from the serial icterus indices and volume of packed red cell determinations. However, the initial decrease in volume of packed cells which took place shortly after the titer decrease suggests a possible transient hemolytic episode because of antibody absorption by Pi_1 red cells. This point will require further investigation in view of the fact that Ashby studies were not performed, and urobilinogen excretion in urine and stools was not determined.

That hemodilution was the most likely basis for the sudden titer decrease is suggested by the fact that blood typing of the anti-Pi_1 pigs subsequent to the transfusion of large volumes of Pi_1 blood revealed considerably weaker agglutination than was present prior to the experiment. In addition, there occurred a progressive concomitant rise in the volume of packed red cells after the initial temporary fall.

Summary. Multiple transfusions of Pi_1 blood from one pig into two other pigs of anti-Pi_1 specificity resulted in the formation of high titers of "blocking" antibodies of anti-Pi_1 specificity. The implications in experimental hemolytic syndromes and in selective fetal death in the pig are discussed.

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An Improved Assay for "Strepogenin" Based on Essential Nature of Material for *Lactobacillus bulgaricus* 09. (18016)

LEMUEL D. WRIGHT, JOSEPH S. FRUTON, KATHERINE A. VALENTIK AND HELEN R. SKEGGS

From the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa., and the Department of Physiological Chemistry, Yale University, New Haven, Conn.

"Strepogenin" has been applied as a name to a factor or group of closely related factors essential for the growth of certain Group A streptococci(1,2) or stimulatory for the growth of a variety of lactic acid bacteria (2-4). Partial hydrolysates of certain purified proteins have been shown to have high "strepogenin" activity(2,4,5). Recent work has suggested that the amino acids serine,

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3. Pollack, M. A., and Lindner, M., *J. Biol. Chem.,* 1943, v147, 183.

4. Wright, L. D., and Skeggs, H. R., *J. Bact.,* 1944, v48, 117.

5. Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.,* 1945, v67, 1734.

glycine, and glutamic acid might be involved in the "strepogenin" structure(6,7). It appears possible that a variety of "strepogenins" exist, having one parent or basic structure to which other amino acids are combined.

Although "strepogenin" is an absolute growth factor essential for some of the Group A streptococci, growth obtained with these strains is not abundant. Many investigators have resorted to the use of an assay based on growth stimulation of *Lactobacillus casei* for the estimation of "strepogenin" activity. The response of *Lactobacillus casei* to "strepogenin" is not specific, however, since it has been shown that under defined conditions asparagine and glutamine as well as a variety of synthetic peptides have "strepogenin" activity(6-10).

Investigations on the nature of an unknown growth factor required by *Lactobacillus bulgaricus* 09 have shown that the growth-promoting activity of natural materials can be duplicated with synthetic orotic acid(11). The basal medium used in these studies contained, in addition to the usual components of a semi-purified medium, norit-treated tryptic digest of casein, yeast extract, and vitamin B_{12}. When it became possible to grow *Lactobacillus bulgaricus* 09 with orotic acid in this medium, studies were undertaken to determine the extent to which the norit-treated tryptic digest of casein, the yeast extract, and vitamin B_{12} contributed to the growth factor requirements of the organism. It was found that the yeast extract and the vitamin B_{12} were dispensable components of the medium provided adequate amounts of the norit-treated tryptic digest of casein were present. Studies on the nature of the growth factor contributed by the norit-treated tryptic digest of casein have shown that the material has the properties and distribution of "strepogenin." Under the conditions to be described essentially no growth, even on prolonged incubation, is obtained with *Lactobacillus bulgaricus* 09 in the absence of "strepogenin." In the presence of adequate amounts of "strepogenin", however, good turbidity and high acid production result.

"Strepogenin" has not yet been isolated and identified. One reason for lack of progress undoubtedly lies in the non-specific nature of the *Lactobacillus casei* assay usually employed. The use of *Lactobacillus bulgaricus* 09 appears to offer definite advantages over the *Lactobacillus casei* procedure and may be of aid to those concerned with the isolation or biochemistry of "strepogenin."

Procedure. The organism employed was *Lactobacillus bulgaricus* 09 of the Cornell collection.* The organism was maintained by daily transfer in sterile skim milk supplemented with 1% tryptose (Difco). The incubation temperature was 37°C. Inocula for seeding microbiological assays were prepared

TABLE I.
Composition of Double Strength Basal Medium.*

Acid-hydrolyzed norit-treated vitamin-free casein	1.0	g
Glucose	4.0	"
Sodium acetate (anhydrous)	1.2	"
L-Tryptophane	20	"
L-Cystine	20	"
Orotic acid	4	"
Adenine	1	"
Guanine	1	"
Xanthine	1	"
Uracil	1	"
Salts A	1	ml
Salts B	1	"
Thiamine chloride	200	γ
Pantothenic acid	200	"
Riboflavin	200	"
Nicotinic acid	200	"
Pyridoxine	400	"
Pyridoxal	50	"
Folic acid	50	"
PABA	100	"
Biotin	1	"
Vitamin B_{12}	0.04	"
Tween 80	0.2	ml
pH 5.6-5.8 and dilute to	100	"

* 5 ml double strength medium used per tube. Addition is made to 5 ml of water or suitable aliquot of material under test.

* We are indebted to Dr. I. C. Gunsalus for this culture.

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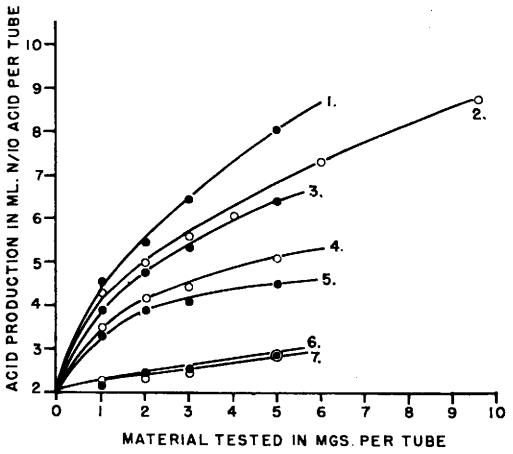

FIG. 1.

The response of *Lactobacillus bulgaricus* 09 to various proteins and protein digests. (1) Tryptic digest of vitamin-free casein. (2) Tryptic digest of vitamin-free casein treated twice with norit. (3) Undigested vitamin-free casein. (4) Tryptic digest of gelatin. (5) Tryptic digest of crude egg albumin. (6) Undigested gelatin. (7) Undigested crude egg albumin.

by dispersing 0.2 ml of a 24-hour culture from milk in 10 ml of sterile saline. One drop per assay tube was the routine inoculum used. Tests were incubated at 37°C for 48-72 hours. Relative growth was determined either by titration of the acid produced using 0.1 N NaOH with bromthymol blue as the indicator, or by measurement of turbidity in a photoelectric colorimeter.

The basal medium had the composition given in Table I. Although the organism does not appear to have a requirement for vitamin B_{12} this factor has been included in the basal medium in an attempt to increase the specificity of the response to "strepogenin" since Welch and Wilson have reported(12) that norit-treated tryptic digests of vitamin-free casein contain a factor capable of replacing vitamin B_{12} for *Lactobacillus leichmannii.*

In the assays that were carried out with *Lactobacillus casei* a medium essentially that of Landy and Dicken(13) with the asparagine level increased to 5 mg/tube was used.

12. Welch, A. D., and Wilson, M. F., *Arch. of Biochem.*, 1949, v22, 486.

13. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, v27, 1086.

The tryptic digests of the various proteins described in Fig. 1 were prepared as follows: One gram of each protein was suspended in 10 ml 0.8% $NaHCO_3$. Twenty mg of trypsin (Difco 1:250) were added to each protein suspension and the mixtures incubated under benzene at 37°C for 48 hours. The undigested proteins were treated similarly prior to assay except that the trypsin was omitted. The results summarized in Table II were obtained on digests prepared with crystalline trypsin. In this instance one tenth the quantities employed in the earlier series were used.

The synthetic peptides that were studied were sterilized by autoclaving separately in water at 120°C for 10 minutes and assayed by aseptic addition to previously autoclaved media prepared in the usual manner.

Results and discussion. The response of *Lactobacillus bulgaricus* 09 to a variety of proteins or enzymatic digests is shown in Fig. 1.

Lactobacillus bulgaricus 09 responds almost as well to undigested casein as it does to a tryptic digest. Norit treatment did not appreciably reduce the "strepogenin" activity of a tryptic digest of casein. Gelatin and crude egg albumin have little growth-promoting activity for *Lactobacillus bulgaricus* 09. Tryptic digestion increased the "strepogenin" activity of these products.

The "strepogenin" activities of a variety of protein digests determined with the proposed assay with *Lactobacillus bulgaricus* 09 and with the *Lactobacillus casei* procedure are summarized in Table II. Agreement between the two methods of assay is reasonably good. Thus it would appear that under the conditions employed the two organisms respond to the same factor, or factors. The assays with *Lactobacillus bulgaricus* 09 were much more satisfactory, however, with respect to reproducibility and the absence of "irregular" tubes and "drift" at varying assay levels.

Asparagine and glutamine (sterile filtration, aseptic addition to previously autoclaved assay) were tested at levels up to 100 γ/tube. Neither compound stimulated *Lactobacillus bulgaricus* 09. Woolley reported(7) that glutamine stimulates *Lactobacillus casei* in

TABLE II.
"Strepogenin" Activities of Various Tryptic Digests.

Material studied	*Lactobacillus bulgaricus* 09 assay*	*Lactobacillus casei* assay*
Casein (Labco vitamin-free)	1.0	1.0
Twice norited casein (Labco vitamin-free)	0.53	0.55
Ribonuclease (crystalline)	0.67	—
Trypsin (crystalline)	1.2	1.3
Gelatin (Difco)	0.36	0.31
Protamine (purified)	0	—
β-Lactoglobulin (crystalline)	0.78	0.70

* Results are calculated in terms of trypsinized vitamin-free casein assigned arbitrarily an activity of 1.0.

the "strepogenin" assay at levels of about 3-30 γ/tube.

The following synthetic peptides have been examined at levels up to 4000 γ/tube: Glycylglycine, glycylglycylglycine, L-alanylglycylglycine, L-alanyl-L-alanine, L-leucylglycine, glycyl-L-leucine, glycylglycyl-L-leucylglycine, L-leucylglycylglycine, glycyl-L-glutamic acid, L-isoglutamine, glycylglycyl-L-glutamylglycine, α-L-glutamyl-L-glutamic acid, L-serylglycine, L-seryl-L-alanine, L-seryl-L-serine, L-serylglycyl-L-glutamic acid, L-seryl-L-alanyl-L-glutamic acid, glycyl-L-phenylalanine, L-glutamyl-L-phenylalanine, glycyl-L-tyrosine, L-tyrosylglycine, L-glutamyl-L-tyrosine, glycyl-L-glutamyl-L-tyrosine, glycyl-L-tryptophane, L-leucyl-L-tyrosine, glycyl-L-proline, glycylglycyl-L-proline, glycyl-L-prolyglycine, L-prolyl-L-glutamic acid, L-prolyl-L-tyrosine, glycyl-L-hydroxyproline, L-hydroxyprolylglycine, DL-methionylglycine, L-methionylglycylglycine, L-methionyl-L-methionine, L-methionyl-L-tyrosine.

At the levels tested none of the peptides showed activity. Woolley has reported(6,7) that DL-serylglycylglutamic acid has some activity in the *Lactobacillus casei* test over a range of about 200-3000 γ/tube. Certain other synthetic peptides have a lower order of "strepogenin" activity. The activity of serylglycylglutamic acid has been confirmed by Krehl and Fruton(14) using L-serylglycyl-L-glutamic acid. Thus it would appear that the *Lactobacillus bulgaricus* 09 assay is more specific than the *Lactobacillus casei* assay since in the present study such peptides as well as asparagine and glutamine had no growth-promoting properties.

A thorough study of recently identified growth factors and vitamins in the proposed assay for what appears to be "strepogenin" has not been made. Through the courtesy of Dr. G. Fraenkel a concentrate of vitamin B_T (15) which, like "strepogenin", is not well adsorbed on norit, has been tested and found to be inactive in the present assay. Most, if not all, of the uncharacterized bacterial growth factors and vitamins that have been described in the recent literature are adsorbed on norit or have not been found to be associated with purified proteins and consequently would not be expected to promote growth in the present assay.

Summary. A microbiological method for the determination of "strepogenin" activity is presented that depends upon the *essential* nature of the material for a strain of *Lactobacillus bulgaricus*. The method is believed to possess advantages over existing methods that depend upon the *stimulation* of the growth of *Lactobacillus casei*.

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