QUANTITATIVE DETERMINATION OF THE BACTERIOSTATIC EFFECT OF THE SULFONAMIDE DRUGS ON PNEUMOCOCCI

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Determination of the susceptibility of various microorganisms to the bacteriostatic action of the sulfonamide drugs requires a test which is relatively simple to perform, clear and easy to read, and with which readily reproducible results can be obtained. From the clinical standpoint an *in vitro* test has certain advantages as compared with *in vivo* methods. Attention has been drawn by a number of investigators to certain of the factors affecting *in vitro* tests with this group of drugs. Among the variables may be mentioned the nature of the medium employed (Long and Bliss, 1939), the character of the fluid used in making dilutions for the inoculum, the age of the culture (Finkelstone-Sayliss, Paine and Palruk, 1937; Green, 1940; and Woods and Fildes, 1940) and the size of the inoculum (Colebrook, Buttle and O'Meara, 1936; Nitti, Bovet and Depierre, 1937; Domagk, 1937; Rosenthal, 1937; Stamp, 1939; and Fleming, 1940).

The present paper deals with several sources of error in the procedures commonly used and describes an *in vitro* method which, in the studies thus far made, has yielded consistent results in the determination of the relative susceptibility of certain bacteria to the action of the sulfonamide drugs. The results obtained by the use of this technique for testing the relative susceptibility to sulfapyridine of different strains of the disease-producing types of pneumococci will be presented in a subsequent paper.

DILUTING FLUID

The fluid used for diluting the culture of pneumococcus for the proper inoculum is important. It must not of itself be injurious to the bacterium and furthermore must contain no sulfonamide inhibitor. Diluting the culture in water, physiological saline or solutions of buffers either kills some of the cells or so injures them that a prolonged lag phase may occur. If plain peptone broth is used, sufficient inhibitor may be introduced along with the inoculum so as to alter the end point of the bacteriostatic test. The most satisfactory diluting fluid has been found to be an inhibitor-free liver infusion containing 2 per cent rabbit serum. This medium is described in a subsequent section of the present paper.

AGE OF CULTURES USED FOR INOCULATION

Several investigators have reported that the age of the culture used as an inoculum effects the outcome of the bacteriostatic tests. It has been stated that young cultures, during the logarithmic phase of growth, are more susceptible to the action of the sulfonamide drugs than are the cells from older cultures. The reported difference in the susceptibility of young and old cells to sulfon-

amide drugs parallels older observations on the lower resistance of young cells to various other unfavorable agents. For example, in 1910 Schultz and Ritz showed that cells of *Escherichia coli* during the lag phase and in the early logarithmic phase of growth are much more susceptible to heat than in the late logarithmic phase. This observation has been repeatedly confirmed (Winslow and Walker, 1939). Sherman and Albus (1923) reported the greater resistance of older cells of *E. coli* and *Proteus vulgaris* not only to heating but to chilling, hypertonic solutions of sodium chloride and phenol. The greater susceptibility of young cells of *E. coli* to distilled water has been described by Hershey (1939) and to ultraviolet light by Gates (1929–30). In addition, Bayne-Jones and Sandholzer (1933) have demonstrated that young cells are more readily attacked by bacteriophage than are the cells from older cultures.

Variations in the susceptibility of young and old bacterial cells to the action of the sulfonamide drugs may be related to the content of sulfonamide inhibitor in cultures of different ages, and may also be due to variations in the organisms themselves. The presence of sulfonamide inhibitors in bacterial cultures has been demonstrated in a number of laboratories (Stamp, 1939; Green, 1940; Woods and Fildes, 1940; Fleming, 1940; MacLeod, 1940; and MacLeod and Mirick, 1941). The presence of these substances unquestionably affects the outcome of the bacteriostatic tests.

In the present studies, 8- to 10-hour cultures grown in charcoal-adsorbed peptone broth containing defibrinated rabbit blood have been used. At this period of growth cultures of pneumococcus are at the peak of the logarithmic phase. Reproducible results have been obtained with cultures of this age.

SIZE OF THE INOCULUM

A concentration of a sulfonamide drug which is bacteriostatic for a small inoculum of a particular microorganism may have little or no effect if a larger inoculum is used for seeding (Colebrook, Buttle and O'Meara, 1936; Nitti, Bovet and Depierre, 1937; Domagk, 1937; Rosenthal, 1937; Stamp, 1939; Fleming, 1940). However, because of the variations in the amount of sulfonamide inhibitor present in different lots of peptone broth it has not previously been possible to determine accurately the effect of varying the size of the inoculum. When sulfapyridine was used in routine bacteriostatic tests with pneumococci of various types it was found that the results were usually the same whether an inoculum of 10^{-4} ml. or 10^{-5} ml. of culture was used, and the end points were never more widely separated than a two-fold dilution of the drug. These inocula fall within the range of 40,000 to 3,000 cells as determined by colony counts of poured plates. Safe limits are 5,000 to 20,000 cells. Since identical results were almost invariably obtained within these limits it was possible to simplify the method of preparing the dilutions.

A 2 mm. loopful of a fully grown 8- to 10-hour blood-broth culture of pneumococcus is placed in 5 ml. of liver infusion. The suspension is mixed thoroughly and 0.1 ml. is seeded into the 2 ml. volume of the medium used in the bacteriostatic tests. This inoculum contains between 5,000 and 10,000 organisms.

Bacteriostatic tests performed with cultures diluted in this fashion gave the same results as were obtained when decimal dilutions of culture were made in the ordinary manner.

If these relatively large inocula (5,000 to 20,000 cells) are used in bacteriostatic tests carried out in plain peptone broth, the differences in the susceptibility of various strains of pneumococci to the sulfonamide drugs, and the differences in the bacteriostatic potency of the different sulfonamide drugs on the same strain of bacterium are often obscured. The combined effect of the inhibitor in the medium and the large inoculum is such that growth of a relatively susceptible strain may take place even in the presence of the highest concentrations of the drug that can be dissolved in the medium. Bacteriostatic tests in peptone broth must therefore be carried out with a very small seeding of bacterial cells so that the bacteriostatic effect of a sulfonamide drug may be detected within the limits of its solubility. However, the use of a small inoculum is unsatisfactory for several reasons. In the first place, a very small inoculum of a freshly isolated strain of pneumococcus will often not grow in ordinary artificial medium until the strain has been repeatedly subcultured in this medium. During this process of adaptation some of the characteristics of the freshly isolated strain are obviously modified, and perhaps among others its susceptibility to the sulfonamide drugs. It seems desirable, when testing bacteria for their sulfonamide susceptibility, that the tests be carried out as soon as possible after isolation of the organism. If a larger inoculum is used freshly isolated strains may be tested without requiring a preliminary period of adaptation to artificial media.

Little is known at present concerning the phenomenon of drug resistance in different strains of bacteria. It is possible that the development of drug fastness is the result of a gradual change which takes place in the individual bacterial cells as a result of exposure to the drug. On the other hand drug-fastness may be a manifestation of natural selection, any culture being a variable mixture of drug-susceptible and drug-resistant cells. Until these matters are better understood it seems important that studies of the relative sulfonamide susceptibility of bacterial strains be made upon an adequate sample of the population.

THE MEDIUM

One of the chief sources of error is due to the presence of inhibitors of the sulfonamide compounds in the common bacteriological culture media. MacLeod (1940) has described a satisfactory technique for detecting the presence of sulfonamide inhibitor by the use of a smooth strain of *E. coli* grown in a synthetic medium. Lockwood (1938, 1940) has shown that peptone is inhibitory to the bacteriostatic action of sulfanilamide. More recently it has been demonstrated that different peptones and even different lots of the same brand of peptone contain varying amounts of sulfonamide inhibitor. For this reason it is often difficult to obtain reproducible results in bacteriostatic tests performed with different lots of the ordinary media containing peptone. Moreover, another important ingredient of culture media, namely meat infusion as customarily prepared, also contains sulfonamide inhibitor. It is obvious, therefore, that to

obtain inhibitor-free media the use of these components must be avoided, at least in the form ordinarily employed. For these reasons it seemed important to develop a medium capable of supporting the maximal growth of the more fastidious microorganisms which, at the same time, would be as free as possible of substances known to inhibit the bacteriostatic effect of sulfonamide drugs.

For this purpose a method was devised for preparing extracts of animal organs under conditions yielding the essential nutritive and growth-accessory factors without releasing the drug-inhibitory substances which are liberated or formed during the processes of tissue autolysis. It was found that an infusion of fresh calf or beef liver may be so prepared as to fulfill these requirements and to provide a medium which supports the rapid and profuse growth of pneumococci without the addition of peptone. The preparation of the liver infusion has been described elsewhere in detail (MacLeod, 1940).

Fresh beef or calf liver is obtained at the slaughter house as soon as possible after the death of the animal. The liver is immediately plunged into boiling water and kept at this temperature for 5 to 10 minutes in order to destroy the autolytic ferments. After mincing in a meat chopper the tissue is suspended in twice its weight of tap water, including that used in boiling the organ originally. The pH is adjusted to 4.5 with N/1 HCl, and the infusion heated slowly to 80°C. over a boiling water bath. The liver pulp is then removed by filtration through paper, the pH adjusted to 7.8 with N/1 NaOH, filtered again through fine-grained paper and the fluid then sterilized by filtration through Pasteur-Chamberland candles, bougie B. Filtration is facilitated by warming the infusion to 35°–40°C.

When prepared from calf liver the infusion is clear, golden-yellow in color and strongly fluorescent. Similar infusions made from beef liver have tended to be opalescent, due apparently to the presence of lipids. A seeding of pneumococci or Group A hemolytic streptococci containing only 5 to 10 viable cells grows readily when inoculated into 2 to 5 ml. of infusion. If stored in the dark and in the cold with only a small surface exposed to air, the infusion retains its growth-promoting qualities for a considerable period of time. However, if exposed to light at room temperature, the infusion loses much of its fluorescence within 48 hours with a concomitant loss of its ability to support the growth of small inocula of pneumococci. The growth-promoting qualities of the infusion may be restored completely by the addition of a reducing agent such as sodium thioglycollate (0.1 mgm. per ml.), although the fluorescence does not return. The addition of the sodium thioglycollate at the time the infusion is prepared tends to stabilize the medium and to prevent the formation of deleterious products of oxidation.

Occasional strains of penumococcus have been encountered which do not grow readily in the infusion unless serum is added. To avoid this difficulty, fresh normal rabbit serum in final concentration of 2 per cent has been added routinely. This serum is free of sulfonamide inhibitor, and in addition serves to stabilize the infusion.

In the bacteriostatic tests a volume of 2.0 ml. of medium has been used

throughout. The various concentrations of sulfapyridine have been obtained by making appropriate dilutions in the liver infusion of a 1:2,000 stock saline solution sterilized by heating in a boiling water bath for 5 to 10 minutes.

The content of reducing sugars in different lots of liver infusion has varied between 0.4 and 0.5 per cent. Because of the high carbohydrate content the pH of the medium reaches the acid death point for pneumococcus shortly after maximum growth has taken place. This fact does not interfere with reading the end point in the bacteriostatic tests, and the inhibitor-free liver infusion has been found to be entirely satisfactory for performing these tests. The high sugar content and subsequent acid production in the liver infusion do, however, make this medium unsuitable for storing cultures of pneumococci and for growing the culture to be seeded in the bacteriostatic tests.

REMOVAL OF SULFONAMIDE INHIBITOR FROM PEPTONE BROTH

As previously mentioned, the common bacteriological media prepared from muscle infusion and containing peptone, are unsuitable for bacteriostatic tests because of their content of inhibitor. Media of this sort, however, are desirable for the storage of cultures and for growing cultures to be used for the inoculation of the liver infusion, since the low sugar content does not permit the production during growth of sufficient acid to cause rapid death of the organisms. However, if peptone broth culture is used for inoculation, sufficient sulfonamide inhibitor may be carried over into the liver infusion to affect the outcome of the bacteriostatic tests, even though the serial dilutions are made in the inhibitor-free liver infusion. Accordingly, methods were sought for removing the inhibitor from peptone broth by the use of a selective adsorbent.

The reaction of plain broth prepared with Pfanstiehl's peptone is adjusted to between pH 5.0 and 5.5 with HCl, and for each 100 ml. of medium, 2 grams of powdered charcoal are added. The mixture is then brought to the boiling point over an open flame, and immediately filtered through paper. The reaction is readjusted with NaOH so that the final pH will be 7.8 after sterilization in the Arnold.

The charcoal-adsorbed broth is considerably lighter in color than the original unabsorbed medium, and most of the sulfonamide inhibitor is removed. Moreover, the ability of the adsorbed medium to promote the growth of small inocula of pneumococci is at the same time greatly enhanced. An inoculum of 5 to 50 cells in 5.0 ml. of medium usually grows readily, whereas before adsorption an inoculum of from 1 to 10 million cells is frequently necessary to initiate growth.

In a study of the oxidation-reduction potentials of bacteriological media Dubos (1929 a and b) has shown that peptone broth contains certain substances which become oxidised upon exposure to air, and so alter the oxidation-reduction potential of the medium that the growth of bacteria occurs only when a large inoculum is used. When a medium which has become oxidized by exposure to air is subsequently reduced either by boiling or by the addition of cysteine or thioglycollic acid, the growth-promoting properties are greatly improved. However, when again exposed to air, the autoxidizable substances become

re-oxidized quickly and interfere with the growth of small inocula. In a further investigation of this problem Dubos (1930) showed that various brands of commercial peptone contain different amounts of oxidizable substances which exert a bacteriostatic effect on certain microorganisms. The addition of reducing agents annuls this effect. The bacteriostatic substances may be removed from commercial peptone by acid precipitation, and media prepared with the purified material are capable of supporting the growth of very small inocula of pneumococci.

The improvement in the growth-promoting properties of peptone broth after adsorption with charcoal is much greater than that which occurs when broth is merely heated for the same period in the absence of charcoal. Moreover, even if adsorption is carried out in the cold, the growth-promoting qualities of the medium are greatly improved although not so much as when adsorption is carried out in hot solution.

In order to compare the effect of heat and charcoal adsorption upon the growth-promoting properties of a lot of plain broth prepared with Pfanstiehl's peptone, the reaction was adjusted to pH 5.2 with N/1 HCl, and various portions of the broth treated as described below.

Portion 1. 2 grams of charcoal were added for each 100 ml. of broth. The mixture was brought to a boil over an open flame and immediately filtered through paper. After cooling, the reaction was adjusted to pH 7.8 and the adsorbed broth sterilized by heating in a boiling water bath for 20 minutes.

Portion 2. Instead of carrying out adsorption in hot solution, this procedure was done at room temperature, contact with charcoal being maintained for the same length of time as in the case of Portion 1. After filtration through paper the reaction was adjusted to pH 7.8 and the broth sterilized by filtration through a Berkefeld "V" candle.

Portion 3. In this instance no charcoal was added, but the broth was otherwise subjected to the same procedures as in the case of Portion 1.

The ability of each treated portion and of the original untreated broth to support the growth of varying inocula of Pneumococcus Type I was tested. The results of these tests are shown in table 1.

From table 1 it can be seen that adsorption of plain peptone broth with charcoal in hot solution improves greatly its capacity to promote the growth of small inocula of pneumococci. In the original untreated broth a 10^{-2} ml. inoculum was necessary to inititate growth whereas after adsorption the medium supported the growth of an inoculum of 10^{-8} ml. The difference between these portions of the same lot of broth cannot be explained on the basis of the heating to which the adsorbed broth was subjected, since heat alone in the absence of charcoal improved the growth-promoting properties of the broth only moderately. Moreover, when the broth was adsorbed with charcoal at room temperature and sterilized by filtration, its nutritive properties were greatly improved, indicating that the bacteriostatic substance present in broth is adsorbed by charcoal at pH 5.2 and that the improvement in the broth is not due solely to the heating used in the adsorption procedure. The superiority of broth adsorbed in hot solution to that

adsorbed at room temperature is probably due to more efficient adsorption at the higher temperature. It is worthy of note that charcoal-adsorbed broth even when exposed to air retains its growth-promoting properties for a prolonged period and becomes oxidized very slowly, if at all.

It should be emphasized that adsorption must be carried out between pH 5.0 and 6.0 in order to remove at the same time both the growth-retarding and sulfonamide-inhibiting substances. Below pH 5.0 adsorption removes from the medium certain of its growth-promoting properties, and above pH 6.0 removal of the sulfonamide inhibitor is much less complete than at more acid reaction.

Although the sulfonamide inhibitor and the growth-inhibitory substances are removed from broth by the same procedure, it is not inferred that the two sub-

TABLE 1

Effect of the growth-promoting properties of charcoal-adsorbed broth and heated broth

MEDIUM	GROWTH OF PNEUMOCOCCUS TYPE I*							
	Inoculum in ml.†							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Portion 1 adsorbed with charcoal in hot solution and sterilized by boiling	+	+	+	+	+	+	+	+
Portion 2 adsorbed with charcoal at room temperature and sterilized by filtration	+	+	+	+	+	+	−	−
Portion 3 heated at pH 5.2, readjusted to pH 7.8 and sterilized by boiling. Unadsorbed	+	+	+	+	−	−	−	−
Original untreated broth	+	+	−	−	−	−	−	−

* Readings of growth were made after incubation at 37°C. for 24 hours.

† Dilutions of culture for inoculation were made in plain broth adsorbed with charcoal in hot solution. Inoculum of 10^{-7} ml. contained 80 pneumococci as determined by plate count.

stances are of the same nature. The evidence at present indicates that these two principles are distinct. For example, most of the growth-retarding effect of oxidized broth can be annulled by the addition of a reducing agent such as sodium thioglycollate, whereas the sulfonamide inhibitor is not affected.

BACTERIOSTATIC TESTS IN DIFFERENT CULTURE MEDIA

Comparative bacteriostatic tests with Pneumococcus Type I were carried out in peptone broth, in peptone broth adsorbed with charcoal in hot solution, and in the liver infusion. The differences in the degree of bacteriostasis caused by sulfapyridine added to the various media are shown in table 2. Inasmuch as normal rabbit serum in final concentration of 2 per cent has been added routinely to the liver infusion, in the present experiments the same amount of serum was added to charcoal-adsorbed and unadsorbed peptone broth. This amount of

serum improves greatly the growth-promoting properties of the peptone broth, and permits the use of small inocula. The normal rabbit serum was shown to be free of sulfonamide inhibitor by *E. coli* test in a synthetic medium, as previously reported (MacLeod, 1940).

The degree of growth was estimated by comparing the gross turbidity of the tubes after incubation at 37°C. for 24 hours. This was found to be a satisfactory growth period, since even with more prolonged incubation the results are almost invariably the same as at 24 hours.

As shown in table 2 the bacteriostatic effect of sulfapyridine varies greatly in different culture media. In liver infusion bacteriostasis was complete in dilutions of sulfapyridine as high as 1:200,000 whereas the same degree of bacteriostasis in unadsorbed peptone broth requires a concentration of 1:5,000. However, after charcoal-adsorption bacteriostasis requires concentrations of the drug considerably less than in the untreated broth indicating that adsorption has removed most

TABLE 2

Bacteriostatic effect of sulfapyridine on pneumococcus type I in different culture media

MEDIUM*	GROWTH OF PNEUMOCOCCUS TYPE I†							
	Concentration of sulfapyridine							
	M/1,250 1:5,000	M/2,500 1:10,000	M/5,000 1:20,000	M/12,500 1:50,000	M/50,000 1:200,000	M/125,000 1:500,000	M/250,000 1:1,000,000	0
Peptone broth.....	−	++	+++	++++	++++	++++	++++	++++
Charcoal-adsorbed peptone broth....	−	−	−	+	++++	++++	++++	++++
Liver infusion.....	−	−	−	−	−	−	++	++++

* 2 per cent of normal rabbit serum added to each of the various media.

† ++++ = maximum growth; + = slight growth; − = no growth.

Inoculum: 4,000 cells.

The final readings were made after incubation at 37°C. for 24 hours.

of the sulfonamide inhibitor. The concentration of sulfapyridine required for bacteriostasis in the charcoal-adsorbed broth is only moderately greater than that necessary in the liver infusion.

It is important, however, that the medium used for bacteriostatic tests be completely free from sulfonamide inhibitor, since only under these conditions can the results obtained with different drugs or different bacterial strains be compared. As previously shown (MacLeod, 1940) the content of sulfonamide inhibitor varies in different brands of commercial peptones and in infusions of different organs. Complete freedom from inhibitor is most important in the tubes containing higher dilutions of the sulfonamide drugs, since a very small amount of inhibitor will entirely annul the effect of small concentrations of the drug, although in the presence of high concentrations of drug the effect of a small amount of inhibitor may not be so apparent. No lot of liver infusion has been used unless shown by previous testing with the *E. coli* technique to be free of sulfonamide inhibitor.

COMPARISON OF BACTERIOSTATIC TESTS WITH VARIOUS SULFONAMIDE DERIVATIVES IN THE PRESENCE AND ABSENCE OF DRUG INHIBITOR

The differences in the bacteriostatic potency of various sulfonamide drugs may be almost completely masked by the presence of sulfonamide inhibitor in the medium in which bacteriostatic tests are made. This effect is shown in table 3.

It can readily be seen that the striking differences in the bacteriostatic effect of equimolar concentrations of sulfanilamide, sulfadiazine, sulfapyridine, and sulfathiazol on a strain of Type I Pneumococcus, are almost completely masked when the test is performed in ordinary culture medium. These actual differences in the bacteriostatic potency of the four drugs are sharply defined however when inhibitor-free liver infusion is used.

TABLE 3

Effect of the presence of drug inhibitor in media upon the bacteriostatic action of various sulfonamide derivatives

MEDIUM	SULFONAMIDE DRUG	GROWTH OF PNEUMOCOCCUS TYPE I*							
		Concentration of drug							
		M/1000	M/2000	M/4000	M/8000	M/16,000	M/32,000	M/64,000	0
Peptone broth + 2 per cent rabbit serum	Sulfanilamide	++++	++++	++++	++++	++++	++++	++++	++++
	Sulfadiazine	+	++++	++++	++++	++++	++++	++++	++++
	Sulfapyridine	−	+++	++++	++++	++++	++++	++++	++++
	Sulfathiazole	−	++	++++	++++	++++	++++	++++	++++
Liver infusion + 2 per cent rabbit serum	Sulfanilamide	−	+++	++++	++++	++++	++++	++++	++++
	Sulfadiazine	−	−	−	++	++++	++++	++++	++++
	Sulfapyridine	−	−	−	−	−	++++	++++	++++
	Sulfathiazole	−	−	−	−	−	−	++++	++++

* ++++ = maximum growth; + = slight growth.
Inoculum: 7,600 cells.
Readings of growth were made after incubation at 37°C. for 24 hours.

SUMMARY

A technique has been described for estimating the bacteriostatic effect of sulfonamide drugs upon pneumococci. By this method certain of the variables which affect the outcome of these tests may be avoided. It has been shown that the most important source of error in bacteriostatic tests is due to the presence of sulfonamide inhibitor in the usual bacteriological media. This is particularly true in that different lots of the same peptone contain different amounts of sulfonamide inhibitor.

The question of the fluid used in diluting the culture to obtain a suitable inoculum is discussed briefly. In the present study liver infusion has been used since it is not only free from sulfonamide inhibitor and growth-inhibiting substances, but also causes no damage to the bacterial cells.

In carrying out the test a relatively large inoculum of 5,000 to 20,000 cells has been used. By this means a more representative sample of the bacterial popula-

tion is taken, and furthermore if a large inoculum is used strains of pneumococcus may be tested immediately after isolation from the patient, and without the necessity of prolonged adaptation to artificial media. The use of cultures of a standard age in performing bacteriostatic tests with sulfonamides is discussed. This is important since it has been shown by various investigators that older cells are in general less susceptible than young cells to the action of many deleterious physical and chemical agents.

The preparation and use of an inhibitor-free liver infusion are described. This infusion will support the luxuriant growth of various fastidious microorganisms without the addition of peptone, and has been found satisfactory as a culture medium for the performance of the bacteriostatic tests.

A technique is described whereby most of the sulfonamide inhibitor may be removed from plain peptone broth by boiling it with charcoal at an acid reaction. The growth-promoting properties of the broth are also greatly improved by this process due to the simultaneous removal of substances which inhibit growth. Charcoal-adsorbed peptone broth has been found satisfactory for growing the cultures to be used as inocula in the bacteriostatic tests.

By employing these various modifications of technique, readily reproducible results may be obtained in determining the susceptibility of freshly-isolated strains of pneumococci to the action of sulfonamides. Clear cut differences in the potency of various sulfonamide derivatives may also be demonstrated.

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