THE COMBINED ACTIONS OF CHLORAMPHENICOL AND OF BACTERICIDAL ANTIBODY PLUS COMPLEMENT ON SALMONELLA TYPHOSA*

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(Received for publication, November 4, 1953)

Although specific antibody and complement have long been known to produce appreciable bactericidal effects on Gram-negative organisms, *in vitro*, the role of this phenomenon in immunity to infections with these organisms is still not clear (1). Similarly, certain antibiotics, including chloramphenicol, produce significant inhibition of the growth of Gram-negative bacteria in the test tube, and, in favorable instances, also exert a chemotherapeutic effect on infections such as typhoid fever. The quantitative correlations between these two actions of the drug are, however, not entirely defined nor is it known in satisfactory detail whether, in addition to the drug, immunity factors such as antibody, complement, and phagocytes play a significant part in recovery.

In a recent investigation (2) it has been possible to determine with some precision the quantitative relations among antibody, complement, and bacteria which affect the bactericidal reaction. In a separate series of investigations in our laboratory, attention has been directed to the development of more precise methods of determining the susceptibility of bacteria to antibiotics, and to the interpretation of the data in terms of modes of action, at least on a populational level (3).

The convergence of these two lines of investigations has now permitted the establishment of a model *in vitro* system for the quantitative study of some of these therapeutic factors, and their interactions, with a greater precision than has been available with the methods used hitherto. Although important new information can be obtained, the approach is still inadequate so long as factors such as the participation of phagocytic cells are neglected. It is hoped, however, that the latter, in particular, may be included in a proposed extension of our procedures.

* Supported by a grant from the National Science Foundation. The preliminary method was developed under a grant from the National Institutes of Health, United States Public Health Service.

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EXPERIMENTAL

The general plan in all experiments was to study the inhibitory actions of the antibiotic and of the specific antibody plus complement, separately, and then in various combinations. The organisms used were the Ty2 and 0901 strains of *Salmonella typhosa*. Detailed descriptions of the materials and methods will be found in our other studies cited below.

The basic features of each experiment were:

1. A reaction period, during which the organisms were exposed to the inhibitory agent or agents.

2. The relative numbers of organisms surviving this period were then determined by means of a turbidimetric growth assay of a subculture. The latter depended on the fact that the larger the number of viable organisms remaining, the more rapidly would the subculture grow in a given assay period.

Bactericidal Action of Antibody and Complement.—The technique employed was an extension of an earlier procedure (4). The details of the revised test, the variables affecting it, and its application to some problems in the serology of *S. typhosa* have been described recently (2). In brief, however, the test involves a series of test tubes. The first contains only broth, the others contain, in addition, a constant (usually an excess) amount of complement, and a variable amount of the appropriate rabbit antiserum. Sufficient magnesium ion is added to all tubes to provide the optimum for bactericidal activity (2); all tubes are brought to constant volume with a diluent. An accurately measured amount of culture is next added to all tubes and the set is incubated in a water bath at 37°C. for the desired reaction period (in these experiments, 60 minutes). The assay of the surviving organisms may then be made by subculturing an aliquot, or more conveniently, 3 volumes of broth may be added directly to each tube. This effectively terminates the reaction, both by dilution of the antibody and complement, and by an anticomplementary action on the latter. It also serves to eliminate, for practical purposes, any contribution of residual, non-viable organisms to the final turbidity since considerable multiplication must ensue during this second incubation stage before the cultures reach readable densities. Further details on the controls employed are noted below.

Recording of Readings.—After the set of tubes has reached a suitable reading range (with care being taken that the principal control is still in the log-growth phase) it is chilled in an ice water bath to check further growth, and the optical densities of the tubes are read in a Coleman spectrophotometer (wave length–650 mμ; standard–uninoculated broth control) (2).

The simplest treatment of the data is based on the assumption that the relative optical densities represent the relative numbers of organisms present and (assuming a constant lag) that these in turn are directly proportional to the number of viable organisms present at the end of the reaction period. The *apparent percentages of growth* can then be obtained by dividing the turbidimetric reading (in optical density units) for each experimental tube by the optical density of the inoculated broth control, and multiplying by 100. Tubes controlling the separate effects of antibody or of complement should not differ significantly from the inoculated broth control standard.

Although the apparent percentage growths thus obtained are reproducible experimental quantities, and can be used directly for many purposes, particularly for comparative titrations, they do not represent immediately the relative numbers of organisms surviving the bactericidal action of antibody and complement, or the inhibitory action of an antibiotic.

This may be readily demonstrated by inoculating a series of plain broth tubes with graded numbers of organisms and incubating under the test conditions. The optical densities obtained do not fall on a straight line of 45° slope, but rather form a curve, with disportionately low readings for the larger initial inocula. This is probably due to the accumulation of acid or other metabolic products during growth, so that the leading tubes are soon slowed below the initial rate (2). Nevertheless, by means of calibration curves these apparent percentages of growth—the simple ratios of experimental and control densities (× 100)—can be corrected to yield the true relative numbers of organisms. For convenience the latter are designated *percentages of growth (corrected)*. All values reported in this paper have been so corrected.

Linear Representation of Data.—In experiments involving the inhibition of bacterial growth by antibody plus complement (2), or by an antibiotic (3), or by the combined actions of these, a plot of the percentages of growth against the logarithms of the amounts of inhibiting agent results generally in a sigmoidal curve. This is, of course, but a special instance of a widespread biological dosage-response relationship. As is well known, the analytical treatment of sigmoidal curves is cumbersome and a great simplification can be effected by the probit transformation, which yields a linear plot (5). In practice this can be carried out with ease by plotting the percentage growth on special but readily available graph papers,[1] ruled with normal curve deviates for the ordinate percentages, and logarithmic spacings for the drug or serum dosages. In addition to the advantages of a linear plot, such graphs also yield a new parameter—the slope. Two dosage response lines must be parallel if a unique numerical value is to be assigned to an increase in drug resistance; in all other instances the comparison is dependent on the percentage inhibitions selected.

Inhibition by an Antibiotic: Chloramphenicol.—The procedure here was similar to the above-with the substitution of graded amounts of the antibiotic in place of the antibody and com, plement. It thus resembled our turbidimetric antibiotic assay (3), except that to provide comparable conditions the foregoing schedule of incubation, followed by dilution and secondary assay incubation, was followed. It should be noted, in contrast to the bactericidal procedure, that while the activity of the antibiotic is reduced by the secondary dilution it does not cease, and the final turbidities reached are affected by its presence during the two stages of incubation. The concentrations recorded are those of the reaction stage.

The susceptibility (or alternatively considered, the resistance) of a culture can be estimated conveniently and accurately from the amount of antibiotic required to reduce the growth to 50 per cent of that of the control (3). This can be read off by inspection, from the (probit or normal deviate) percentage growth *versus* log drug concentration plot mentioned. The absolute reproducibility of the endpoints is indicated by a series of titrations made with the Ty2 cultures, on 6 separate days, over a period of 2 months. The 50 per cent endpoints were 1.2, 1.4, 1.4, 1.1, 1.2, and 1.2 μg., from which may be computed an average of 1.25, and an average deviation of ±0.10, or 8 per cent. These determinations were all made on broth-grown inocula; there are indications that suspensions from agar slants may differ from these in resistance but our series was too short to support this adequately. Until further information is available, the inocula for series to be compared should all be prepared as uniformly as possible.

Combined Actions: Antibody, Complement, and Antibiotic

These were all present together from the beginning of the reaction period, before addition of the test organisms. In all other respects the conditions were as for the assays of the separate actions. In some experiments the amounts of

[1] Such as logarithmic normal paper, No. 32.476, Codex Book Co., Norwood, Massachusetts.

antibody and complement were kept constant, while the amounts of the antibiotic were varied in the set. In other experiments these conditions were reversed. In all cases control titrations of the separate activities were made along with each set.

Antibiotic as Variable.

In these experiments, performed with the Ty2 strain, the quantities of guinea pig complement and anti-typhoid rabbit serum present in most of the tubes were constant. The two levels chosen were sufficient to reduce the growth by about 33 per cent, or 46 per cent; *i.e.*, growth was 67 per cent, or 54 per cent, respectively, of that of the broth control. Variable amounts of chloramphenicol were also added in the experimental tubes, and in a control series which contained complement but lacked antibody.

From the data given in Table I and Fig. 1 it can be seen that the observed effect of antibody and complement plus the antibiotic is, within the experi-

TABLE I

Observed and Calculated Inhibitions by Chloramphenicol in Presence and Absence of Bactericidal Antibody and Complement

(For data of Fig. 1)

Test organism: *S. typhosa*, strain Ty2.

Chloramphenicol present	No antiserum added	Amount of 1:3000 dilution of antiserum added			
		0.30 ml.		0.50 ml.	
	$P = P_2$	*P* observed	*P* calculated	*P* observed	*P* calculated
μg./ml.					
0	0*	0.33*	—	0.46*	—
0.17	0.28	0.52	0.52	0.64	0.61
0.25	0.42	0.56	0.61	0.69	0.69
0.38	0.56	0.64	0.71	0.79	0.76
0.57	0.68	0.75	0.79	0.84	0.83
0.84	0.80	0.85	0.87	0.88	0.89
1.25	0.88	0.89	0.92	0.91	0.94
1.88	0.91	0.92	0.94	0.95	0.95
Average deviation between observed and calculated points, *per cent*		4.1		1.9	

P is the observed growth in the presence of antibiotic, expressed as the fraction of the control growth in broth.

The expected inhibition is calculated from equation (1) of text:

$$P = P_1 + P_2(1 - P_1)$$

in which P_1 is the inhibition due to the constant level of antiserum present in the set and P_2 is the inhibition due to varying level of antibiotic present in the set.

* This is also the value of P_1 for this column (fraction of inhibition due to constant level of antiserum present). Note that for column 2, $P_1 = 0$.

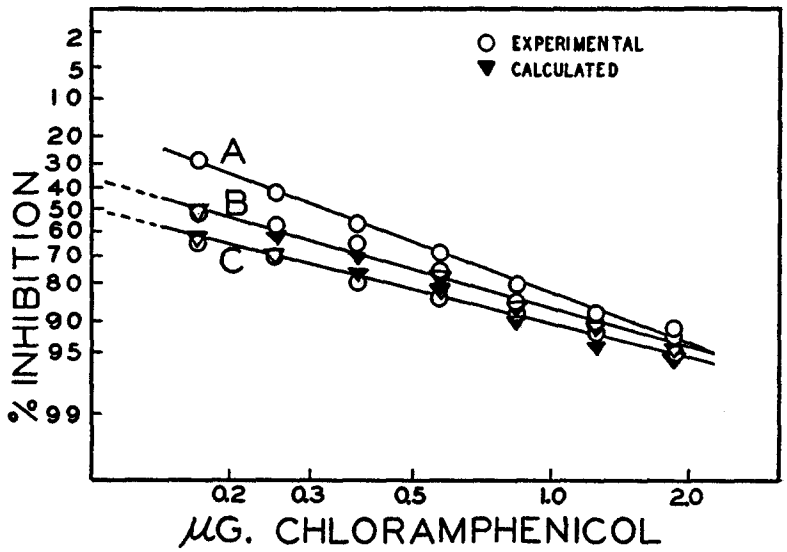

FIG. 1. Inhibition of *S. typhosa*, strain Ty2. Curve A: effect of chloramphenicol alone. Curve B: the same in the presence of 0.30 ml. antiserum dilution and complement. Curve C: the same, in the presence of 0.50 ml. antiserum dilution. Note: Because of the logarithmic abscissae, and thresholds of activity, the curves will approach closely but will not extrapolate exactly, in the region presented, to the zero control values.

mental error, precisely that expected from a summation of the individual inhibitory effects.

Antibody as Variable.

In this series, illustrated in Fig. 2, complement was, as usual, held constant while variable amounts of antibody were added.

The inhibition of growth due to the killing of a portion of the initial inoculum increased with added serum, both in the control series which lacked antibiotic (curve A), and in the presence of amounts of antibiotic (0.25 or 0.50 μg.) which had they been added alone would have resulted in inhibitions of 43 per cent (curve B) or 65 per cent (curve C).

Calculated Points.—The results expected on the hypothesis of joint, independent actions may be calculated in simple fashion by either of two methods. The first assumes that agent I—for example, antibody, plus complement—reduces the initial inoculum uniformly by a given percentage. Thus, for the first illustration above, the control tube containing complement plus the smaller amount of antiserum, 0.30 ml., had only 67 per cent of the growth given by the control containing broth alone (or broth plus complement, without serum). This condition should apply in all the other tubes in this set. We

may next consider the effect of agent II—here chloramphenicol. A certain level of the latter, such as 57 μg., resulted, in a control experiment, in an inhibition of 68 per cent (32 per cent growth). It is characteristic of both of these types of agents that although the absolute numbers of organisms killed or inhibited will vary directly with the inoculum size, the percentage reduction will be, to a first approximation, a constant independent of the inoculum. Hence we may calculate that agent II will reduce by 68 per cent the number of organisms escaping agent I. This new expected value, 67 per cent × 32 per cent = 21 per cent, of the original inoculum, corresponds very well with

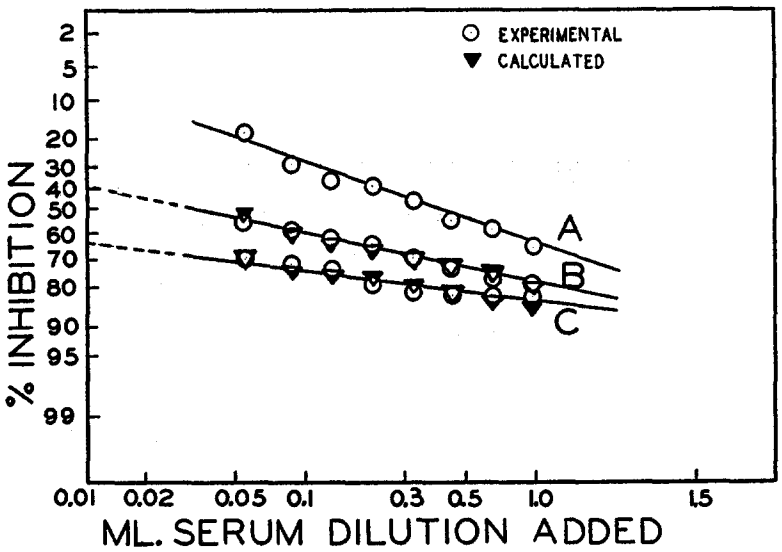

FIG. 2. Inhibition of *S. typhosa*, Ty2. Curve A: effect of antiserum (plus complement). Curve B: the same, in the presence of 0.25 μg. chloramphenicol. Curve C: the same, in the presence of 0.50 μg. chloramphenicol.

the observed value, 25 per cent. Alternatively, this latter value may be expressed as an inhibition of 75 per cent.

The calculation, in terms of inhibitions, is expressed succinctly by an equation given by Finney (reference 5, page 136).

$$P = P_1 + P_2 (1 - P_1) \quad (1)$$

in which P is the total mortality (or inhibition) and P_1 and P_2 are the mortalities (inhibitions) resulting from agents I and II applied separately. A calculation for the data of Fig. 1 is given in Table I; the computed values are also presented in Fig. 1. The average deviation for all points between the expected and observed inhibitions is 3.1 per cent. A similar calculation for the data of Fig. 2 is given in Table II; the average deviation, for all points, is 2.8 per cent.

Alternatively, as a second method of calculation, one may compute percentages of growth utilizing as denominator the optical density for the control tube containing the agent common, in constant quantity, to all tubes in that set. This method, applied to the data of Fig. 1, would reduce the calculated points for curves B and C to points lying along curve A.

TABLE II

Comparison of Observed and Calculated Inhibitions after Bactericidal Action of Antibody and Complement, at Two Levels of Chloramphenicol

(For data of Fig. 2)

Antiserum dilution (1:1500) added	Inhibition				
	Curve A (no drug)	Curve B (0.25 μg. drug)		Curve C (0.50 μg. drug)	
	Observed	Observed	Calculated	Observed	Calculated
ml.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	0	43	—	65	—
0.05	18	54	53	70	70
0.08	29	58	60	72	75
0.12	36	62	64	74	77
0.18	38	64	65	80	77
0.27	45	69	69	82	80
0.40	54	74	74	83	83
0.60	58	76	76	83	85
0.90	65	79	80	84	87
Average deviation between observed and calculated points, *per cent*		3.0		2.6	

Drug-Resistant Organisms

Chloramphenicol-Resistant S. typhosa.—It was of interest to determine whether the susceptibility to the bactericidal action of antibody and complement would be altered by a change in the level of susceptibility to an antibiotic. To reduce the hazards for laboratory infection with a drug-resistant strain, the relatively avirulent 0901 strain of *S. typhosa* was chosen; it had been established previously that this strain is suited for quantitative measurement of bactericidal action (2).

The resistant strain was selected by growing the stock culture of the 0901 strain on agar plates containing a gradient of chloramphenicol (6). The parental strain and the resistant variant isolated from it after several passages were titrated against the antibiotic, and also against an appropriate rabbit antiserum (2) and complement. This variant (resistant strain I) proved to have a chloramphenicol resistance, in terms of the relative amounts of drug required to

reach the 50 per cent endpoints, almost exactly twice that of the parental strain (1.91 and 2.05 in duplicate titrations). The amounts of antibody required for the 50 per cent inhibition endpoint, in the bactericidal tests were however, experimentally identical for the two strains (Table III).

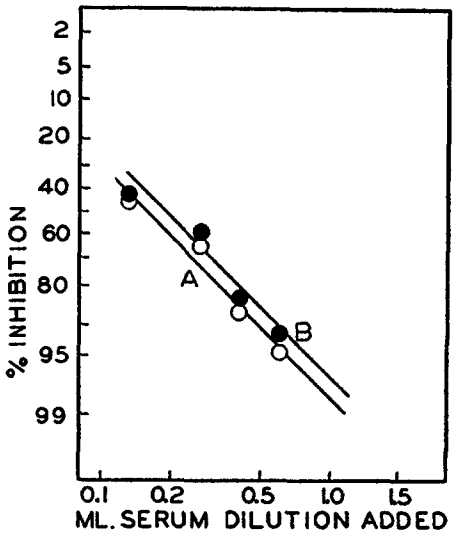

FIG. 3. Susceptibility to bactericidal action of antibody and complement. Curve A: parental strain of *S. typhosa*, 0901. Curve B: chloramphenicol-resistant variant II.

TABLE III

Relative Susceptibilities of Parental and Chloramphenicol-Resistant Strains of S. typhosa 0901 to Antibody (plus Complement), and to Chloramphenicol

Endpoints = 50 per cent inhibition of growth.

Agent titrated	Endpoint for CM-resistant variant / Endpoint for parental strain	
	Resistant Strain I	Resistant Strain II
Chloramphenicol	2.0*	4.2
Antiserum (with complement)	1.05*	0.89

* Averages of duplicate determinations.

By continued culture on drug-agar a second variant (resistant strain II) was isolated which was approximately fourfold more tolerant to the drug than was the parental strain. Nevertheless, its susceptibility to the bactericidal action of antibody and complement appeared to be that of the parental strain, within the experimental error of a single titration (Table III). It will be noted from the parallelism of the lines for the comparable pairs (Fig. 3) that although

the 50 per cent endpoint was chosen for numerical tabulation, any arbitrary endpoint would have served as well.

DISCUSSION

There have been a number of reports that in infections in which either specific antibody or antibiotic, separately, have therapeutic effects the combination may have additive value (7–9). In some instances the combined effect either *in vitro* or *in vivo* may be sufficiently greater than those of the individual components so as to suggest synergism. Some of the reports cited involve Gram-positive organisms and thus presumably do not include bactericidal effects. In all instances it is difficult to relate the observed *in vivo* effects to the *in vitro* model systems which we have investigated, particularly when data are lacking as to the relation between a given percentage reduction in the infecting dosage and the recovery rate.

Of immediate pertinence to our data is the report of Hounie (10) that low concentrations of chloramphenicol (1:5,000,000–1:2,000,000) which by themselves do not have appreciable effects, may increase the sensitivity of organisms to the bactericidal actions of blood. Miller and Foster (11) have reported analogous properties for penicillin.

The availability of quantitative assay methods of some sensitivity has permitted the examination of possible synergistic or antagonistic effects between a bactericidal system composed of specific antibody and complement and an antibiotic, chloramphenicol, active against the test organisms. Under the conditions here employed the only effect observed was, however, that of joint but independent action; *i.e.*, the total effect could be predicted quantitatively from a knowledge of the activities of the separate components. It is of interest to note that although Finney (5) discussed the possible applications of formula (1) above, he remarked that up to 1952 he had found no experimental data to illustrate its use; the present data would, however, appear to be satisfactorily expressed by it. As has already been implied above, the derivation of this formula assumes that the organisms escaping the action of the first agent are inhibited by the second agent in the same percentage as would have been the case had the second agent been added initially.

The rabbit antiserum employed against the Ty2 strain contained both anti-O and anti-Vi antibodies; recent evidence (2) indicates that the particular slope of curve A, Fig. 1, is a reflection of the fact that both antibodies are active here.

The probit or normal deviate plot employed provides a linear representation of the data. It is not to be expected, however, for this particular scale that curves B and C (Fig. 1) would be parallel to each other or to curve A. They are nevertheless related in that each original datum—the percentage survival—for the points of curve B is a constant fraction of the value for the point on

curve A lying immediately above it. Since the smaller amount of antiserum resulted in a constant inhibition of 33 per cent, the percentage survival obviously is 67 per cent; the corresponding values for curve C are 46 per cent and 54 per cent. These fixed percentages of inhibition (or survival), and the characteristics of the normal deviate rulings, result in a tilting away of the lines, which increases with the magnitude of the constant inhibition incorporated.

As has been noted above, the bactericidal action of antibody and complement is almost entirely confined to the reaction period since the subsequent dilution for the assay effectively reduces it to a negligible fraction. The activity of the antibiotic is not terminated by any process analogous to an anticomplementary action, but is merely decreased by the dilution at a rate which should parallel that of curve A, Fig. 1, except for secondary effects. No separate determination of the inhibition by the antibiotic during the assay stage itself need be made, however, since curve A provides a complete control for the antibiotic portion of the more complex inhibitions detailed in curves B and C. As far as the point of these experiments is concerned, the effective period of antibiotic activity is that of the reaction period; any subsequent antibiotic activity in the absence of antibody and complement is merely a slight experimental complication which can adequately be allowed for.

The experiments with the 0901 strain of *S. typhosa* are of interest in demonstrating that in these instances increases in resistance to chloramphenicol of from two- to fourfold are not accompanied by any appreciable change in susceptibility to the bactericidal action of anti-O antibody and guinea pig complement. It is of value to compare this result with the experiments of Servant (12), who found that the total extractable polysaccharide content of *S. typhosa*, strain 0901, did not change (within 2 per cent) when the organism was "developed" for resistance to dihydrostreptomycin, although other strains of *S. typhosa*, *S. enteritidis*, and *E. coli* decreased in total extractable polysaccharide by as much as 300 per cent on becoming resistant to this antibiotic. Unless there are important individual differences attending the development of specific resistances to the various antibiotics, Servant's data would indicate that the 0901 strain of *S. typhosa* may be somewhat atypical for our purposes. Unfortunately, his paper was not noted until after the present experiments had been completed and the point could not be investigated further at this time.

It would appear valid to assert from these experiments, despite their obvious limitations and exploratory character, that in recovery from typhoid fever the presence of an antibiotic such as chloramphenicol should not affect whatever therapeutic role may ultimately be assigned to the serum bactericidal components, so long as the organisms do not become drug-resistant. In what may be a special case, even the latter has been shown to be without deleterious

effect on the bactericidal action of antibody and complement. Further quantitative experiments on a variety of drug-resistant strains are obviously needed to test the generality of this second conclusion. If the resistant organisms contain significantly variable amounts of specific polysaccharides, such experiments may also contribute to our understanding of the bactericidal process.

SUMMARY

Quantitative determinations, employing turbidimetric growth assays, have been made of the inhibitory actions of choramphenicol, and of specific antibody plus complement, separately, and in combination. In experiments with the Ty2 strain of *S. typhosa* the combined activities of the two groups of reagents have been predicted, within 3 per cent, from the activities of the separate components, on the hypothesis of joint, independent action (additive inhibitions). A fourfold increase in resistance to chloramphenicol of the 0901 strain of *S. typhosa* has been shown to have little, if any, effect on its susceptibility to the bactericidal action of anti-O antibody and complement. Indirect evidence indicates that the latter may not be a general effect but may vary with the particular strain.

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