Brit. J. Pharmacol. (1957), 12, 521.

DETECTION AND MEASUREMENT OF ANTAGONISM TO FOLIC ACID

BY

H. O. J. COLLIER, J. J. GRIMSHAW AND PATRICIA L. HUSKINSON

From the Research Division, Allen and Hanburys Ltd., Ware, Hertfordshire

(RECEIVED JULY 29, 1957)

Methods of testing competitive antagonism in bacterial systems are considered and evaluated with particular reference to antifolic acids. Four antifolic acids—pteridine O/129, methotrexate, CB2295 and CB2335—were examined for competitiveness and potency in three test systems—*Leuc. citrovorum* and folinic acid, *Str. faecalis* and folinic acid, and *Str. faecalis* and pteroylglutamic acid. For comparison, phenol and formaldehyde were studied in the *Leuc. citrovorum* and folinic acid system. Interpretation of results was based on families of log. concentration/growth curves obtained in each system in the presence and absence of each antagonist. These curves were usually simple sigmoids. From these families of curves, *p*A values were derived and reciprocal growth/reciprocal concentration curves were drawn. Pteridine O/129 was fully competitive in all systems, while methotrexate was fully competitive in the *Str. faecalis* and folinic acid system, but did not give simple sigmoids in the other two systems. CB2295 showed some competitive features in all and CB2335 in the two systems in which it was active. It is concluded that the methods adopted, based on those used in vertebrate pharmacology, provide a stringent test of competitiveness in bacterial systems.

In screening potential antifolic acids (a term used here to refer to an antagonist of any vitamin of the folic acid group), Collier and Phillips (1954) and Timmis, Felton, Collier, and Huskinson (1957) employed a conventional microbiological method, which involved measuring growth of *Leuconostoc citrovorum* or *Streptococcus faecalis* at two or more levels of folic acid in the presence of varying amounts of antagonist. Though useful for screening, the method failed to provide a satisfactory measure of the degree of antagonism or competitiveness. To explore these properties further, we have developed from previous studies on antipurine action (Collier and Huskinson, 1957; Collier, 1957) a different method, which is the subject of the present paper. In this, we have measured growth of the test organism in response to geometrically increasing concentrations of folic acid, in the presence and absence of antagonist. The resulting log concentration/growth curves (CG curves) have been analysed by techniques used in vertebrate pharmacology (Gaddum, 1937; Schild, 1947; Chen and Russell, 1950; Ariëns, van Rossum, and Simonis, 1956).

MATERIALS AND METHODS

Four antifolic acids were studied: methotrexate (A-methopterin; Franklin, Belt, Stokstad and Jukes, 1949), 2:4-diamino-6:7-di*iso*propyl pteridine (O/129; Collier, Campbell and Fitzgerald, 1950), 2:4:6-triamino-5-benzeneazopyrimidine (CB2295; Timmis *et al.*, 1957) and 2:6-diamino-8-1′-naphthyl-8-azapurine (CB2335, Timmis *et al.*, 1957). Two non-specific inhibitors, formaldehyde *B.P.* and phenol *B.P.*, were also used.

Bacterial Growth.—Three systems were studied, namely, *Leuc. citrovorum* and folinic acid, *Str. faecalis* and folinic acid, and *Str. faecalis* and pteroylglutamic acid (PGA). Microbiological methods used were those described by Timmis *et al.* (1957), except that dilutions were arranged so that in any one test folinic acid or PGA was present in geometrically decreasing concentrations, while antagonist remained at constant concentration. Within a series of tests, the concentration of antagonist used was increased in geometric steps.

Analysis of CG Curves.—The basic unit of analysis was the family of curves obtained with one system and one antagonist. At least two replicate tubes were used in order to obtain any one point on a curve. Within a family, the mean maximal height of each curve obtained in the presence of antagonist was compared by a *t* test with that of the control curve obtained in its absence. In computing the mean maximal height all the appropriate replicates were used (see Figs. 1 and 2). "Parallelism" of each curve with the control was tested by regression

analysis after linearizing the central portions of the curves. The transformation used was

$$y' = \frac{y}{y_{max} - y}$$

where y is the response in ml. 0.1 N NaOH at a given concentration (x) in ng./ml. folic acid, and y_{max} is the maximum response obtained, since Gaddum (1937) showed that a plot of log. y′ against log. x was linear for the central portion of sigmoid dose-response curves. Constancy of interspace (that is, horizontal distance) between successive pairs of curves obtained in presence of antagonist was tested by deriving mathematically the molar ratios of antagonist to agonist at half-maximal control response, which is the point of minimum error.

In addition, CG curves were examined by the graphical method of Woolf (see Haldane, 1957) and of Lineweaver and Burk (1934), in which reciprocal of dose of agonist is plotted against reciprocal of response for each curve in a family (reciprocal concentration/reciprocal growth curves, RCG curves).

Degree of antagonism may be expressed as the molar ratio described above or as a *p*A value (Schild, 1947). In a particular family of transformed curves, *p*A values were obtained from those curves that had passed the test for parallelism with the control. The horizontal shifts from the control line in log. concentration of folic acid were plotted against negative log. molar concentration of antagonist (log shift curves). Straight lines were fitted by eye and, from these, pA_2 and pA_{10} values were read off by interpolation or extrapolation.

Results

Leuc. citrovorum *and Folinic Acid.*—In this system, CB2335 was inactive. The families of CG curves obtained with O/129, methotrexate, CB2295, phenol and formaldehyde are shown in Figs. 1 to 5, the protocols for O/129 being given in Table I. With the exception of two curves obtained in the presence of higher concentrations of methotrexate, all curves in this system were simple sigmoids. In the two compound curves a break, significant in the regression analysis, occurred at a concentration of 0.625 ng./ml. folinic acid.

The families of curves illustrated in Figs. 1 to 5, including both sections of the two compound curves, were analysed by the methods described above. This analysis (Table II) showed that all curves obtained with O/129 were parallel with the control and of the same height. The molar ratios of antagonist to agonist and the interspace were constant. With methotrexate, the simple curve and the total compound curves were similar in height to the control, and all except part of one compound curve were parallel. The molar ratios determined at concentrations <0.625 ng./ml. folinic acid were not constant, but fell in a way consistent with a geometric series, and the interspace was constant. With CB2295 the CG curves were parallel to and of the same height as the control, but the molar ratios were neither constant nor in geometric series, nor was the interspace constant. With phenol and still more with formaldehyde, curves were vertically depressed and not parallel

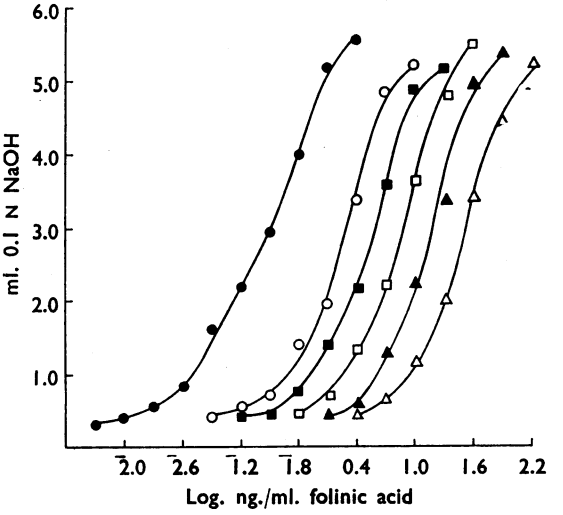

FIG. 1.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of O/129. ●——●, No antagonist; ○——○, 0.078 μg./ml. O/129; ■——■, 0.156 μg./ml. O/129; □——□, 0.312 μg./ml. O/129; ▲——▲, 0.624 μg./ml. O/129; △——△, 1.248 μg./ml. O/129.

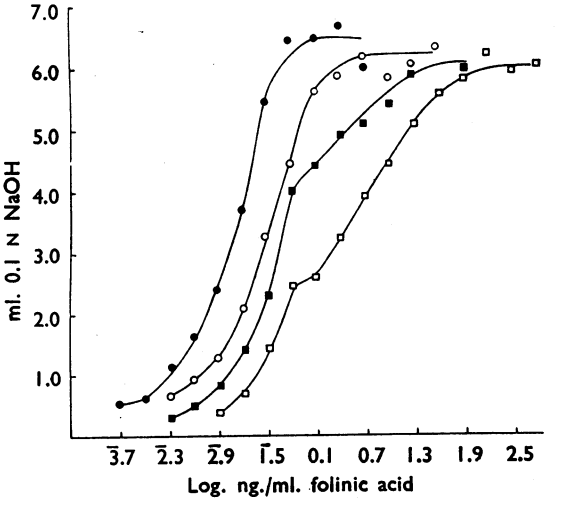

FIG. 2.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○——○, 0.078 μg./ml. methotrexate; ■——■, 0.312 μg./ml. methotrexate; □——□, 1.248 μg./ml. methotrexate.

TABLE I

RESPONSE OF *LEUC. CITROVORUM* TO FOLINIC ACID IN THE PRESENCE AND ABSENCE OF O/129

Titres in ml. 0·1 N NaOH

μg./ml. O/129	ng./ml. Folinic Acid																
	160	80	40	20	10	5	2·5	1·25	0·625	0·3125	0·156	0·078	0·039	0·0195	0·0097	0·0048	0
1·248	5·4 4·9	4·6 4·2	3·9 2·9	1·8 2·2	0·9 1·3	0·7 0·6	0·4 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4						0·4 0·4
0·624		5·3 5·4	5·0 4·9	3·1 3·6	2·3 2·1	1·3 1·2	0·5 0·6	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4					0·4 0·4
0·312			5·5 5·4	4·6 5·0	3·6 3·7	2·0 2·4	1·3 1·3	0·6 0·8	0·4 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4				0·4 0·4
0·156				5·3 5·0	4·7 5·0	3·8 3·4	2·1 2·2	1·2 1·6	0·8 0·7	0·5 0·4	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4			0·4 0·4
0·078					5·7 4·7	5·0 4·7	3·7 3·1	2·1 1·8	1·4 1·4	0·7 0·7	0·6 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4		0·4 0·4
0							5·8 5·3	5·1 5·3	3·8 4·2	3·0 2·9	2·3 2·1	1·7 1·5	0·8 0·9	0·6 0·5	0·5 0·5	0·4 0·4	0·4 0·4

TABLE II

LEUC. CITROVORUM AND FOLINIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295, PHENOL AND FORMALDEHYDE

— =Not tested. In the column headed Parallelism, + =no significant deviation from parallelism, $P>0.05$; 0=significant deviation from parallelism, $P<0.05$.

Antagonist	Conc. of Antagonist (μg./ml.)	Slope		Height				Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	P	
O/129 ..	0	0·967	0·850–1·086	—	100·0	—	—	—
	0·078	1·050	0·847–1·254	+	90·7	1·36	>0·1	78·7
	0·156	1·190	1·046–1·333	+	90·1	2·30	>0·05	78·9
	0·312	1·227	1·041–1·412	+	98·2	0·39	>0·4	79·1
	0·624	1·200	1·050–1·350	+	96·4	0·78	>0·2	79·2
	1·248	1·158	1·086–1·230	+	92·8	1·13	>0·1	75·8
Methotrexate	0	1·060	0·718–1·402	—	100·0	—	—	—
	0·078	0·934	0·826–1·041	+	96·9	0·79	>0·2	95·2
	0·312	1·104	1·025–1·183	+	—	—	—	190·8
	0·312	0·563	0·140–0·986	—	94·8	0·96	>0·1	—
	1·248	1·131	0·926–1·337	+	—	—	—	382·4
	1·248	0·506	0·478–0·536	0	95·1	0·71	>0·2	—
CB2295 ..	0	1·117	0·819–1·415	—	100·0	—	—	—
	0·195	1·081	0·967–1·194	+	111·5	—	—	868
	0·780	1·099	0·987–1·211	+	106·1	—	—	1,741
	3·120	1·211	1·102–1·320	+	96·2	0·86	>0·2	1,389
	12·480	2·222	1·203–3·242	+	—	—	—	2,123
Phenol ..	0	1·994	1·817–2·172	—	100·0	—	—	—
	2,000	1·539	1·380–1·697	0	89·5	3·46	<0·01	—
	2,500	2·094	1·890–2·297	+	52·9	—	—	—
	3,125	—	—	—	0	—	—	—
Formaldehyde	0	0·983	0·837–1·129	—	100·0	—	—	—
	57·1	0·754	0·636–0·873	+	66·2	11·42	<0·001	—
	61·5	—	—	—	48·6	—	—	—
	66·7	—	—	—	25·7	—	—	—
	72·7	—	—	—	8·7	—	—	—
	100·0	—	—	—	0	—	—	—

TABLE III

*p*A VALUES FOR SOME ANTIFOLIC ACIDS IN THREE TEST SYSTEMS

Bacterial Species	Growth Factor	O/129			Methotrexate			CB2295			CB2335		
		pA_2	pA_{10}	pA_2–pA_{10}	pA_2	pA_{10}	pA_2–pA_{10}	pA_2	pA_{10}	pA_2–pA_{10}	pA_2	pA_{10}	pA_2–pA_{10}
Leuc. citrovorum	Folinic acid	7·02	6·35	0·67	6·85	5·47	1·38	6·11	5·40	0·71	—	—	—
		6·64	6·04	0·60	9·82	9·42	0·40	6·20	5·94	0·26	6·98	6·57	0·41
Str. faecalis	PGA	8·38	8·02	0·36	10·69	9·83	0·86	6·62	6·10	0·52	8·02	7·36	0·66

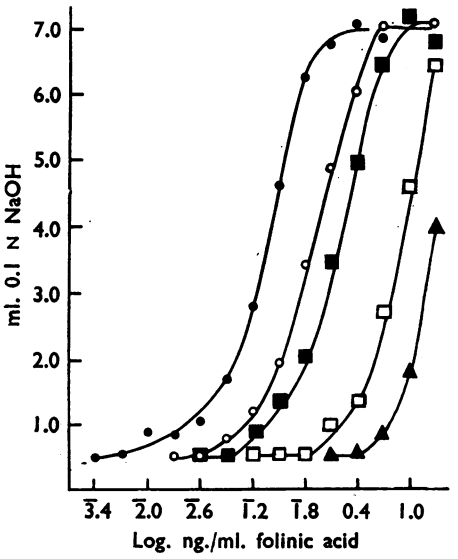

FIG. 3.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of CB2295. ●——●, No antagonist; ○——○, 0.195 μg./ml. CB2295; ■——■, 0.78 μg./ml. CB2295; □——□, 3.12 μg./ml. CB2295; ▲——▲, 12.48 μg./ml. CB2295.

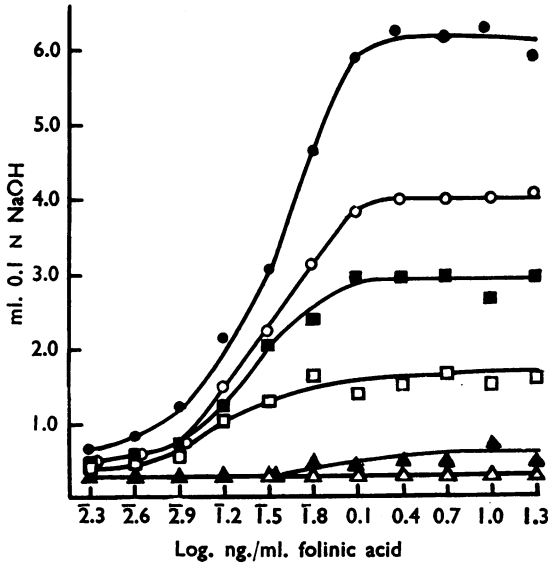

FIG. 5.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of formaldehyde. ●——●, No antagonist; ○——○, 57.1 μg./ml. formaldehyde; ■——■, 61.5 μg./ml. formaldehyde; □——□, 66.7 μg./ml. formaldehyde, ▲——▲, 72.7 μg./ml. formaldehyde; △——△, 100 μg./ml. formaldehyde.

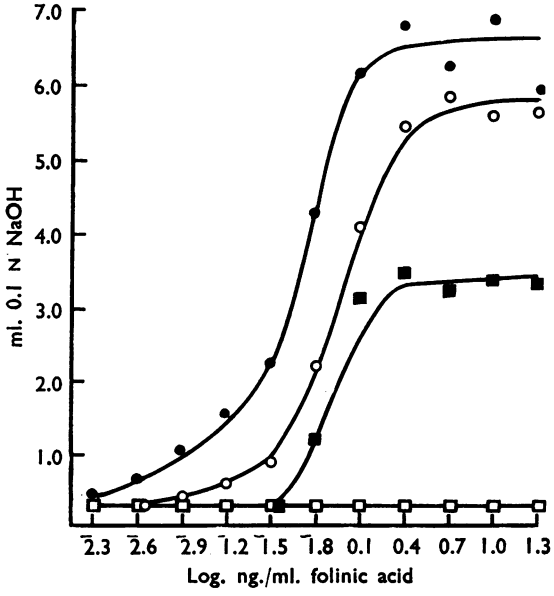

FIG. 4.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of phenol. ●——●, No antagonist; ○——○, 2,000 μg./ml. phenol; ■——■, 2,500 μg./ml. phenol; □——□, 3,125 μg./ml. phenol.

with controls. The interpretation of these results in relation to competitiveness will be discussed below.

In the family of RCG curves obtained for O/129 (Fig. 6) by the method of Lineweaver and Burk (1934), the six lines met at the ordinate. CB2295 presented a similar picture. With formaldehyde (Fig. 7) we obtained a series of almost parallel lines. The figure obtained with phenol was essentially similar to that of formaldehyde, but the lines showed a greater change in slope.

Fig. 8 gives the log. shift curves for O/129, CB2295 and for methotrexate at concentrations <0.625 ng./ml. folinic acid. From such curves are derived the *p*A values summarized in Table III.

Str. faecalis *and Folinic Acid.*—The curves obtained with methotrexate and CB2335 (Figs. 9 and 10) were simple sigmoids. So also were those with CB2295 and O/129, which are not illustrated. The analysis of all four families is presented in Table IV. All curves passed the test for parallelism and for similarity of height, compared with their respective controls. With methotrexate the molar ratios did not vary appreciably, with O/129 they increased in a way consistent with a geometrical series, and with CB2295 and CB2335

these ratios were irregular. RCG curves were drawn for all four antifolic acids in this system and did not differ in type from Fig. 6. The *p*A values in Table III are derived from log. shift curves similar to those illustrated in Fig. 8.

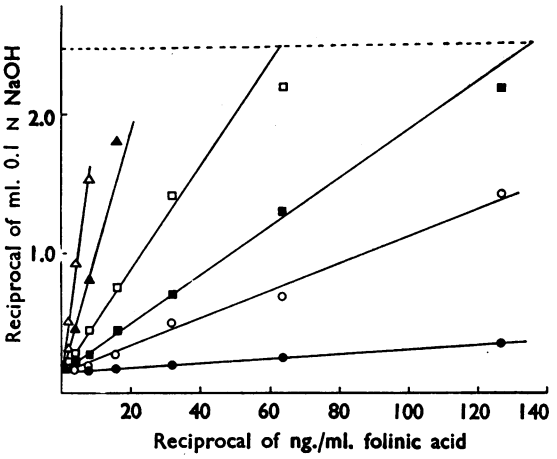

FIG. 6.—*Leuc. citrovorum* and folinic acid. Reciprocal concentration/reciprocal growth curves obtained in the presence and absence of O/129. Symbols as Fig. 1. The broken line indicates a value of 1/tube blank.

Str. *faecalis and PGA.*—O/129, CB2295 (Fig. 11) and CB2335 gave families of typical sigmoid curves analysed in Table V. Most CG curves passed the test for parallelism with their controls, but all families showed a drop in height at high concentrations of antagonist. The molar ratios for O/129 and CB2335 decreased apparently geometrically and those for CB2295 were irregular. RCG curves for these three substances resembled Fig. 6. Their *p*A values (Table III) were derived

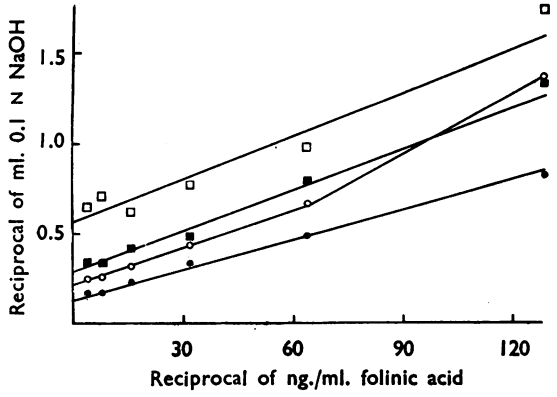

FIG. 7.—*Leuc. citrovorum* and folinic acid. Reciprocal concentration/reciprocal growth curves obtained in the presence and absence of formaldehyde. Symbols as Fig. 5.

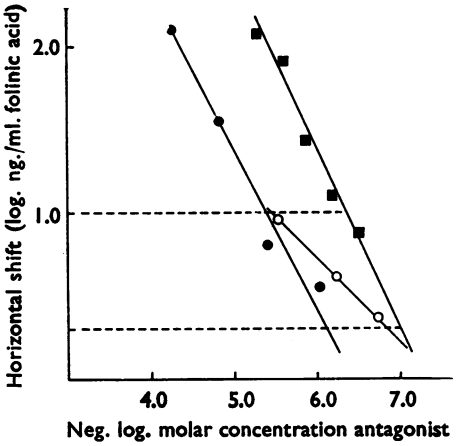

FIG. 8.—*Leuc. citrovorum* and folinic acid. Log. shift neg. log. molar concentration curves obtained with antifolic acids and estimation of pA_x values. The broken lines intersect the ordinate at log 10 and log 2; their intercepts with the curves are at points corresponding on the abscissa to the pA_{10} and pA_2 values. ●———●, CB2295; ○———○, methotrexate; ■———■, O/129.

from log. shift curves similar to those illustrated in Fig. 8.

The curves of methotrexate (Fig. 12) differed from those of the above substances in several features. All were compound, showing two maxima, and portions of the curves obtained at 0.078 and 0.312 ng./ml. methotrexate fell to the left of the control line. Except for these two portions, each part of the curves was tested separately for parallelism. The curve at 0.312 ng./ml. was not tested for height, because we thought that it was incomplete; but all other compound curves were not significantly lower than the control. The log. shifts were obtained from the second ascending portions of the compound curves at half-maximal control growth, and the *p*A values thus derived are given in Table III.

Discussion

The fundamental concept of competitive interaction rests on competition between two substances for one receptor system. Competitive antagonism occurs where one of these substances has little or no intrinsic activity (Ariëns *et al.*, 1956). Applying this definition, it can be shown that with a competitive antagonist CG curves should be parallel to the control and of constant height. Ariëns *et al.* have shown that, for geometrically increasing doses of antagonist, the interspace between successive CG curves obtained in the presence of a competitive antagonist should be constant. Where the same numbers of mole-

TABLE IV

STR. FAECALIS AND FOLINIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295, AND CB2335

See Table II for explanation of symbols in column headed Parallelism.

Antagonist	Conc. of Antagonist (ng./ml.)	Slope			Height			Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	*P*	
O/129 ..	0	1·083	0·957–1·211	—	100·0	—	—	—
	78	0·978	0·738–1·218	+	99·0	0·149	>0·5	469
	156	1·124	1·022–1·227	+	96·3	0·795	>0·2	505
	312	1·116	1·040–1·193	+	99·0	0·149	>0·5	568
Methotrexate	0	0·901	0·763–1·040	—	100·0	—	—	—
	0·0975	0·973	0·694–1·607	+	96·5	1·029	>0·1	0·506
	0·390	1·061	0·725–1·397	+	97·6	0·611	>0·2	0·535
	1·560	0·945	0·724–1·166	+	99·5	—	—	0·563
CB2295 ..	0	0·976	0·672–1·279	—	100·0	—	—	—
	195	0·820	0·596–1·045	+	107·5	—	—	901
	780	0·850	0·573–0·948	+	93·2	1·417	>0·1	1,193
	3,120	0·738	0·573–0·903	+	97·4	0·359	>0·3	689
CB2335 ..	0	0·942	0·830–1·053	—	100·0	—	—	—
	15·6	0·998	0·762–1·233	+	98·2	0·447	>0·5	464
	62·5	1·019	0·839–1·199	+	97·6	0·518	>0·3	733
	250	1·004	0·774–1·232	+	95·6	0·660	>0·2	733
	1,000	0·968	0·793–1·141	+	101·0	—	—	915

cules of agonist and of antagonist can combine with one receptor unit, constancy of interspace is shown by a constant molar ratio of agonist to antagonist. Where the numbers of competing molecules are unequal, the interspace remains constant but the molar ratios lie in a geometric series.

When the interspace between CG curves is constant, the slope of the corresponding RCG curves increases constantly. Even if the interspace is not constant, provided the CG curves are parallel with their control and of the same height, the lines of the RCG plot will intersect on the ordinate, although their increase in slope will not be constant.

When the interspace between CG curves is constant, the lines obtained in log. shift plots will be

TABLE V

STR. FAECALIS AND PTEROYLGLUTAMIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295 AND CB2335

See Table II for explanation of symbols in column headed Parallelism.

Antagonist	Conc. of Antagonist (ng./ml.)	Slope			Height			Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	*P*	
O/129 ..	0	1·354	1·136–1·553	—	100·0	—	—	—
	1·25	1·134	0·873–1·394	+	95·1	1·679	>0·05	2·57
	2·5	1·227	1·110–1·344	+	94·8	1·860	>0·05	1·82
	5·0	0·805	0·699–0·912	0	73·1	9·589	<0·001	1·24
	10·0	1·063	0·661–1·466	+	77·0	5·158	<0·001	0·80
Methotrexate	0	1·190	0·900–1·480	—	100·0	—	—	—
	0·078	1·156	0·703–1·609	+	100·2	—	—	0·132
	0·312	0·686	0·468–0·903	+	—	—	—	0·047
	1·248	0·994	0·273–1·715	+	—	—	—	—
	1·248	2·654	1·873–3·435	0	107·1	—	—	0·035
	4·992	2·049	1·575–2·524	0	—	—	—	—
	4·992	1·965	1·239–2·691	+	106·8	—	—	0·031
CB2295 ..	0	1·386	0·972–1·799	—	100·0	—	—	—
	78	0·753	0·510–0·996	+	97·3	1·549	>0·05	300
	312	0·908	0·625–1·190	+	96·0	1·842	>0·05	338
	1,248	0·894	0·501–1·288	+	93·1	3·750	<0·01	170
	4,992	—	—	—	32·0	—	—	—
CB2335 ..	0	1·278	1·118–1·437	—	100·0	—	—	—
	9·75	1·540	1·369–1·711	+	100·0	—	—	13·1
	19·5	1·007	0·818–1·195	+	90·1	4·76	<0·001	12·4
	39·0	1·445	0·961–1·929	+	95·0	2·95	<0·01	11·6
	156·0	—	—	—	31·1	—	—	—

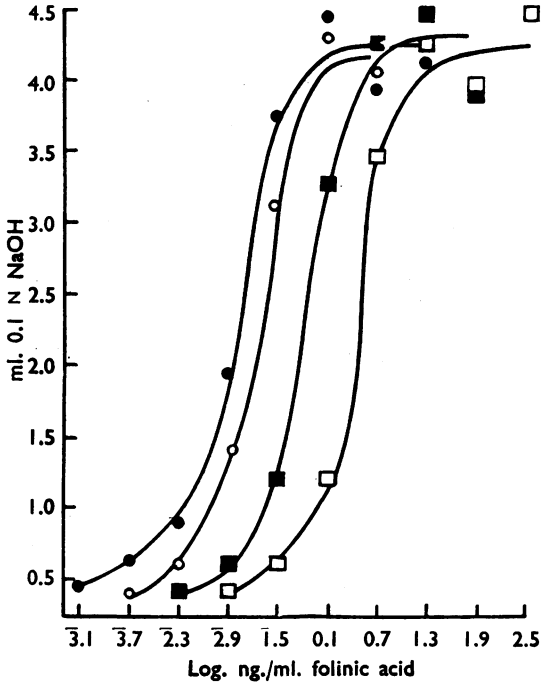

FIG. 9.—*Str. faecalis* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○——○, 0.0975 ng./ml. methotrexate; ■——■, 0.390 ng./ml. methotrexate; □——□, 1.560 ng./ml. methotrexate.

rectilinear. Where this condition did not hold, the best straight lines were fitted by eye, in order to obtain *p*A values (Fig. 8). Schild (1947) has shown from the mass action equation of Gaddum (1937) that " a first order reaction requires a nine-fold increase of antagonist corresponding to a five-fold increase of active drug." This gives the equation

$$pA_2 - pA_{10} = 0.95$$

It is clear, however, that the value of 0.95 will only be realized in a situation where one molecule of

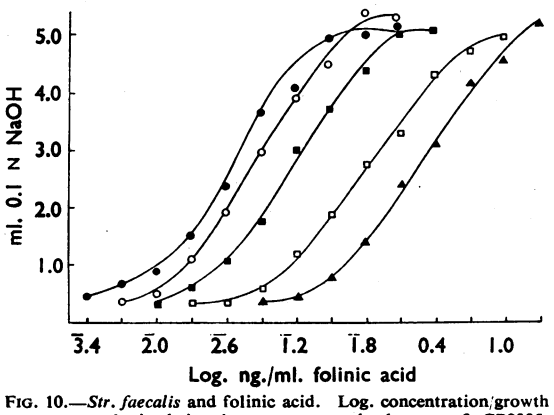

FIG. 10.—*Str. faecalis* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of CB2335. ●——●, No antagonist; ○——○, 62.5 ng./ml. CB2335; ■——■, 250 ng./ml. CB2335; □——□, 1,000 ng./ml. CB2335.

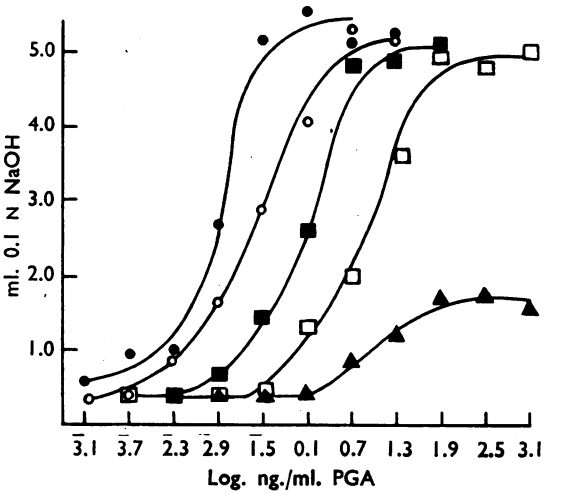

FIG. 11.—*Str. faecalis* and PGA. Log. concentration/growth curves obtained in the presence and absence of CB2295. ●——●, No antagonist; ○——○, 78 ng./ml. CB2295; ■——■, 312 ng./ml. CB2295; □——□, 1,248 ng./ml. CB2295; ▲——▲, 4,992 ng./ml. CB2295.

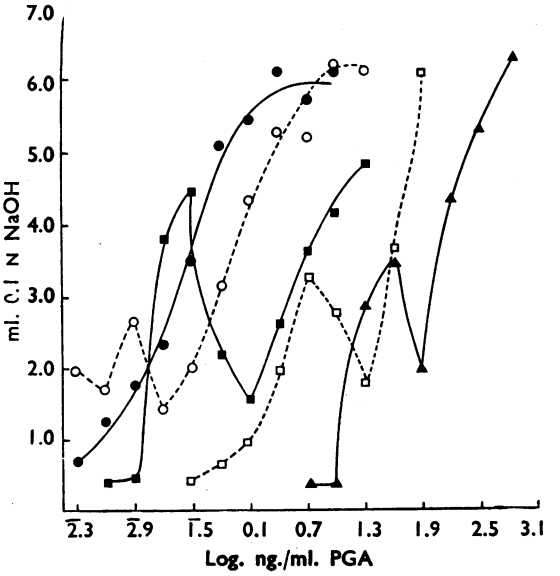

FIG. 12.—*Str. faecalis* and PGA. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○------○, 0.078 ng./ml. methotrexate; ■——■, 0.312 ng./ml. methotrexate. □------□, 1.248 ng./ml. methotrexate; ▲——▲, 4.992 ng./ml. methotrexate.

agonist or one of antagonist combine with one receptor unit. Marshall (1955) found that atropine and acetylcholine gave a $pA_2 - pA_{10}$ value of 0.73 on guinea-pig ileum. Although he inferred from this that their relationship was not competitive, this need not be so, as Timms (1956) points out and the present discussion makes clear.

Of the compounds examined, only O/129 fulfilled in all three systems the criteria of competitive antagonism discussed above. The $pA_2 - pA_{10}$ values, however, were less than 0.95, which may be explained by supposing that the relationships between O/129 and folic acid with the receptors are not of the first order.

In the *Str. faecalis* and folinic acid system, methotrexate fulfilled the criteria of competitive antagonism. In both the other systems, however, it presented a complicated picture. With *Leuc. citrovorum* and folinic acid, breaks appeared in some of the CG curves, although corresponding effects were not detected with the earlier method of investigation (Collier and Phillips, 1954). It was possible, however, to obtain *p*A values from the log shifts of the parallel portions of the curves. The $pA_2 - pA_{10}$ value was much greater than unity, which was exceptional. In the *Str. faecalis* and PGA system, methotrexate at low concentrations slightly stimulated growth (Fig. 12), which suggests that it acts at more than one of the receptors concerned with the utilization of PGA. This may help to account for the unusual shape of the CG curves.

CB2295 and CB2335 exhibit some competitive features, but generally lack that of constant interspace over the concentration ranges tested. The two compounds are clearly antifolic acids, which may well be fully competitive over some ranges of concentration. The drop in the maxima of the CG curves at high concentrations seen with CB2295, CB2335, and O/129 in some systems indicates toxicity unconnected with folic acid.

Phenol shows some degree of parallelism, but no other competitive features. Formaldehyde provides a series of curves of decreasing maxima without horizontal shift, except for a slight initial one. It thus exemplifies inhibition that is not competitive.

Our findings show that the technique of testing competitiveness developed in vertebrate pharmacology can be applied to bacterial systems. They emphasize the necessity for full tests of this type before relationships can be characterized as competitive. They also show that chemical structure and potency can be guides to competitive action. Thus, both compounds which were fully competitive in at least one system (O/129 and methotrexate) are pteridines and were also the most potent inhibitors tested. CB2295 and CB2335, which show some features of competitiveness, are pyrimidines and were of moderate potency. Phenol and formaldehyde, which were not competitive, do not resemble folic acid in structure and were of relatively low potency.

We wish to thank Miss J. A. Moore and Miss J. Wallace for technical assistance. We are indebted to Professor F. C. Happold for the culture of *Str. faecalis* (R) and to the American Cyanamid Company for methotrexate (" A-methopterin ") and the calcium salt of folinic acid (" Calcium leucovorin ").

REFERENCES

Ariëns, E. J., van Rossum, J. M., and Simonis, A. M. (1956). *Arzneim. Forsch.*, **6**, 282.
Chen, G., and Russell, D. (1950). *J. Pharmacol.*, **99**, 401.
Collier, H. O. J. (1957). *Pharmacol. Rev.*, **9**, 264.
—— Campbell, N. R., and Fitzgerald, M. E. H. (1950). *Nature, Lond.*, **165**, 1004.
—— and Huskinson, P. L. (1957). *Ciba Foundation. Symposium on the Chemistry and Biology of Purines*, p. 146. London: Churchill.
—— and Phillips, M. (1954). *Nature, Lond.*, **174**, 180.
Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H. (1949). *J. biol. Chem.*, **177**, 621.
Gaddum, J. H. (1937). *J. Physiol.*, **89**, 7P.
Lineweaver, H., and Burk, D. J. (1934). *J. Amer. chem. Soc.*, **56**, 658.
Marshall, P. B. (1955). *Brit. J. Pharmacol.*, **10**, 354.
Schild, H. O. (1947). Ibid., **2**, 189.
Timmis, G. M., Felton, D. G. I., Collier, H. O. J., and Huskinson, P. L. (1957). *J. Pharm., Lond.*, **9**, 46.
Timms, A. R. (1956). *Brit. J. Pharmacol.*, **11**, 273.
Woolf. B. (1932). See Haldane, J. B. S. (1957). *Nature, Lond.*, **179**, 832.