TABLE II—COMPARISON OF TRITIATION[a] AND HOMOLYTIC AROMATIC SUBSTITUTION[b]

Reaction	% *ortho* Substitution				
	$PhCH_3$	PhCl	$PhOCH_3$	$PhNO_2$	PhCOOH
Tritiation	54, 56	50	40	58	58
Phenylation	71	62	67	62.5	...
Methylation	56.5	64	74	65.5	...
	meta/para Substitution Ratio				
Tritiation	1.92, 1.34	2.10	1.22	3.44	1.04
Phenylation	1.32	1.71	1.20	0.36	...
Methylation	1.56	2.27	1.36	0.21	...

[a] From *References 2, 12, 13, 14.* [b] Fieser, L. F., and Fieser, M., "Advanced Organic Chemistry," Reinhold Publishing Corp., New York, N. Y., 1961, p. 638.

with methane in the gaseous phase. They concluded that methane incorporates tritium by several mechanisms, all involving heterolytic reactions of positively charged transient species. It would be difficult to apply these mechanisms to more complex systems, particularly to a condensed phase system such as chlorphenesin carbamate.[8] However, it seems unlikely that an electrophilic reaction mechanism could explain the distribution of tritium on the aromatic ring of chlorphenesin carbamate or the other aromatic compounds discussed above.

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[8] It is interesting to note that the distribution of tritium in chlorobenzene is essentially the same whether it is tritiated in the gaseous phase or in the presence of excess liquid chlorobenzene (13).

Drug Standards

Outline of Details for Official Microbiological Assays of Antibiotics

By AMIEL KIRSHBAUM and BERNARD ARRET

Tables are presented listing pertinent data required for 57 different microbiological assays, including those for all antibiotics approved for human use in the United States.

In 1959, the authors published an "outline" for assaying antibiotics (1). Development of new methods, modifications and improvements of old ones, and development of methods for antibiotics which have since been discovered necessitate a revision and updating of that publication. As in the earlier article, pertinent information has been tabulated as a ready guide for the analyst performing microbiological assays of antibiotics. Complete details for the assays are not given since these tabulations are prepared for those already familiar with the basic methods. Procedural information can be found in the Code of Federal Regulations (2).

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PREPARATION OF MICROBIAL SUSPENSIONS

Since the earlier publication, a uniform procedure for preparation of microbial suspensions has been

TABLE I—TEST ORGANISMS FOR VARIOUS ASSAYS

		ATCC No.
(*A*)	*Staphylococcus aureus*	6538P
(*B*)	*Sarcina subflava*	7468
(*C*)	*Sarcina lutea*	9341
(*D*)	*Staphylococcus epidermidis*	12228
(*E*)	*Saccharomyces cerevisiae*	9763
(*F*)	*Bordetella bronchiseptica*	4617
(*G*)	*Bacillus cereus* var. *mycoides*	11778
(*H*)	*Bacillus subtilis*	6633
(*I*)	*Klebsiella pneumoniae*	10031
(*J*)	*Escherichia coli*	10536
(*K*)	*Streptococcus faecalis*	10541
(*L*)	*Micrococcus flavus*	14452
(*M*)	*Microsporum gypseum*	14683
(*N*)	*Sarcina lutea*	9341a

described (3). A modification of this procedure is used for preparing and standardizing most of the microbial suspensions, as follows.

Maintain the test organism on slants of medium No. 1 and transfer to a fresh slant once a week. Using 3 ml. of U.S.P. saline T.S., wash the organism from the agar slant (which has been incubated for 24 hr. at 32–35°) onto a large agar surface of medium No. 1 such as that provided by a Roux bottle containing 300 ml. of agar. Incubate for 24 hr. at 32–35°. Wash the resulting growth from the nutrient surface, using 50 ml. of sterile U.S.P. saline T.S.

Determine the dilution of the suspension with saline that will give a 25% light transmission reading at a wavelength of 580 mμ in a spectrophotometer

TABLE II—MEDIA

Medium	Ingredients	Gm.
(*1*)[a]	Peptone	6
	Pancreatic digest of casein	4
	Yeast extract	3
	Beef extract	1.5
	Dextrose	1
	Agar	15
	Distilled water to make	1000 ml.
	pH 6.55 ± 0.05 after sterilization	
(*2*)[a]	Same as medium *1*, except omit pancreatic digest of casein and dextrose	
(*3*)[a]	Peptone	5.0
	Yeast extract	1.5
	Beef extract	1.5
	Sodium chloride	3.5
	Dextrose	1.0
	Monobasic potassium phosphate	1.32
	Dibasic potassium phosphate	3.68
	Distilled water to make	1000 ml.
	pH 7.0 ± 0.05 after sterilization	
(*4*)[a]	Same as medium *1*, except omit pancreatic digest of casein	
(*5*)[a]	Same as medium *2*, except the pH is adjusted so that it is 7.9 ± 0.1 after sterilization	
(*8*)[a]	Same as medium *2*, except the pH is adjusted so that it is 5.65 ± 0.05 after sterilization	
(*9*)[a]	Pancreatic digest of casein	17.0
	Papaic digest of soy bean	3.0
	Sodium chloride	5.0
	Dibasic potassium phosphate	2.5
	Dextrose	2.5
	Agar	20
	Distilled water to make	1000 ml.
	pH 7.25 ± 0.05 after sterilization	
(*10*)[a]	Same as medium *9*, except use 12 Gm. instead of 20 Gm. of agar. Boil to dissolve the ingredients; then add 10 ml. of polysorbate 80.	
(*11*)[a]	Same as medium *1*, except the pH is adjusted so that it is 7.9 ± 0.1 after sterilization.	
(*13*)[a]	Peptone	10
	Dextrose	20
	Distilled water to make	1000 ml.
	pH 5.65 ± 0.05 after sterilization	
(*18*)[b]	Same as medium *11*, except boil to dissolve the ingredients; then add 20 ml. of polysorbate 80.	
(*19*)[b]	Peptone	9.4
	Yeast extract	4.7
	Beef extract	2.4
	Sodium chloride	10.0
	Dextrose	10.0
	Agar	23.5
	Distilled water to make	1000 ml.
	pH 6.1 ± 0.1 after sterilization	
(*20*)[b]	Dextrose	40.0
	Peptone	10.0
	Chloramphenicol	0.05 (activity)
	Agar	15.0
	Distilled water to make	1000 ml.
	pH 5.65 ± 0.05 after sterilization	
(*21*)[b]	Dissolve 10 mg. of cycloheximide per ml. of distilled water and sterilize by filtration through a membrane filter having a porosity of 0.22 mμ. Aseptically, add 20 ml. of the sterile solution to each liter of medium *20* which has been autoclaved and cooled to 50°.	

[a] Numbers correspond to those used in "Assay Methods of Antibiotics" Grove, D. C., and Randall, W. A., Medical Encyclopedia Inc., New York, N. Y., 1955, p. 220. [b] Numbers assigned by authors.

TABLE III—BUFFERS

Buffer		
I	**1% Phosphate Buffer, pH 6.0 ± 0.05**	
	Dibasic potassium phosphate	2 Gm.
	Monobasic potassium phosphate	8 Gm.
	Distilled water to make	1000 ml.
III	**0.1 M Phosphate Buffer, pH 7.9 ± 0.1**	
	Dibasic potassium phosphate	16.73 Gm.
	Monobasic potassium phosphate	0.523 Gm.
	Distilled water to make	1000 ml.
IV	**0.1 M Monopotassium Phosphate Buffer, pH 4.5 ± 0.05**	
	Monobasic potassium phosphate	13.6 Gm.
	Distilled water to make	1000 ml.
VI	**10% Phosphate Buffer, pH 6.0 ± 0.05**	
	Same as I, except use 10 times as much of each salt.	
X	**0.2 M Phosphate Buffer, pH 10.5 ± 0.1**	
	Dibasic potassium phosphate	35 Gm.
	10 *N* sodium hydroxide	2 ml.
	Distilled water to make	1000 ml.

such as a Bausch & Lomb Spectronic 20, using a round cell with an inside diameter of 1.2 cm.; a Beckman model B spectrophotometer, using a 1.0-cm. square cell; or any other suitable instrument. Use U.S.P. saline as the blank for all instruments. Determine the amount of suspension to be used by test plates or test broth (2, 3).

The test organisms used for the various assays are listed in Table I. The media and buffers are listed in Tables II and III, respectively.

DETAILS OF ASSAY PROCEDURES

Pertinent information for cylinder plate assays is given in Table IV and for turbidimetric assays in Table V.

The third column of the tables, titled "Inoculum," lists the dilution of the stock suspension necessary to obtain 25% light transmission and, in parentheses, the volume of suspension to be added to each 100 ml. of seed medium for plate assays or broth for turbidimetric assays. Those volumes followed by *Footnote b* are added from a dilution of the stock suspension; those without the footnote are added directly from the stock suspension. For example, for penicillin G, the suspension requires a 1 + 19 dilution to give 25% light transmission, and 1.0 ml. of the stock suspension is used per 100 ml. of seed agar. It is stressed that these values are guides only, and each laboratory should perform its own standardization.

For those assays to which the uniform procedure

TABLE IV—PLATE DIFFUSION ASSAYS

Antibiotic	Test Organism	Inoculum	Medium Base	Medium Seed	Initial Soln. of Std.	Buffer for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Ampicillin	*C*	1 + 39 (0.5)	*11*	*11*	100 mcg./ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35
Amphomycin	*L*	1 + 34 (0.5)	*2*	*1*	100 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	37
Amphotericin B	*E*	1 + 29 (0.2)	None	19[a]	1000 mcg./ml. in dimethylsulfoxide	X	0.51, 0.7, *1.0*, 1.4, 1.96	30
Bacitracin	*B*	1 + 29 (0.3)	*2*	*1*	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Bacitracin	*L*	1 + 29 (0.3)	*2*	*1*	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Carbomycin	*C*	1 + 39 (0.2)	*11*	*11*	10,000 mcg./ml. in methanol. Then dilute to 100 mcg./ml. in distilled water	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Cephaloridine	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Cephalothin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Chloramphenicol	*C*	1 + 39 (2.0)	*2*	*1*	10,000 mcg./ml. in ethanol	I	32, 40, *50*, 62.5, 78.1	32–35
Chlortetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.01 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Cloxacillin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Sodium colistimethate	*F*	1 + 19 (0.1)	*9*	*10*	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37
Colistin	*F*	1 + 19 (0.1)	*9*	*10*	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37
Cycloserine	*A*	1 + 19 (0.04)	*2*	*1*	1000 mcg./ml. in distilled water	I	32, 40, *50*, 62.5, 78.1	30

(*Continued on next page.*)

TABLE IV—(*Continued.*)

Antibiotic	Test Organism	Inoculum	Medium Base	Medium Seed	Initial Soln. of Std.	Buffer for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Dactinomycin	*H*	Sec. 148u.1	*5*	*5*	10,000 mcg./ml. in methanol	III	0.5, 0.71, *1.0*, 1.41, 2.0	37
Dicloxacillin	*A*	1 + 19 (0.1)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Dihydrostreptomycin	*H*	Sec. 141c.101	*5*	*5*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Erythromycin	*C*	1 + 39 (1.5)	*11*	*11*	10,000 mcg./ml. in methanol	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Gentamicin	*D*	1 + 14 (1.5)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Griseofulvin	*M*	Sec. 148g.1	*20*	*21*	1000 mcg./ml. in dimethylformamide[d]	III	3.2, 4.0, *5.0*, 6.25, 7.81	30 (48 hr.)
Kanamycin	*A*	1 + 19 (0.4)[b]	*11*	*11*	1000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Lincomycin	*C*	1 + 39 (1.5)	*11*	*11*	1000 mcg./ml. in distilled water	III	1.28, 1.6, *2.0*, 2.5, 3.12	32–35
Methicillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	6.4, 8.0, *10.0*, 12.5, 15.6	32–35
Nafcillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	1.28, 1.6, *2.0*, 2.5, 3.12	32–35
Neomycin	*A*	1 + 19 (0.4)[b]	*11*	*11*	1000 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35
Neomycin	*D*	1 + 14 (1.0)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Novobiocin	*D*	1 + 14 (4.0)	*11*	*11*	10,000 mcg./ml. in ethanol	III	0.32, 0.4, *0.5*, 0.625, 0.781	32–35
Nystatin	*E*	1 + 29 (1.0)	None	*19*[a]	1000 mcg./ml. in dimethylformamide[d]	VI	12.8, 16, *20*, 25, 31.2	30
Oleandomycin	*D*	1 + 24 (1.0)	*11*	*11*	10,000 mcg./ml. in ethanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Oxacillin	*A*	1 + 19 (0.3)	*2*	*1*	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Oxytetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Paromomycin	*D*	1 + 24 (2.0)	*11*	*11*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Penicillin G	*A*	1 + 19 (1.0)	*2*	*1*	1000 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Phenethicillin	*C*	1 + 39 (0.5)	*11*	*11*	1000 units/ml. in III	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35
Phenoxymethylpenicillin	*A*	1 + 19 (1.0)	*2*	*1*	1000 units/ml. in methanol	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35
Polymyxin	*F*	1 + 19 (0.1)	*9*	*10*	10,000 units/ml. in distilled water	VI	6.40, 8.0, *10.0*, 12.5, 15.6	37
Streptomycin	*H*	Sec. 141c.101	*5*	*5*	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37
Tetracycline	*G*	Sec. 141c.201	*8*	*8*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30
Triacetyloleandomycin	*D*	1 + 24 (1.0)	*18*	*18*	1000 mcg./ml. in 80% isopropanol	X	9.6, 12, *15*, 18.8, 23.4	37
Tylosin	*N*	1 + 39 (1.0)	*11*	*11*	10,000 mcg./ml. in methanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35
Vancomycin	*G*	Sec. 148s.1	*8*[c]	8	1000 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	30
Viomycin	*H*	Sec. 148t.1	*5*[c]	*5*	1000 mcg./ml. in III	III	32, 40, *50*, 62.5, 78.1	37

[a] No base layer is used. Add 8 ml. of inoculated seed layer only. [b] Use indicated dilution of stock suspension. [c] Use 10 ml. instead of 21 ml. [d] Solution is further diluted in solvent to 20 times the final concentration of each concentration of the standard curve so that each final dilution is 1 + 19 with indicated buffer.

does not apply, reference is made to the appropriate section of the Antibiotic Regulations (2).

In Table IV, under the column headed "Medium," there are subheadings "Base" and "Seed." Unless otherwise specified, 21 ml. of base agar and 4 ml. of seed agar should be used for each plate. For turbidimetric assays in Table V, 9 ml. of broth should be used.

The column marked "Initial Soln. of Std." indicates the method of getting the standard into solution. Unless a concentration of 1000 mcg. or units or less per milliliter is indicated, the solution

TABLE V—TURBIDIMETRIC ASSAYS

Antibiotic	Test Organism	Inoculum	Medium	Initial Soln. of Std.	Diluent for Further Dilutions	Final Concn. for Std. Curve, units or mcg./ml.	Incubation Temp., °C.
Candicidin	*E*	1 + 29 (0.2)	*13*	1000 mcg./ml. in dimethylsulfoxide	Distilled water	0.03, 0.042, *0.06*, 0.084, 0.12	25
Chloramphenicol	*J*	1 + 19 (0.7)	*3*	10,000 mcg./ml. in ethanol	I	2.0, 2.24, *2.5*, 2.79, 3.12	37
Chlortetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.01 *N* HCl	IV	0.036, 0.047, *0.060*, 0.077, 0.099	37
Demethylchlortetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.06, 0.077, *0.10*, 0.129, 0.167	37
Dihydrostreptomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in distilled water	Distilled water	24, 26.7, *30*, 33.6, 37.5	37
Doxycycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.08, *0.10*, 0.125, 0.156	37
Gramicidin	*K*	Overnight broth culture (1.0)	*3*	1000 mcg./ml. in 95% ethanol	95% ethanol	0.028, 0.034, *0.040*, 0.048, 0.058	37
Methacycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.01 *N* methanol soln. prepared with concentrated hydrochloric acid	IV	0.036, 0.047, *0.060*, 0.077, 0.099	37
Oxytetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Rolitetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in methanol	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Streptomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in distilled water	Distilled water	24, 26.7, *30*, 33.6, 37.5	37
Tetracycline	*A*	1 + 19 (0.1)	*3*	1000 mcg./ml. in 0.1 *N* HCl	IV	0.146, 0.187, *0.240*, 0.308, 0.395	37
Triacetyloleandomycin	*I*	1 + 24 (0.1)	*3*	1000 mcg./ml. in 80% isopropanol	I	15, 19.5, *25*, 32, 41.2	37
Tyrothricin	*K*	Overnight broth culture (1.0)	*3*	1000 mcg./ml. in 95% ethanol	95% ethanol	0.14, 0.17, *0.20*, 0.24, 0.29	37
Viomycin	*I*	1 + 24 (0.1)	*3*	5000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37

should immediately be further diluted with the indicated buffer to obtain a stock solution of convenient concentration.

The entries under "Final Concn. for Std. Curve" are self-explanatory, except that the italicized concentration in each instance is the reference concentration. Authentic standards are supplied by the U.S.P. or N.F. for those antibiotics listed in these compendia.

SUMMARY

Five tables have been presented listing pertinent data required for 57 different microbiological assays, including those for all antibiotics approved for human use in the U. S. For those analysts already familiar with basic procedures, the tables give all of the information necessary for the performance of the assays.

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