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THE USE OF THE CHICK EMBRYO IN THE SEARCH FOR ANTITUBERCULOUS DRUGS

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The need, in searching for new antituberculous drugs, for a simple screening test capable of giving results more in accord with those obtained in animals than the ordinary *in vitro* test has been emphasized repeatedly (e.g., Feldman, 1946). Lee and Stavitsky (1947) described the use of the chick embryo for the purpose, using a virulent strain of the human tubercle bacillus injected intravenously, with assessment of the effect of drugs by histological examination of the liver. Intravenous injection of the embryo is technically difficult, the associated trauma causes the death of a considerable proportion of embryos, and the histological assessment is time-consuming. These factors make the method unsuitable for the examination of large numbers of compounds. It has been found (Francis, 1946 ; Francis, Peters, and Davies, 1947) that if streptococci are placed on the chorio-allantois of the chick-embryo, drugs with only "antiseptic action," such as acriflavine, have little action if injected into the yolk-sac, whilst penicillin and the sulphonamides are effective. An attempt was therefore made to apply this simplified technique in the search for antituberculous drugs. In preliminary unpublished experiments it was found that virulent mammalian tubercle bacilli produced much larger lesions than did avirulent bacilli, *Mycobacterium johnei,* or the vole acid-fast bacillus. These results would be expected, but it was surprising to find that virulent or avirulent avian bacilli produced only small lesions. The relatively low virulence of the avian strain was confirmed when it was found that after the intravenous injection of 1 mg. of moist bacilli a virulent avian strain took on the average 6.6 days to kill chick embryos and a virulent bovine strain 3.8 days. At the time, however, the lesions produced by virulent mammalian strains on the chorio-allantois were not considered sufficiently regular for routine testing. *Myco. phlei* and *Myco. butyricum* grew in the chorio-allantois but did not give satisfactory responses to known drugs. A series of tests with the avirulent human strain L.48 (National Collection of Type Cultures) showed that a suspension of 0.5 mg. of a 10- to 12-day culture on Löwenstein's medium multiplied well five days after inoculation on the chorio-allantois and a reduction of growth could be demonstrated with the standard antituberculous drugs. Later the avian strain S.H.I. was found to be more sensitive towards certain drugs in the test, and is now used in place of strain L.48.

METHODS

The following routine procedure has been adopted after various trials. Twelve-day-old chick embryos, prepared by the method of Beveridge and Burnet (1946), receive on the chorio-allantois an inoculation of 0.5 mg. (moist weight) of a strain of the avian bacillus (S.H.I.) previously grown for 12 days on Löwenstein's medium. The infecting dose of organisms is suspended by grinding in a ball mill for a short time (Martin, 1946) in 0.1 ml. of 5% broth-saline, and delivered by the McLintock automatic syringe used for tuberculin-testing cattle (McLintock, 1948). About 20 minutes later an aqueous solution or suspension of a drug is injected into the yolk-sac, with a 14-gauge needle, through a small hole drilled in the shell towards the narrow end of the egg. Ten eggs are used per group and a single dose of 18 mg. of drug per egg is given. Should this prove toxic, the dose is reduced to 9, 3, or 1 mg. in subsequent experiments. After five days' incubation the membranes from each group are harvested and ground in saline, using 0.6 ml. per membrane. The suspensions are randomized, and smears made and stained. The number of acid-fast organisms is then assessed as described below and the results obtained with the various compounds compared with the controls. A standard "active" substance is always included, usually *p*-aminosalicylic acid.

The following notation is used for the assessment of smears:

0.5 Less than five acid-fast bacilli in most fields.
1.0 10–20 acid-fast bacilli in most fields.
1.5 Few acid-fast bacilli in most fields.
2.0 Moderate number of acid-fast bacilli in all fields.
2.5 Fairly numerous acid-fast bacilli in all fields.
3.0 Numerous acid-fast bacilli in all fields.

TABLE I

ASSESSMENT BY TWO OBSERVERS OF FOUR SERIES OF SMEARS PREPARED FROM FALLING TWOFOLD DILUTIONS, IN CHORIO-ALLANTOIC SUSPENSIONS, FROM AN ORIGINAL SUSPENSION WITH AN ASSESSMENT OF 2.5

	1/1	1/2	1/4	1/8	1/16	1/32
Observer A	2·0 2·0 2·5 2·5	1·5 2·0 2·0 2·0	1·0 1·5 1·5 1·5	0·5 1·0 1·0 1·0	0·5–0 0·5 0·5 0·5	0·5–0 0·5–0 0·5–0 0·5
Average	2·25	1·88	1·38	0·88	0·44	0·31
Observer B	2·5 2·5 2·5 2·5	2·0 2·0 2·0 2·0	1·5 2·0 2·0 2·0	1·0 1·5 1·5 1·5	0·5 1·0 1·0 1·0	0·5 0·5 0·5 1·0
Average	2·5	2·0	1·88	1·38	0·88	0·63
Average of averages	2·38	1·94	1·63	1·13	0·66	0·47

It is shown below that differences of "0.5" in the assessment correspond to approximate twofold differences in the number of organisms.

Various trials were performed (with strain L.48) to test the accuracy of the method of assessment. Two observers made the same reading on 266 of 366 smears, and the readings differed by plus or minus 1.0 on only four occasions. They had, however, previously compared readings, and about the same error was made by one person on re-reading smears or when duplicate smears were made from a series of suspensions. The error was not increased if suspensions were diluted with an equal volume of saline before the smears were made. This was because a standard background was chosen for assessing the number of organisms. When, however, falling twofold dilutions of a suspension having an initial reading of 2.5 were prepared in a suspension of normal chorio-allantoic membrane, decreasing scores were obtained. All smears were assessed by two observers and the readings are shown in Table I. It will be seen from this table that one observer was reading higher than the other, but otherwise there was good agreement, and the average results show that our notations of 2.5, 2.0, etc., correspond approximately to twofold differences in the number of organisms. Smears from eggs kept in the refrigerator gave readings of about 0.5, and so a reading of 2.5 indicates that there had been a thirtyfold multiplication of organisms.

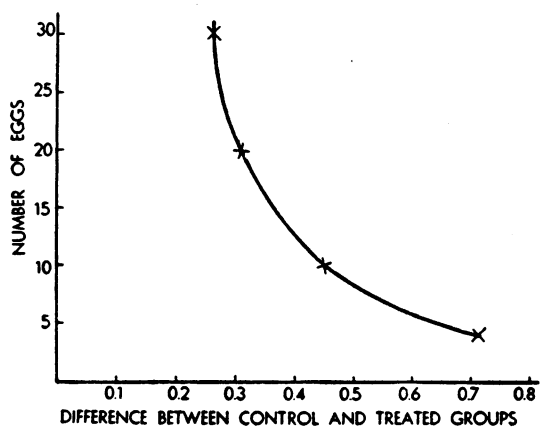


FIG. 1.—Difference between scores of control and treated groups of various sizes required for significance.

From an examination of the results of a number of tests our colleague, Dr. O. L. Davies, was able to supply the information for us to construct the curve shown in Fig. 1, and this is now used to decide whether any result is significant. It will be seen that, with a difference of 0.7 in the average readings between the control and treated groups, only five eggs are required for significance, and, with an average difference of 0.3, 21 eggs. A compound is not, however, reported as "active" unless it produces a significant effect in two, or usually three, tests.

Originally the number of organisms on the chorio-allantois of dead as well as live embryos were estimated, and as a rule the results corresponded with those in the live embryos. Presumably this is because most drugs with only an "antiseptic" action cannot diffuse out of the yolk-sac even after death of the embryo. However, a number of compounds have been observed to give spurious positive results in dead embryos only, and "effects" in dead embryos are now disregarded.

Choice of Test Organism.—Routine tests were carried out for some time using the strain L.48. However, comparative tests with a number of strains of mycobacteria showed that the virulent avian strain S.H.I. was rather more sensitive in *in vitro* tests towards compounds known to show antituberculous activity in animals (Table II). It was then shown that the S.H.I. strain was slightly but consistently more sensitive to the thiosemicarbazone No. 6057, *p*-aminosalicylic acid, and to streptomycin in the embryo, and it was decided to use this strain in preference to strain L.48. It will also be seen from Table II that the saprophytic mycobacteria were much more resistant than the tubercle bacilli to the thiosemicarbazones and *p*-aminosalicylic acid. This would explain the insusceptibility of these organisms to some of the known antituberculous drugs in the chick embryo.

RESULTS

The action of known antituberculous drugs is shown in Table III. All are active, but it is clear that the embryo test does not place these compounds in the same order as they are placed by tests in animals (cf. the weak action of streptomycin).

Up to the present, about 700 speculative compounds have been examined in the chick embryo of which some 50 have shown a signi-

TABLE II

DILUTIONS OF VARIOUS SUBSTANCES WHICH CAUSE 50% INHIBITION OF DIFFERENT STRAINS OF ACID-FAST BACTERIA ON LOWENSTEIN'S MEDIUM

(All figures × 10^{-6} except those marked by an asterisk, which are × 10^{-3})

	Human Tubercle Bacilli—Decreasing Order of Virulence from Left to Right						Virulent Bovine Tubercle Bacilli A.N.5	Avian Tubercle Bacilli			Saprophytes	
	905	Human "C"	H.37 (Blackley)	H.37 Ra	Saranac	L.48		S.H.I. (Virulent)	Sheard (Virulent)	Atten. A/30	*M. phlei*	*M. butyricum*
4:4'-Diaminodiphenyl sulphone	0·06	0·07	0·5	0·06	0·08	0·01	3·8	0·06	0·5	0·09	0·06	0·05
Streptomycin	1·2	0·7	1·0	1·7	0·58	0·15	4·7	0·1	0·2	0·6*	1·3	0·9
Thiosemicarbazone† No. 8388	3·04	2·2	4·2	1,590	4·7	0·03	8·6	0·6	0·07	4·6*	2·4*	0·25*
Thiosemicarbazone† No. 6057	476	233	824	1,590	662	0·36	426	2·9	0·73	6·0*	1·4*	0·25*
p-Aminosalicylic acid ..	181	127	662	307	209	0·02	1,590	0·01	0·36	0·49	1·0*	0·25*

The figures are the average of 3 tests. The main purpose of the test was to compare the reaction of different strains, so the partial loss of activity of streptomycin during inspissation of Lowenstein's medium was not important.

† See Table III for formulae.

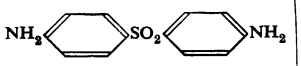
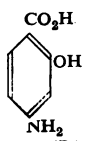
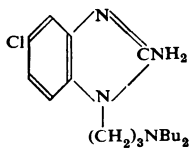
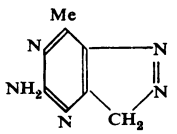
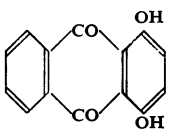
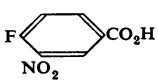
ficant positive effect. A selection of these latter compounds is given in the first part of Table III. The "active" compounds have been examined in the mouse by the method described by Martin (1946), and with the exception of No. 7438 none showed a significant degree of activity in this animal. It is evident that many more compounds are active in the embryo than in the mouse. With some drugs this may be due to rapid excretion in the mouse, or to a higher relative toxicity in this species. Nevertheless it is of interest that the last five compounds recorded in Table III, selected because of their structural relationship with *p*-aminosalicylic acid, are all inactive both in the mouse and chick embryo assays. Further, the use of the embryo test has led to the discovery of "activity" in the mouse in one novel chemical type. This relates in particular to compound No. 7438 (6-amino-4-methyl-2:3:5:7-tetra-azaindene) originally prepared in these laboratories by Dr. F. L. Rose for general investigation as an agent likely to interfere either with the synthesis or metabolism of purines, to which it is structurally related. This substance has been examined in about 100 embryos, and at a dose level of 6 mg. the mean difference of score between treated and control subjects was 0.39, which is highly significant. As a result, extensive investigations with compound No. 7438 have been carried out in several species of experimental animals, and the results will form the basis of later and more detailed communications. For the present purpose, it is sufficient to report that the mean survival time of mice infected intravenously with 0.75 mg. of *Myco. tuberculosis* (strain 905), and each given 10 mg. of the drug in the food daily, was extended by 6.3 days beyond that of a similarly infected but untreated control group. An increase in survival time of 2.8 days (at $p=0.05$) was required for significance in this particular test.

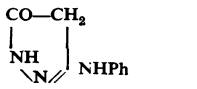
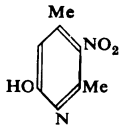
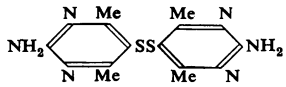
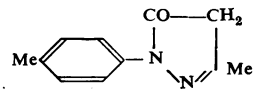
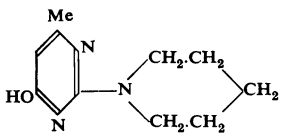
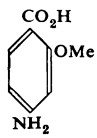
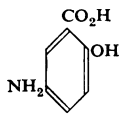
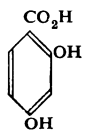
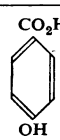
DISCUSSION

The test described in this paper detects the "activity" of the known antituberculous drugs and has led to the recognition of one new type of compound active against tuberculosis in animals. The test does not place these compounds in the same order of activity as they are placed by tests in animals. This is not surprising, since it has been shown (Hoggarth and Martin, 1950) that even in mice relative activity is frequently dependent upon the precise conditions under which the test is conducted. Generally, only one dose of compound has to be given and an answer is obtained after five days. These are important advantages over any test in animals, as the number of compounds that can be examined depends on the time, and amount of compound required, for each test. The test has the disadvantage that many compounds active in the chick embryo are inactive in the mouse, but it is still a better method of screening compounds than *in vitro* tests. This is probably because, unlike the latter, it eliminates compounds which do not pass through the living membranes without killing the embryo. The importance of giving the infective agent by one route and the drug by another is illustrated by the results of Francis (unpublished), who showed that when acriflavine, proflavine, or Nitroakridine (Hurst, Peters, and Melvin, 1950; Rasmussen and Stokes, 1951) were injected on to the chorio-allantois or even into the allantoic cavity they significantly reduced the number of pocks produced by the fowl-pox virus on the chorio-allantois, but they were quite inactive when inoculated into the yolk-sac. Lee and Stavitsky (1947) injected embryos

TABLE III

THE ACTIVITY OF VARIOUS COMPOUNDS AGAINST A TUBERCULOUS INFECTION IN THE CHICK EMBRYO

No.	Compound	Dose (mg. per Embryo)	No. of Embryos	Mean Score†
371		0·11 0·33 1·0	13 20 16	0·77* 0·8* 0·45*
8005	 (PAS)	3 9	9 14	0·72* 0·76*
—	Streptomycin	1 3 6	22 25 18	0·06 0·39* 0·63*
6057		1	281	0·76*
6082		1 3	29 29	0·31* 0·23
8388		1	4	0·7*
	Sodium tauroglycocholate	9 18	8 17	0·82* 0·65*
	Sodium deoxycholate	9 18	13 8	0·59* 1·15*
5330		1 3	13 19	0·26 0·32*
7438		6	108	0·39*
7697		9	21	0·36*
8092		3 9	5 4	0·3 1·03*

No.	Compound	Dose (mg. per Embryo)	No. of Embryos	Mean Score†
9102		18 18	28 25	0·29* 0·63*
9115		18	28	0·58*
9313		9 18	12 2	0·83* −0·6
9432		18	16	0·38*
9696		18	17	0·51*
1871		18	6	0·26
8009		18	9	−0·08
8290		9	7	−0·42
8736		18 27	3 5	0·59 0·07
8738		18	9	−0·27

† By "mean score" is meant the difference between the mean scores of treated and control groups, and an asterisk indicates that the difference is significant.

intravenously and gave drugs into the yolk-sac or on to the chorio-allantois, so their technique satisfied this criticism although it is considered to be unsuitable for the routine screening of compounds. Emmart (1946) reported experiments which purported to show the *in vivo* activity of compounds in the chick embryo. The dose of compound that killed 50% of embryos was mixed with 0.2 ml. of a suspension of tubercle bacilli, incubated overnight, and then inoculated on to the chorio-allantois. Highly significant reductions were obtained in the number of pocks which developed, but the technique does not provide a true test of activity *in vivo*.

The proportion of compounds showing a positive effect in routine testing (i.e., 50 in 700) is of the same order as that found in similar tests with other infecting organisms. Thus we have found that, with *Salm. dublin* as infecting agent in embryos, 36 out of a series of 650 compounds showed a significant positive effect, with *Corynebacterium pyogenes* 59 out of 919, and with the causal organism of fowl-pox 8 out of 800. In these tests, organisms were injected on to the chorio-allantois and drugs into the yolk-sac. With rickettsiae both drug and organism are often injected into the yolk-sac (Francis, 1952), but, as already stated, this is considered to reduce the significance of the test. It is considered that the test using tubercle bacilli has the same general significance as tests against other organisms commonly performed in the chick embryo, and constitutes a useful step in the search for antituberculous drugs.

SUMMARY

1. A technique is described for testing the antituberculous activity of speculative compounds in which the chorio-allantoic membrane of the chick embryo is infected with an avian strain of the tubercle bacillus. A standard dose of a compound (reduced in subsequent tests, if toxic) is injected into the yolk-sac and the effect is measured by examination of smears made from the ground membrane after five days' incubation.

2. The test detects the activity of the standard antituberculous drugs, but gives positive results with more compounds than can be shown to have " activity " in animals. This is true of tests against other organisms in the chick embryo, and the test against tubercle bacilli is considered to have the same general validity as routine screening tests commonly used against other organisms in the chick embryo and to constitute a useful step in the search for antituberculous drugs. It has led to the discovery of the antituberculous activity of a new type of compound (6-amino-4-methyl-2:3:5:7-tetra-azaindene).

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