15572

A Method for Routine Determination of Streptomycin Levels in Body Fluids.*

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The rational employment of antibiotics for the successful treatment of infectious diseases involves the establishment of curative dosage schedules. Optimum dosage schedules are usually predicated on a correlation between the *in vitro* sensitivity of the infecting organism and maintenance of requisite blood levels as estimated by assays of blood and urine.

Exploratory clinical study of streptomycin thus far has indicated its effectiveness in the treatment of some diseases due to organisms not inhibited by penicillin. There is therefore need for a simple, reliable quantitative method of assaying streptomycin in body fluids for purposes of dosage standardization.

Most streptomycin assay methods hitherto described[1,2] employ as test organisms *Escherichia coli, Bacillus subtilis* or *Staphylococcus aureus* which are too insensitive for microdeterminations.

Donovick *et al.*[3] employing *Klebsiella pneumoniae No. 41* as test organism in a screened 1% tryptone broth have demonstrated inhibition by as little as 0.05 unit of streptomycin per ml of test broth. The technic described by these authors, however, seems to be too laborious to be applicable as a routine clinical procedure.

Our method for determining penicillin levels in body fluids[4] has proved consistent and reliable in more than 4000 assays. The technic was modified and adapted for similar estimations of streptomycin, utilizing as standard a streptomycin sensitive organism grown in appropriate media. *Klebsiella pneumoniae No. 41,*† which grows luxuriantly in simple media and is readily inhibited by streptomycin, seemed ideally suited as a test organism.

Klebsiella pneumoniae No. 41 was inoculated in various media having a wide range of growth-promoting properties in order to discover suitable conditions for culture. The basic concept of the method calls for the most simple, least-enriched medium which will insure adequate growth. Media for levels determination although relatively deficient, must compare favorably with routine nutrient media in its growth-promoting properties. Conditioned in this manner the test organisms are made more susceptible to the action of streptomycin thereby rendering the assay more sensitive. Furthermore a sensitive level test makes possible higher dilutions of body fluids thus eliminating or reducing (1) the growth stimulus of such fluids and (2) any serological interference due to contained native antibodies.

Growth of Klebsiella pneumoniae No. 41 in Different Media at Various Concentrations. All media investigated were first prepared in distilled water in 1% concentration and adjusted to pH 7.2, filtered hot and autoclaved at 15 pounds for 20 minutes.

Serial, 2-fold dilutions of the 1% media were prepared with sterile water (2 cc in each of 12 test tubes representing a concentration range of 1% to 0.0005%). The 12 tubes were each seeded with 0.1 cc of a 4-hour culture of *Klebsiella pneumoniae No. 41* grown in brain heart infusion broth and diluted to 10^{-4} with the medium employed in the test. The stock culture of *Klebsiella pneumoniae No. 41* was maintained

* Aided by a grant from the John L. Smith Fund for Medical Research of the Jewish Hospital of Brooklyn.

1 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

2 Waksman, S. A., and Reilly, H. J., *J. Infect. Dis.*, 1944, **75**, 150.

3 Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

4 Rosenblatt, P., Alture-Werber, E., Kashdan, F., and Loewe, L., *J. Bact.*, 1944, **48**, 599.

† We are indebted to Dr. G. Rake of the Squibb Institute for Medical Research for a culture of this organism.

TABLE I.
Growth of *Klebsiella pneumoniae No. 41* in Different Media at Various Concentrations.

	Media						
	Amigen	Yeast	Neopeptone	Bactotryptone	Savita	Bactopeptone	Beef extract
Growth	%	%	%	%	%	%	%
Absent (neg.)	.004	.004	.008	.008	.004	.016	.063
Retarded (1 +)		.008	.016	.016	.008, .016	.031	
Dense (4 +)	.008	.016	.031	.031	.031	.063	.125

on blood agar plates which were stored at 5°C. All tubes were incubated 18 hours and then tabulated turbidometrically for presence and density of growth. The gradations were designated as negative, 1+ or 4+, indicating respectively absent, retarded or dense growth.

It may be noted (Table I) that *Klebsiella pneumoniae No. 41* can be grown luxuriantly in any of the following relatively deficient media, namely, 0.008% amigen, 0.01% yeast extract, 0.031% neopeptone, 0.031% bactotryptone, 0.031% savita, 0.063% bactopeptone or 0.125% beef extract.

Streptomycin Sensitivity of Klebsiella pneumoniae No. 41 in Different Media at Various Concentrations. Serial 2-fold dilutions of streptomycin‡ were set up in 1%, 0.5%, 0.25% and 0.125% concentrations of savita, bactotryptone and neopeptone, each concentration in 12 tubes covering a streptomycin range of one unit to 0.0005 unit per ml with a 13th tube as control. Each tube was seeded with 0.1 cc of a 10^{-4} dilution of a 4-hour culture of *Klebsiella pneumoniae No. 41* grown in brain heart infusion broth. All tubes were incubated for 18 hours and then read for inhibition or growth.

The results (Table II) clearly demonstrate that media of deficient nutritional value render *Klebsiella pneumoniae No. 41* more sensitive to the *in vitro* action of streptomycin. The metabolic changes so induced conceivably make the organisms more susceptible to streptomycin. Studies of the extent of metabolic interference with the bacterial cell caused by continuous propagation in deficient media are under investigation.

‡ We wish to thank Mr. John L. Smith of the Charles Pfizer Company, who supplied the streptomycin used in this study.

TABLE II.
Streptomycin Sensitivity of *Klebsiella pneumoniae No. 41* in Different Media at Various Concentrations.

		Reaction to streptomycin—units per ml	
Media	%	Inhibition	Growth
Neopeptone	1.	.25	.125
	.5	.125	.063
	.25	.0625	.032
	.125	.016	.008
Bactotryptone	1.	.25	.125
	.5	.0625	.032
	.25	.016	.008
	.125	.004	.002
Savita	.5	1.0	.5
	.25	.5	.25
	.125	.125	.063

Method of Determining Streptomycin in Body Fluids. The media used in our current standard levels determination are 0.5% and 0.25% neopeptone which meet the requirement of being simple and relatively deficient though still capable of sustaining adequate growth of the test organism.

Sufficient clear serum for the assay can be obtained from 10 ml of blood. Sera under investigation are stored at 5°C until used at which time they are diluted 1:10, 1:20, 1:40 or 1:100 in the test medium. If levels between 0.16 unit per ml and 1.6 units per ml or higher are expected 0.5% neopeptone is employed. If a more sensitive endpoint is desirable the assays are performed with 0.25% neopeptone.

Aliquots of urine specimens are Seitz filtered as soon after voiding as possible and

TABLE III.
Diagram of Test.

	Undiluted or diluted serum									
Amt of serum	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
" " broth	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0

To each tube add 1 ml neopeptone broth seeded with *Klebsiella pneumoniae No. 41* (4-hour culture diluted 10^{-4}).

	Standard: 0.8 units/ml									
Amt of standard	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
" " broth	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0

To each tube add 1 ml neopeptone broth seeded with *Klebsiella pneumoniae No. 41* (4-hour culture diluted 10^{-4}).

stored at 5°C until processed. For assay they are diluted 1:10, 1:100, 1:1000 or, occasionally, 1:10,000 in test broth. The dilution factor for urine is predicated on the anticipated concentration in the urine, the urinary excretory curve being directly related to the time of administration and dose of streptomycin.

Sera are pipetted into 100 mm × 13.5 mm tubes in 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 cc amounts.

A parallel series of tubes containing a standard solution of streptomycin is also prepared. From a stock standard solution stored at 5°C secondary standards containing 0.8 unit per ml are set up in test media, serum and urine. These standards are dispersed into test tubes as follows:

0.1 ml to 1 ml as in the diagram of the test (Table III). To all tubes sufficient neopeptone broth is added to total 1 ml. Two control tubes each containing 1 ml of broth are added to the test. To each of the tubes 1 ml of neopeptone broth seeded with *Klebsiella pneumoniae No. 41* is added, making a total of 2 ml in each tube. The seeded broth is prepared as usual by diluting a 4-hour culture 10^{-4}.

All the tubes are incubated at 37°C overnight (18 hours). Slight growth is disregarded. The readings are very clear and definite as between significant growth and inhibition.

Under the conditions of the test it requires 0.16 unit of streptomycin in 0.5% neopeptone and 0.032 units in 0.25% neopeptone to inhibit *Klebsiella pneumoniae No. 41* per 2 ml. This means that the test organism has a streptomycin sensitivity of 0.08 unit per ml in 0.5% neopeptone and 0.016 unit in 0.25% neopeptone.

In the calculation streptomycin levels are converted into units per ml of body fluid. For example, if 0.5 ml serum is required to inhibit the growth of the test strain and if 0.4 ml of serum permits growth and the test was performed in 0.5% neopeptone, the serum therefore contains 0.32 unit streptomycin per ml.

Calculation:

$$\frac{\text{Streptomycin inhibiting test organism}}{\text{Amount serum inhibiting test}}$$

(a) in 0.5% neopeptone $\frac{0.16}{0.5} = 0.32$ unit streptomycin/ml.

(b) in 0.25% neopeptone $\frac{0.032}{0.5} = 0.064$ unit streptomycin/ml.

Should the serum have been diluted before processing the test, the result must necessarily be multiplied by the dilution factor.

Summary. 1. A simple, easily readable method for the clinical determination of streptomycin in body fluids has been described requiring a minimum amount of test fluid.

2. *Klebsiella pneumoniae No. 41* which grows luxuriantly in simple media and is readily inhibited by streptomycin was adopted as test organism.

3. The assay was made more sensitive by utilizing relatively deficient media which made the test organisms more susceptible to the action of streptomycin.

4. It is possible, because *Klebsiella pneumoniae No. 41* is insensitive to penicillin, to perform streptomycin level determinations in patients who are receiving streptomycin and

penicillin treatment concomitantly.

5. By the use of this method it has been noted that streptomycin is retained in the blood in effective therapeutic amounts for relatively longer periods of time, thereby permitting more convenient spacing of injections. The clinical application of these studies will be made the subject of a subsequent report.

15573 P

Cocarboxylase and the Synthesis of Acetylcholine.

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Biochemical studies of nerves under electrical stimulation or at rest have shown that acetylcholine, regarded for a long time only as a transmitter of nervous impulses at synaptic levels, is present in appreciable amounts in the nerve fiber itself.[1] We have proved that extracts from stimulated nerves produce a more intense action on the dorsal muscle of the leech than extracts from nerves at rest. This finding led us to consider the part played by acetylcholine in the phenomenon of nerve conduction. An analysis of the observed effects enabled us to reach the conclusion that excitation is followed not only by an increase in the amount of acetylcholine in nerves but also by the formation of an unknown substance appearing in the system and capable of sensitizing the reactive muscle. Further work has made clear analogies of action between this unknown substance and thiamin and has enabled us to prove that stimulated nerve liberates an amount of thiamin many times greater than that given off by the nerve at rest.[1] Since the amount of thiamin which sensitizes the muscle of the leech to acetylcholine is much smaller than that which would inactivate cholinesterase, we concluded that the vitamin plays a part in the synthesis of the chemical transmitter.

Thiamin is active in organisms in the form of thiamin pyrophosphate or cocarboxylase. Therefore, it seemed desirable to determine whether this coenzyme may play a part in the synthesis of acetylcholine from choline.

A contracture of the dorsal muscle of the leech may be obtained with relatively high doses of choline. But contrary to the reaction produced by acetylcholine, this effect is not increased by eserine.[2,3] If cocarboxylase is capable of transforming choline into acetylcholine, the mixture of both should exert an action on the muscle which is increased by eserine. Experiments have shown that cocarboxylase alone is without any effect on the leech muscle preparation whether treated with eserine or not. The coenzyme, however, increases the action on the muscle of a contracture-producing concentration of choline in the absence of eserine. This increase is even greater after the muscle has been treated with eserine. With threshold doses of choline, at which cocarboxylase has no visible effect, eserine has brought about a clear reinforcement. Thus, the muscle may aerobically and in the presence of cocarboxylase transform choline into an ester the action of which is increased by eserine. These are the biological properties of acetylcholine.

How may these facts be interpreted? We know that cocarboxylase intervenes at a particularly important level in the metabolism of carbohydrates. It catalyzes the decarboxylation of pyruvic acid so that acetic acid is produced. From the work of Banga, Ochoa and Peters[4,5] it also appears that this de-

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[3] Minz, B., *Arch. f. exp. Path.*, 1932, **168**, 292.

[4] Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, 1939, **33**, 1980.

[5] Ochoa, S., *J. Biol. Chem.*, 1941, **138**, 751.