centrifuged without much delay for 30 minutes at medium speed. The sediment is spread evenly on a number of slides which have been thinly coated with Mayer's albumen. The smears thus prepared are allowed to stand only for a short time until they begin to show dryness around the edge and then they are immersed in a solution of equal parts of 95% alcohol and ether.

After a fixation of at least 15 minutes the slides are carried through 80%, 70%, and 50% alcohols to distilled water.

The staining is as follows:

Harris Hematoxylin (which has been prepared without acetic acid, and diluted with an equal amount of distilled water) for 6 minutes.

Rinse in distilled water.

Dip in 0.5% HCl (aqueous solution) 6 times.

Place in running tap water for 6 minutes.

Rinse in distilled water, 50%, 70%, 80%, and 95% alcohols.

Stain in OG6 for 1½ minutes.

Rinse in 95% alcohol, 2 changes.

Stain in EA65† (procedure No. 267) for 1½ minutes or in EA36[7] (procedure No. 268).

Rinse in 95% alcohol (3 changes); absolute alcohol and xylol, equal amounts; xylol; mount with a coverslip.

Be sure that smears are never allowed to dry at any time.

Summary. Pregnancy causes distinct morphologic changes in the epithelium of the urinary tract which are reflected in the cytology of the "urine sediment smear." There is an appearance of certain characteristic cell forms such as cells of the "navicular" type corresponding to those described in the vaginal smear of pregnancy. The urine sediment smear provides, thus, a simple, rapid and dependable method for diagnosing pregnancy. It appears to be superior to the vaginal smear in that its cytology shows greater uniformity and is more distinctive. Its practical value as a routine diagnostic procedure and its applicability in the early diagnosis of pregnancy are still to be determined.

† EA65 is the same as EA36 with the difference that the Light Green is one-half strength (0.25% instead of 0.5%). It has the advantage of giving a lighter and more transparent staining which is very desirable in cancer diagnosis. The differentiation between acidophilic and basophilic cells is better with the EA36 (or EA50). This differentiation is more important in vaginal, endocervical, and endometrial smears. Therefore, EA36 (or EA50) is preferable for vaginal, endocervical, and endometrial smears, whereas EA65 is better adapted to other types of smears. Either one of these two stains can be used for all types of smears.

7 Papanicolaou, G. N., *Science*, 1942, **95**, 438.

16268

Streptomycin as an Essential Nutrilite.

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In consideration of the mode of action of streptomycin one of the possibilities explored has been that of its functioning as a metabolite antagonist. Little evidence of this has been forthcoming, however, and it was therefore with interest that one read the report of Miller and Bohnhoff[1] on their so-called B variant meningococcus. This variant (mutant) resembled the A variant (mutant) in that both were resistant to, or developed in high concentrations of, streptomycin. The B mutant differed from the A in that it required streptomycin for multiplication either *in vitro*, or *in vivo* in the mouse, while the A mutant did not. The B mutant never

1 Miller, C. P., and Bohnhoff, M., *Science*, 1947, **105**, 620.

emerged in the presence of streptomycin concentrations less than 40 γ/ml, but once secured, its growth was promoted by 5γ/ml but not less. Rare back-mutation to non-streptomycin-requiring forms occurred. A recent paper by Kushnick, Randles, Gray and Birkeland deals with variants (mutants) of *E. coli, P. aeruginosa* and *B. subtilis* requiring streptomycin.[2] All mutants showed this growth stimulation when glucose was present in the medium. In the case of *P. aeruginosa* sodium formate could replace the glucose. The case of *B. subtilis* was unusual in that the streptomycin-requiring mutants grew only under anaerobic conditions. Many papers in the literature[3,4] demonstrate that the antibacterial action of streptomycin is greatly reduced by anaerobic conditions.

It has been possible to confirm the findings of Miller and Bohnhoff as regards streptomycin-requiring mutants of meningococci. These authors obtained such variants from 16 of 18 strains tested. They do not mention type distribution. In our case the mutants developed with a Type II (No. 763) and a Type II-*a* strain (No. 1054) but not, in limited tests, with a Type I strain (No. 1168).* The medium we have used is a beef heart, glucose agar containing 4% fresh citrated rabbit blood[5] on which the meningococcus grows readily. On such medium 2 colony forms have appeared similar to those described by Miller and Bohnhoff, but such difference in colony morphology has not been associated in our hands with as sharp differentiation as they found into colonies resistant to streptomycin but not requiring it as contrasted to colonies both resistant to and requiring the antibiotic. Furthermore, our mutant strains showed much less stability than that indicated by Miller and Bohnhoff

TABLE I. Serial Transfers of Mutant Strains of *E. coli*.

Strains D56				Strain D57			
Transfer No.	Units/ml* streptomycin	Back-mutants†	Colony type	Transfer No.	Units/ml* streptomycin	Back-mutants†	Colony type
1	5	Frequent	Undifferentiated	1	20	Frequent	Undifferentiated
2	20	,,	,,	2	80	,,	,,
3	80	,,	Small	3	640	,,	,,
4	80	None	,,	4	2560	None	,,
5	320	Frequent	Undifferentiated	5	5120	Very few	Small
6	80	Very few	Small	6	1280	Frequent	Undifferentiated
7	40	Frequent	,,	7	320	,,	,,
8	40	Very few	,,	8	160	,,	,,
9	80	,, ,,	,,	9	40	,,	,,
10	20	Frequent	Undifferentiated	10	40	None	Small
11	160	None	Small	11	20	,,	,,
12	160	,,	Undifferentiated	12	20	,,	,,
13	40	,,	,,	13	10	,,	Large
14	10	,,	,,	14	40	Very few	Undifferentiated
15	10	,,	,,	15	10	,, ,,	,,

* Concentration of streptomycin in units per ml on which colony grew and from which transfer was made.
† Frequency of colonies on control streptomycin-free medium; *i.e.*, reversion to parent type no longer requiring streptomycin as essential metabolite.

2 Kushnick, T., Randles, C. I., Gray, C. T., and Birkeland, J. M., *Science*, 1947, **106**, 587.

3 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

4 Bondi, A., Deitz, C. C., and Spaulding, E. H., *Science*, 1946, **103**, 399.

* My thanks are due to Dr. Sara E. Branham of the National Institute of Health, Bethesda, Md., for these strains.

5 Rake, G., *J. Exp. Med.*, 1934, **59**, 553.

TABLE II.
Optimal Concentrations of Streptomycin for *E. coli* Mutants.

Strain 56, 6th passage		Strain 57, 13th passage	
Streptomycin pool III Concentration/ml	Growth on plate	Streptomycin Concentration/ml	Growth on plate
20 u	+	5 u	0
40 u	+++	10 u	3 colonies
80 u	+++	20 u	+
160 u	++	40 u	4 colonies
320 u	0	80 u	0

and apparent back-mutation was too frequent to allow these strains to be used readily as a tool for further study. It may be that such unsatisfactory behavior is due, in part at least, to the fact that colonies for transfer were, after the first few transfers, selected from plates containing high concentrations of streptomycin (800 units/ml or greater).

At the same time that the work with meningococci was started we began investigation of 4 strains of *E. coli* of our own isolation. In limited tests one strain (H36) did not produce streptomycin-requiring mutants on beef heart agar[5] (without rabbit blood), or on a fluid medium of the same beef heart infusion without the agar. The other 3 did. In the case of one strain, D11, there was still a marked tendency to back-mutation although it was less than in the case of the meningococcus mutants. This strain was maintained mostly by transfer from colonies growing at the higher concentrations of streptomycin (3,200 units/ml or greater). Both large and small colony types occurred but again there was no correlation of any such morphological appearance with a demand or no for streptomycin as an essential metabolite.

In the case of the two other strains of *E. coli*, D56 and D57, the later transfers in particular were made from colonies growing on agar with lower concentrations of streptomycin. As can be seen in Table I back-mutation became less frequent and the correlation of small colony with necessity for streptomycin was fairly good. The strains tended towards stability.

It is thus apparent that selection of the streptomycin-requiring mutants in pure form occurs more readily by colony picking from plates of lower, rather than of higher, concentrations of streptomycin. The exact reason for this is not clear but probably these mutants, though relatively resistant to streptomycin, are not as resistant as the mutants which, while growing in concentrations of streptomycin as high as 100,000 units/ml[6] and possibly metabolizing the antibiotic, do do not find it essential for their multiplication. Single cell isolation would be necessary for certainty on this point; its probability is borne out by the fact that the streptomycin-requiring mutants, when plated out on increasing concentrations of commercial streptomycin, or streptomycin A, showed an optimum which for the *E. coli* strains was around 20 to 80 units/ml (Table II). However, resistant forms could readily be selected which grew in much higher concentrations of streptomycin (5,000 units/ml or greater). These, which grow freely in absence of streptomycin, show limitation of growth at higher, but not at lower, concentrations of the antibiotic.

All the above work has been carried out on complex media. It was decided to attempt to adapt the mutants to a synthetic medium. After trial of several, that described by Cohen[7] was finally adopted. For our purposes the pH was adjusted to 7.3 with NaOH before sterilization and a final pH of 7.0 was thus achieved. The medium was used mostly as a broth but also as an agar. Streptomycin-requiring mutants were obtained from both D56 and D57 strains of *E. coli* on such media. For most of the subsequent work D57 mutant ($D57_M$), carried in the fluid medium, has been used. As long as the broth cultures are not held under conditions allowing multipli-

[6] Lapedes, D., Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 269.

[7] Cohen, S. S., *J. Biol. Chem.*, 1947, **168**, 511.

TABLE III.
Substances* Active as Essential Metabolites for *E. coli* Mutant D 57_M.

Active	Inactive
Streptomycin A	D-glucosamine HCl
Streptomycin B	Streptodine sulfate[11]
Dihydrostreptomycin A	Pneumococcus polysaccharides‖
N-Acetyl streptomycin A †,[9]	*K. pneumoniae* polysaccharides¶
Streptobiosamine HCl ‡,[10]	Streptamine[11] dihydrochloride
Lipositol§	L-(+)-Arabinose
	N methyl-L-glucosaminic acid[12]

* My thanks are due to Dr. Oskar Wintersteiner and Dr. Josef Fried of the Division of Organic Chemistry, The Squibb Institute for Medical Research, for all the streptomycin and derivative samples; to Dr. H. E. Carter, Department of Chemistry, University of Illinois, and Dr. D. W. Woolley, The Rockefeller Institute for Medical Research, for samples of lipositol; to Dr. J. W. Palmer, Biological Laboratories, E. R. Squibb and Sons, for the polysaccharides of the pneumococcus types and *K. pneumoniae* type B; and to Dr. M. Heidelberger, Department of Medicine, Columbia University, for the polysaccharides of *K. pneumoniae* types A and C.

† Contains 15% streptomycin A.

‡ Activity probably due to residual streptomycin A (see text).

§ Some preparations showed slight activity (see text).

‖ Types 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 14, 18, 19.

¶ Types A, B, and C.

cation to occur for longer than 72 hours back-mutation has been rare and growth has rarely appeared in the control (streptomycin-free) medium within this time. When cultures are held for longer periods, mutants not requiring the antibiotic gradually emerge. For all of the routine passage and selection of mutants, in this work with the synthetic medium, a streptomycin pool designated III has been used. This has the following characteristics: streptomycin A 65%, streptomycin B 30%, other maltol producing material 5%, total streptomycin 64%, inert material 33%, minimal inhibiting concentration (M.I.C.) for *K. pneumoniae* (in the standard biological assay)[8] 0.103 γ/ml, M. I. C. inhibiting *E. coli* (D57 parent strain) 0.155 γ, *K. pneumoniae*/*E. coli* ratio 0.67, streptomycin potency 354 units/mg.

Using this strain $D57_M$ the capacity of many different substances to replace the streptomycin pool III in the medium as essential metabolite has been investigated. The results are shown, in summary, in Table III.

One notes that streptidine and streptamine are inactive. Also inactive are the polysaccharide fractions of pneumococci and *K. pneumoniae*. The latter inactivity is particularly to be noted. We also received and tested 3 samples of polysaccharides of *M. tuberculosis* strain H37 kindly supplied by Dr. M. Heidelberger of the Department of Medicine, Columbia University. These represent the high rotating (fraction C), low rotating (fraction B_{2a}) and serologically inert (fraction B_2C) fractions described elsewhere.[13] All were negative.

As far as the active substances are concerned it is to be noted that lipositol showed very little activity and that such activity as was obtained was not found with every sample. Thus a sample from soybean provided by Dr. D. W. Woolley (see footnote to Table III) was slightly active at 20 γ and 40 γ per ml but not in lower or higher concentration. A soybean lipositol and a brain lipositol labeled

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

[9] Fried, J., and Stavely, H. E., to be published.

[10] Kuehl, F. A., Jr., Flynn, E. H., Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 2096; Fried, J., Walz, D. E., and Wintersteiner, O., *J. Am. Chem. Soc.*, 1946, **68**, 2746.

[11] Carter, H. E., *et al.*, *Science*, 1946, **103**, 53; Fried, J., Boyack, J. A., and Wintersteiner, O., *J. Biol. Chem.*, 1946, **162**, 391; Peck, R. L., *et al.*, *J. Am. Chem. Soc.*, 1946, **68**, 29, 776.

[12] Kuehl, F. A., Jr., Flynn, E. H., Holly, F. W., Mozingo, R., and Folkers, K., *J. Am. Chem. Soc.*, 1946, **68**, 536; Wolfrom, M. L., Thompson, A., and Hooper, R. I., *J. Am. Chem. Soc.*, 1946, **68**, 2343.

[13] Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

TABLE IV.
Relation of Stimulatory and Antibacterial Activity of Different Active Substances.

	Stimulatory activity on $D57_M$ *E. coli*	Antibacterial activity on D57 *E. coli*
Streptomycin pool III	1.0	1.0
Streptomycin A	1.4	1.9
Dihydrostreptomycin A	1.4	2.1
Streptobiosamine HCl	0.06	0.07
N-Acetyl streptomycin A	0.18	
Streptomycin B	0.7	0.54

"water extract," containing 3.9% inositol, from Dr. H. E. Carter (see footnote to Table III) were inactive, but a brain lipositol labeled "ether extract," containing 6.3% inositol, from Dr. H. E. Carter was slightly active at 20 γ to 160 γ but not in lower concentration.

The relative growth promoting capacities on mutant $D57_M$ of the substances labeled active in Table III are shown in Table IV. These activities have been referred to that of the streptomycin pool III as unity and are to be regarded as only approximate since the dilutions tested varied from each other by 2-fold. Also shown are the antibacterial activities of these same substances on the parent strain D57 again referred to the activity of pool III as unity. These values are accurate to within $\pm 5\%$.

It will be noted that the relative values of the growth stimulatory activity agree remarkably well with the antibacterial values of the same substances on the parent strain. The activity of the N-acetyl streptomycin A corresponds to the 15% of streptomycin A which this sample was known to contain. In the case of the sample of streptobiosamine HCl it is not certain whether the activities found are due to a residuum (3.5%) of streptomycin A or whether the compound itself has activity. This is being investigated further. It is of interest that the relative activities of streptomycin B show such good agreement. This would suggest that the same factors may act to decrease the stimulatory activity of streptomycin B relative to A as act to decrease its antibacterial activity on the parent culture. One can perhaps visualize the extra mannose moiety as interfering with the activities of the rest of the molecule.

Summary. Streptomycin-requiring mutants were obtained from 2 of 3 strains of meningococci and 3 of 4 strains of *E. coli.* In the case of 2 *E. coli* strains such mutants grew readily on a synthetic medium in the presence of streptomycin. Such mutants appear more stable when isolated from low rather than from high streptomycin concentrations. In the case of the *E. coli* mutants, they exhibit optimal growth at concentrations from 20 to 80 units/ml of a commercial streptomycin or of pure streptomycin A. Growth of the mutants can be promoted by pure preparations of streptomycin A, dihydrostreptomycin A, and streptomycin B and the relative stimulatory activities of these compounds for the mutant agrees closely with the relative antibacterial activities against the parent strain.

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