3. Paschkis, K. E., Goddard, J., Cantarow, A., Adibi, S., *ibid.*, 1959, v101, 184.

4. Bucher, N. L. R., Scott, J. F., Aub, J. C., *Cancer Res.*, 1951, v11, 457.

5. Islami, A. H., Pack, G. T., Hubbard, J. C., *Surg. Gynecol. Obstet.*, 1959, v108, 549.

6. Friedrich-Freksa, H., Zaki, F. G., *Naturforsch.*, 1954, v96, 394.

7. Annau, E., Manginelli, A., Roth, A., *Cancer Res.*, 1951, v11, 304.

8. Trotter, N. L., *ibid.*, 1961, v21, 778.

9. Rabes, H., Wrba, H., *Klin. Wschr.*, 1961, v39, 206.

10. Zalliacus, H., *Acta Obst. et Gynec. Scand.*, 1959, v38, 737.

11. Omland, G., *Acta Medica Scand.*, 1959, v164, 451.

12. Waldmann, T. A., Levin, E. H., Baldwin, M., *Am. J. Med.*, 1961, v31, 318.

13. McLaughlin, E. D., *Surg.*, 1960, v47, 420.

14. Erslev, A. J., *Blood*, 1955, v10, 954.

15. Borsook, H. A., Graybiel, A., Keighley, C., Windsor, E., *ibid.*, 1954, v9, 734.

16. White, W. F., *Recent Progress in Hormone Research*, 1960, v16, 219.

17. Plzak, L., Fried, W., Jacobson, L. O., Bethard, W. F., *J. Lab. Clin. Med.*, 1955, v46, 671.

18. Hennessey, T. G., Huff, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 436.

19. Novikoff, A. B., *Cancer Res.*, 1957, v17, 1010.

20. Higgins, G. M., Anderson, R. M., *Arch. Path.*, 1931, v12, 186.

21. Snedecor, G. W., *Statistical Methods*, Iowa State Coll. Press, Ames, Iowa, 1956.

22. Jacobson, L. O., Goldwasser, E., *Homeostatic Mechanisms*, No. 10, Brookhaven National Lab., Upton, N. Y., 1957, 110.

Received April 17, 1962. P.S.E.B.M., 1962, v110.

Enzymes of *Neisseria gonorrhoeae* and Other *Neisseria*. (27543)

HENRY TAUBER AND HAROLD RUSSELL

Venereal Disease Research Laboratory, Communicable Disease Center, Public Health Service, Atlanta, Ga.

This study deals with enzyme systems of *Neisseria* thus far not described for these species of bacteria. Tonhazy and Pelczar(1), however, have reported an interesting manometric study concerning oxygen uptake by an isolate of *Neisseria gonorrhoeae* in the presence of citric acid cycle compounds. The present investigation was done to aid in classification and identification of *Neisseria*. The Thunberg technic, using color-forming 2,3,5-triphenyltetrazolium chloride (TPT) as the electron acceptor, has been employed. Included are a lactic dehydrogenase (LDH), a reduced diphosphopyridine nucleotide (DPNH) oxidase system, an alcohol dehydrogenase, an aerobic cysteine oxidase (CyO), an aerobic desulfhydrase (DSH), and an acetate oxidase system. The DPNH oxidase and alcohol dehydrogenase appear to be increased in penicillin resistant *N. gonorrhoeae* isolates.

Materials and methods. The culturing procedure has been described(2). The bacterial cells were washed twice with 4 volumes of sterile 0.85% NaCl and once with sterile distilled water. A portion of washed cells was lyophilized and the remainder was stored frozen at −20°C. For most experiments, bacteria were suspended in distilled water and dispersed in a glass homogenizer. Nitrogen was determined by micro-Kjeldahl procedure. When the formazan method was employed, enzyme activities were expressed as μg of formazan per 100 μg of cell nitrogen. Preparation of a formazan calibration curve has been described(3).

Determination of reduced diphosphopyridine nucleotide oxidase. Fifty mg of wet bacteria were suspended in 10 ml of distilled water and dispersed in a glass homogenizer. In Thunberg tubes were placed 1-ml portions of the material, 1 ml of 0.1 M phosphate buffer of pH 7.5, KCN (1×10^{-2} M) and TPT (2×10^{-3} M). In the side arms of the tubes was placed 0.25 ml of a solution containing DPNH (9×10^{-4} M) and cysteine (4×10^{-3} M) of pH 7.5. Compounds were calculated per final volume of 3 ml. Tubes were

evacuated and placed in a water bath at 37°C. After 10 minutes, the contents of the side arms were added to the tubes. After 30 minutes, the reaction was stopped by immersion of the tubes in an ice bath. Formazan was extracted with 7 ml of cold acetone. The acetone extracts were centrifuged and optical density of the supernatant was measured spectrophotometrically at 520 mμ. Controls were included in all experiments. Freezing and thawing destroys DPNH oxidase.

Determination of lactic dehydrogenase. This enzyme also was measured anaerobically in Thunberg tubes with tetrazolium salt as the indicator. The reaction mixture contained 1 ml (5 or 10 mg, wet) bacterial dispersion, 1 ml of 0.05 M glycine buffer of pH 10.0, and 0.5 ml of lactate solution(4). In the side arms was placed 0.5 ml of a solution containing DPN and TPT in 0.01 M phosphate buffer adjusted to pH 7.5. The concentration of DPN was 9×10^{-4} M and that of TPT was 2×10^{-3} M per final volume of 3 ml. Tubes were evacuated and placed in a water bath at 37°C for 30 minutes. Formazan formation was measured as described for DPNH oxidase.

Reduced triphosphopyridine nucleotide oxidase in Neisseria. The same procedure was employed as for DPNH oxidase. Cysteine and cyanide were required for maximum activity. Here 20 mg of wet bacteria were used per test.

Alcohol dehydrogenase in Neisseria gonorrhoeae. Enzyme activity was determined anaerobically in Thunberg tubes with tetrazolium salt as the electron acceptor. The mixture contained 1 ml (5 mg, wet) bacterial dispersion, 0.5 ml 0.1 M phosphate buffer of pH 7.5, 0.2 ml 14.4 M ethanol and 0.1 ml (2 mg) TPT. In the side arms of the tubes was placed 0.2 ml (0.01 M) DPN in 0.1 M phosphate of pH 7.5. The tubes were evacuated and placed in a water bath at 37°C. After 10 minutes, the content of the side arms was added to the tubes. At the end of 60 minutes, the reaction was arbitrarily graded from 0 to 3.

Cysteine oxidase and cysteine desulfhydrase. For assaying of cysteine oxidase, 80 mg of lyophilized bacteria were dispersed in 10 ml of 0.1 M phosphate buffer of pH 7.5, using a glass homogenizer. In Thunberg tubes were placed 0.5 ml of this suspension, 0.5 ml (8.0 mg) of neutralized cysteine · HCl and distilled water to make a final volume of 6 ml. In side arms were placed filter-paper strips saturated with lead acetate solution (0.1% lead acetate in 0.2% acetic acid). The tubes were placed in a water bath at 37°C, but were not evacuated. After 60 minutes, the mixture was analyzed for disappearance of SH groups as follows: To 0.6-ml aliquots, 1 ml of 1.2% metaphosphoric acid was added and the mixture was centrifuged. Nitrogen was passed through the supernatants in order to remove H_2S. Disappearance of SH groups was determined colorimetrically by the method of Shinohara(5) and by the nitroprusside procedure(6). One of the reaction products, cystine, was identified by reconversion to cysteine by reduction with 5% KCN solution by the usual procedure. Since KCN inhibits the Folin-Marenzi reaction, only the nitroprusside method could be employed in this analysis. H_2S, produced by desulfhydrase, was demonstrated by precipitation of dark brown lead sulfide on the filter-paper strips.

Acetate oxidase system. This enzyme system was demonstrated anaerobically employing the Thunberg technic. The mixture contained 50 mg of wet bacteria in 0.5 ml distilled water, 0.75 ml 1.0 M potassium acetate, and 2 mg TPT in 0.25 ml distilled water. The pH of this mixture was 7.5. Oxidation of acetate did not take place aerobically.

Results and discussion. Only suspensions of wet bacteria were employed. Even brief sonic oscillation destroyed some DPNH oxidase and LDH activity. The LDH requires DPN. Since protons take part in the enzymic reduction of DPN, the subsequent reduction of TPT is greatly dependent upon pH; therefore, LDH was determined at its practical optimum pH of 10.0. The DPNH oxidase requires activation by cysteine. The results, however, were more readily reproduced when both cysteine and KCN were present. Cysteine was not oxidized under these conditions. The optimum pH for this enzyme was 7.5. Linearity was obtained with cell

suspensions producing formazan between optical densities of 0.200 and 1.400. Under anaerobic conditions, DPNH oxidation and lactate oxidation was much faster than under aerobic conditions. On all materials, duplicate assays were in close agreement. DPNH oxidase is a sulfhydryl enzyme. It is inactivated by phenylmercuric acetate provided cysteine is omitted from the reaction mixture.

TABLE I. Lactic Dehydrogenase in *Neisseria*.

Organism	No. of cultures†	LDH range	Avg
*N. gonorrhoeae**	21	111–550	270
N. meningitidis	1	156	156
N. perflava	3	204–293	261
N. sicca KC-181	2	217, 386	202
N. catarrhalis	2	44, 57	51

* Nine isolates.
† No. of cultures prepared at different times.
Values are in μg of formazan per 100 μg of N.

Data in Table I represent averages of at least 2 assays and are recorded as μg of formazan produced per 100 μg of nitrogen. Lactic dehydrogenase and DPNH oxidase activities of a series of *N. gonorrhoeae* isolates and several species of *Neisseria* are shown. Formazan produced in 30 minutes by LDH varied between 111-550 μg for *N. gonorrhoeae* and between 44-386 μg for the other *Neisseria*. For DPNH oxidase (Table II), the

TABLE II. Reduced Diphosphopyridine Nucleotide Oxidase in *Neisseria*.

Organism	No. of cultures†	Oxidase range	Avg
*N. gonorrhoeae**	21	134–1135	513
N. meningitidis	1	237	237
N. perflava	3	347– 723	531
N. sicca KC-181	2	281, 729	505
N. catarrhalis	2	131, 131	131

* Nine isolates.
† No. of cultures prepared at different times.
Values are in μg of formazan per 100 μg of N.

values varied between 134-1135 μg in *N. gonorrhoeae* and between 131-729 μg in the other *Neisseria*. *N. gonorrhoeae* isolates which were most resistant to penicillin contained much more DPNH oxidase than the other isolates and the other species (Table III). The range of formazan values was as follows: For the group with low resistance to penicillin it was 248-877, and for the group with high resistance to penicillin it was 780-1408. Alcohol dehydrogenase also was found to be an indicator of relative resistance to penicillin. The enzyme requires DPN. Typical results are listed in Table IV. The degree of formazan formation was arbitrarily graded from 0 to 3. In penicillin sensitive isolates, alcohol dehydrogenase was present only in very small quantities and detectable only after prolonged incubation. Penicillin resistant isolates were obtained from patients who had failed to respond to therapy with this antibiotic. Procedure for determination of penicillin susceptibility may be found in reference 7. For comparison, we have examined a few additional organisms for LDH and DPNH oxidase. The following values were found: For *E. coli*, LDH 11 μg and DPNH oxidase 15 μg; for *S. aureus* Wood 46, LDH 35 μg and DPNH oxidase 109 μg; for *S. aureus* PS 80, LDH 49 μg and DPNH oxidase 111 μg of formazan per 100 μg of N. These organisms contain much less of these 2 enzymes than the *Neisseria* group contains. All organisms contained only small quantities of TPNH oxidase. The formazan range was 68-101 μg per 100 μg of N for all *Neisseria*, except for *N. sicca* which was 55 μg. Under the same conditions *E. coli* 055:B5 produced 14 μg, *S. aureus* Wood 46 produced 44 μg,

TABLE III. Penicillin Resistance *vs* Reduced Diphosphopyridine Nucleotide Oxidase.

No. of *N. gonorrhoeae* isolates	Range of resistance, u/ml	Range of μg formazan per 100 μg N	Avg
5	.005–.010	248– 877	562
8	.300–.650	780–1480	963

TABLE IV. Penicillin Sensitivity *vs* Alcohol Dehydrogenase.

Exp. No.	*N. gonorrhoeae* isolate	Penicillin, u/ml	Formazan reaction in 60 min.
1	P - 82	.005	0
2	P - 16	.005	0
3	P -286	.01	0
4	P -279	.01	0
5	W- 2	.05	1+
6	P -261A	.05	1+
7	P -271	.5	3+
8	P -141	.6	3+

TABLE V. Aerobic Cysteine Oxidase in *Neisseria*.

	% cysteine oxidized
N. gonorrhoeae F18	35.2
" P1	51.0
" 131	56.5
" 97	60.0
N. meningitidis	29.7
N. sicca KC-181	40.8
N. perflava	53.4
N. catarrhalis	54.8

Four mg lyophil. bacteria; 4 mg cysteine; 1 ml 0.1 M phosphate buffer; pH 7.5; vol 6 ml; reaction time 1 hr.

and *S. aureus* PS 80 produced 48 μg formazan per 100 μg of N.

It was also possible to demonstrate an aerobic cysteine oxidase and an aerobic cysteine desulfhydrase in the *Neisseria*. Both enzyme systems were inhibited by KCN. The 2 enzyme systems were inactive in the absence of air. The optimum pH of the CyO is 7.5. The optimum pH of DSH was not determined, but the experiments were carried out at pH 7.5. There is a 2-fold variation between the CyO activities in different *Neisseria* (Table V). About 65% of the cystine produced in the oxidation was reconverted to cysteine by reduction with KCN.

Table VI shows that there was anaerobic oxidation of acetate in the presence of TPT by *N. sicca* and *N. catarrhalis* and an immediate reaction by *E. coli* and 2 strains of *Staphylococcus aureus*. These organisms, however, react rapidly with much smaller quantities than do the *Neisseria*. The *N. gonorrhoeae* isolates did not oxidize acetate within 240 minutes. There was a very slow reaction (19 hours), however, with a few *N. gonorrhoeae* isolates.

TABLE VI. Acetate Oxidation by Bacteria.

	Formazan reaction in hr		
	2	4	19
N. gonorrhoeae F18	None	None	Pink
" 97	"	"	Red
" P1	"	"	None
N. meningitidis	"	"	"
N. perflava	"	"	Red
N. sicca KC-181	Pink	Pink	"
N. catarrhalis	"	"	"
E. coli 055:B5	Immediate deep red		
S. aureus PS 80	*Idem*		

Fifty mg of wet bacteria; 0.75 ml 1 M K-acetate; 2 mg TPT; vol 1.5 ml; pH 7.5.

Summary. It was possible to demonstrate the existence of a potent reduced diphosphopyridine nucleotide oxidase in all *Neisseria*. This enzyme and alcohol dehydrogenase were increased in relative penicillin-resistant isolates of *N. gonorrhoeae*. Another potent enzyme found was diphosphopyridine nucleotide-linked lactic dehydrogenase. Some organisms contained much less LDH and DPNH oxidase than *N. gonorrhoeae* isolates. Reduced triphosphopyridine nucleotide oxidase was present only in small quantities in all organisms investigated. An acetate oxidizing enzyme system was present in large quantities in *E. coli* and *S. aureus*, in small quantities in *N. sicca* and *N. catarrhalis*, and in traces in a few isolates of *N. gonorrhoeae*. The system was absent from *N. meningitidis*. For the above enzymes, the Thunberg technic with tetrazolium salt as the electron acceptor was employed. All *Neisseria* contained a cyanide sensitive, aerobic cysteine oxidase and a cysteine desulfhydrase. It is hoped that the present investigation will aid in identification and classification of *Neisseria*.

These results were obtained with old stock cultures of *N. gonorrhoeae*. When a large number of fresh isolates was assayed only partial correlation between penicillin resistance and enzyme activity was observed.

We are grateful to Dr. James D. Thayer and Miss Frances W. Field for various *N. gonorrhoeae* isolates. We wish to thank Miss Myrtle L. Thomas, Messrs. Ralph Hester and Walter Guest for skillful technical assistance.

1. Tonhazy, N. E., Pelczar, M. J., Jr., *J. Bact.*, 1953, v65, 368.

2. Tauber, H., Russell, H., *Exp. Med. and Surg.*, 1961, v19, 161.

3. Tauber, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 180.

4. Neilands, J. B., *Methods in Enzymology*, 1955, vI, 449.

5. Shinohara, K., *J. Biol. Chem.*, 1935, v109, 665.

6. Grunert, R. R., Phillips, P. H., *Arch. Biochem.*, 1951, v30, 217.

7. *Gonococcus—Procedures for Isolation and Identification*, U. S. Dept. of H.E.W., Public Health Service, 1960, No. 499.

Received April 18, 1962. P.S.E.B.M., 1962, v110.