PYRIDINE NUCLEOTIDE MICRO-ASSAY BY THE METHOD OF SLATER AND SAWYER

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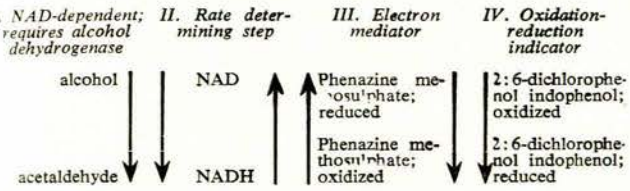
The four reactions which take place in the colorimetric assay procedure of nicotinamide-adenine dinucleotide by the method of Slater and Sawyer,[1] are supposedly linked as follows:



Once calibrated, the proposed assay method appears to demand no further precautions. According to the claims of its authors, there is a direct proportionality between the colour change and the coenzyme concentration. The time over which the reaction continues is therefore a matter of choice. Others have used the method and are evidently satisfied. In our experience, repeated difficulties led to an investigation of the theory and practice of the method as applied to the determination of NAD and NADH.

An investigation into the functioning of the reaction chain showed the following: The prescribed buffering did not hold the pH stable in the presence of the other reagents and tissue extracts. As the reaction rate was little altered between pH 7·4 and 8·1, this inadequacy was of no consequence. Steps II - IV were studied by electrometric titration with pure NADH. Working at pH 7·55 and 24°C, a simple sigmoid titration curve was obtained with a mid-point at + 198mV. A linear fall in optical density of the indicator dye at 600 mμ resulted, 3 μg. NADH/ml. reaction mixture approximately being needed to produce a drop of 0·1 in optical density between 0·8 and zero in a 1 cm. cuvette. In the same reaction mixture the absorption peak of phenazine methosulphate at 388 mμ fell in a not quite linear fashion to the extent of one-third of the fall shown at 600 mμ by the indicator dye. These results show that phenazine methosulphate does not act as a pure mediator but actually undergoes a net change during the reaction. Unfortunately it does not change in a manner which would make it a convenient substitute for the reduction indicator employed. Moreover, it was found that the admission either of light (photoreduction) or oxygen (elevation of potential) severely disturbed the linearity of the change in optical density.

The phenazine methosulphate (Sigma Chemical Co.) used showed 2 sharp absorption peaks, namely at about 259·4 and 387·8 mμ. For convenience of measurement 259·5 and 388 mμ were used, the ratio $\frac{OD_{388}}{OD_{259.5}}$ being about 0·3 for fresh solutions. Photoreduction of phenazine methosulphate by diffuse daylight, acting possibly at 388 mμ, caused some lowering of this peak, but a more marked lowering of the peak at 259·5 mμ also resulted. The above ratio therefore rose. NADH reduction of phenazine methosulphate produced a steep rise at 259·5 mμ and a milder lowering at 388 mμ, causing a fall in this ratio. These changes were not affected by subsequent oxygenation, and the reduction could not be transferred to the indicator dye if the dye was added afterwards.

In contrast to these findings, the indicator dye never showed a linear fall in optical density with time when steps II - IV were coupled with the enzymatic part (I) of the reaction chain, whatever the amount of NAD used within the test range. Nor was there a linear proportionality at any given moment of time with the actual amounts of NAD used. The curves always appeared to be exponential. Furthermore, the NAD to be assayed was not rate-determining. As the amounts of freshly assayed alcohol dehydrogenase per cuvette were increased from 12.500 to 62.500 units, the measured reduction rate was also found to rise. Since the non-enzymatic part of the chain reaction requires only a few tens of effective cycles in part II of the chain to produce a clear fall in part IV, as has been shown above, it follows that the enzymatic part must be operating under an extreme degree of inhibition. Because parts II to IV of the chain show a linear relationship, it seemed likely that inhibition arose between parts I and III of the cycle. This was shown to be the case. By running parts I and II of the reaction with large amounts of NAD, the formation of NADH, as measured at 340 mμ in the presence of phenazine methosulphate, was apparently stopped. Inhibition of the enzyme and reoxidation of NADH could both have been responsible. From other evidence, both must occur, but which possible forms of phenazine methosulphate bring these effects about is not known. When tested in a similar way, parts I and II of the cycle were unaffected by the presence of part IV, the reduction indicator.

It seemed worth deriving a function to connect the mutual effects of those substances which influenced the reaction rate, viz. enzyme, co-enzyme and electron mediator. Each was varied separately at 3 widely-spaced yet workable values, in the absence of light and oxygen. The set of 9 curves thus obtained of optical density against

time was analysed with the aid of a computer. The experimental data from each curve fitted the function $y=a+be^{-ct}$ almost exactly for the calculated values of the 3 constants a, b and c. However, each set of constants was unique for the curve in question, and no simple correction for even 1 variable seemed practicable. Until such a correction is possible, the added effect of other oxidation-reduction systems in a tissue extract under assay cannot be allowed for by this method. It therefore seems that some ostensibly cruder forms of assay[2] may be more satisfactory for certain biological requirements, since apparent improvements bring further problems which it has been the purpose of this note to indicate.

REFERENCES

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2. Findlay, G. H. (1963): S. Afr. J. Lab. Clin. Med., **9**, 73.

THE INCIDENCE OF TRANSMISSIBLE DRUG RESISTANCE FACTORS AMONG STRAINS OF *ESCHERICHIA COLI* IN THE PRETORIA AREA

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Antibiotic-resistant members of the family Enterobacteriaceae, which can transmit their resistance to other members of this family of Gram-negative bacteria, have been isolated in many countries.[1-4] This infectious resistance is controlled by a number of resistance determinants (R-determinants) which are linked on a piece of extrachromosomal genetic material. The transfer of this material to another cell is controlled by a transfer factor (TF). This factor is responsible for the appearance of a conjugation tube between the resistant and sensitive bacteria and also for the transmission of the R-determinants through this tube. The complex of transfer factor attached to resistance determinants is called an R-factor. Characteristics of this system are that the sensitive recipient organism becomes resistant to a number of antibacterial agents simultaneously and that the degree of resistance to some of the antibiotics is remarkably high. Strains of *Salmonella typhimurium* and Citrobacter carrying R-factors have been isolated in Pretoria.[4] In order to gain an impression of the prevalence of R-factors in the Pretoria area, it was decided to investigate locally isolated human strains of *Escherichia coli*.

METHODS AND MATERIALS

Two hundred and seven strains of *Escherichia coli* were isolated from an equal number of patients. One hundred and seventy were from faeces and 37 from urine specimens investigated by the routine section of this laboratory. Gram-negative bacteria which formed non-mucoid red colonies on MacConkey agar were regarded as *E. coli* for purposes of this experiment. A fully sensitive derivative of the multiple drug-resistant *Salmonella typhimurium* and *E. coli strain* E27, previously used,[4] both of which are sensitive to 25 μg./ml. of sulphadiazine, streptomycin, ampicillin, chloramphenicol and tetracycline, were employed as recipients for R-factors in conjugation experiments. The R-factor was eliminated from the *S. typhimurium* strain by means of acriflavine,[4] and this sensitive organism is now called RD42. The liquid medium was Difco Penassay broth and the plating medium was Difco MacConkey agar. Sensitivity to antibiotics was determined by streaking loopfuls of overnight broth cultures onto Penassay base agar, into which appropriate concentrations of the agents had been incorporated. Sulphonamide sensitivity tests were done on a minimal medium[5] in order to overcome interference from sulphonamide inhibitors present in nutrient media.[3] Transfer experiments were done by mixing 1 ml. of overnight broth cultures of resistant donor *E. coli* and sensitive recipient *S. typhimurium* RD42 in 5 ml. of warm broth. The mixture was incubated at 37°C. One ml. of the overnight mixture was added to 10 ml. of warm selenite broth to inhibit the donor *E. coli*, and again incubated. Dilutions of this culture to yield about 200 colonies/plate were then spread on 5 drug-containing MacConkey agar plates. Each plate contained a different drug. After overnight incubation, pale resistant Salmonella colonies were picked off into broth and subsequently tested for their complete spectrum of resistance. Broth cultures of donor and recipient organisms were also plated on drug-free and drug-containing media as controls. Experiments were also done to determine whether the Salmonella strain RD42, which had newly-acquired R-factors from different resistant *E. coli*, was infectious for this property. This was done by mixing the newly-resistant Salmonella with the sensitive *E. coli* E27. These experiments were done as above with the exception that the selenite step was excluded. The resistance-spectrum was also determined for any newly-resistant *E. coli* E27 so produced.

RESULTS

Forty-six of the 207 *E. coli* strains examined were sensitive to 25 μg./ml. of sulphadiazine, streptomycin, ampicillin, chloramphenicol and tetracycline, respectively, and were discarded. The remaining 161 strains were resistant to at least 25 μg./ml. of one or more of the 5 drugs and could be divided into 16 groups according to their pattern of resistance (Table I). The resistance of these strains to sulphadiazine was 100 μg./ml. and resistance to ampicillin and chloramphenicol was 250 μg./ml. Strains resistant to tetracycline grew on agar containing 100 μg./ml., but resistance to streptomycin never exceeded 25 μg./ml.

TABLE I. RESISTANCE PATTERNS AND INCIDENCE OF R-FACTORS IN 207 STRAINS OF *E. coli*

Resistance pattern	*Faeces*	*Urine*
Sensitive	41	5
SuSmACmT	56 (16)	13 (5)
SmACmT	3 (0)	0 (0)
SuSmA T	8 (1)	1 (0)
SuSm	6 (1)	2 (1)
SuSm T	5 (0)	4 (0)
SuSm CmT	2 (1)	2 (0)
Su ACmT	18 (2)	3 (1)
Su Cm	2 (0)	0 (0)
Su	11 (0)	1 (0)
Su ACm	1 (0)	0 (0)
Su CmT	3 (1)	1 (0)
T	4 (0)	1 (0)
Su A T	1 (0)	1 (0)
Su T	4 (1)	0 (0)
A T	2 (0)	1 (0)
SuSmA	3 (0)	2 (0)

Su=sulphonamide, Sm=streptomycin, A=ampicillin, Cm=chloramphenicol and T=tetracycline. Figures in brackets indicate number of strains which possess R-factors.

While donor organisms always grew readily on the drug-containing plates, the recipient controls, plated in pure culture, never showed growth. Twenty-one of the 129