## Interaction of 1-Anilino-8-Naphthalene Sulfonate with Yeast Glyceraldehyde-3-Phosphate Dehydrogenase

In order to evaluate the nature of interactions involved in the binding of $NAD^+$ to glyceraldehyde-3-phosphate dehydrogenase (GAPD) different coenzyme-competitive inhibitors were studied[1,2]. The importance of the adenosine-5-phosphate in the binding of $NAD^+$ was established, whereas nicotinamide mononucleotide was found to bind rather weakly. To get more information about the properties of the $NAD^+$ binding site on the protein surface, it seems of interest to investigate the degree of polarity of this site and the possible participation of hydrophobic regions of the protein in coenzyme binding. For this aim we have used a 'hydrophobic probe', 1-anilino-8-naphthalene sulfonate (ANS), which exhibits low fluorescence in water, but becomes highly fluorescent with a blue shift in emission maximum in non-polar solvents or on binding to hydrophobic regions of proteins[3-5]. As is shown below, yeast GAPD binds ANS competitively with respect to $NAD^+$; the binding being accompanied by changes in the emission spectrum and quantum yield of the dye fluorescence, suggesting the existence of a hydrophobic region in the active site of this enzyme.

*Materials and methods.* GAPD was crystallized from baker's yeast according to the method of Krebs[6]. Fluorescence measurements were performed at room temperature in a spectrofluorometric attachment G-3 for a Hitachi Spectrophotometer EPS-3; excitation was at a wavelength of 350 nm. Fluorescence was measured with right angle optics in 1 cm light path quartz cuvettes. All fluorescence experiments were corrected for fluorescence of dye and enzyme. Quantum yields were determined relative to a standard chinine sulfate solution[7].

*Results and discussion.* Interaction of yeast GAPD with ANS results in a marked enhancement of the fluorescence intensity of the dye and a 30 nm blue shift in its emission maximum (for ANS in glycine buffer the maximum was observed at 520 nm, and for ANS-GAPD complex it shifted to 490 nm). On binding to GAPD ANS is, therefore, transferred to a medium of lower polarity than $0.1\,M$ glycine. Quantum yield of ANS, fully absorbed by GAPD was determined and was found to be 0.05 .Thus, it appears that there are some weakly non-polar regions on GAPD molecule capable of binding ANS. The number of dye binding sites ($n$) and the dissociation constant for the complex (K) were determined for the ANS-enzyme interaction according to the fluorometric method of Weber and Young[8] using the equation:

$$(2)\quad P/XD = 1/n\,(1+K/(1-x)D), \text{ where}$$

P = the total protein concentration, D = dye concentration, $n$ = the number of sites per protein molecule, K = dissociation constant and $x$ is the fraction of dye bound. A plot of $P/xD$ against $1/(1-x)D$ yields values for $n$ and K. The fraction of ANS bound, $x$, is derived from the ratio $x = F/F_0$, where F is the actual observed fluorescence efficiency and $F_0$ – the same when all the dye in solution has been adsorbed. $F_0$ was determined by measuring the fluorescence of a constant amount of ANS (which ranged from $0.7\times10^{-5}$ to $1.05\times10^{-5}M$ in different experiments) in the presence of varying levels of protein and calculating the fluorescence on extrapolation to infinite protein concentration. The molar ratio [ANS]/[protein] was kept below 10:1 in order to eliminate deviations from linearity in the plots due to nonspecific ANS binding.

The results of such an experiment are presented on the double-reciprocal plot (Figure 1), from which the maximum enhancement of ANS fluorescence was obtained. The values $n$ and K could now be determined from the plot given in Figure 2. In this plot the $y$ intercept is the reciprocal $n$, and the $x$ intercept is equal to $-1/K$. In this experiment 4 ANS binding sites per protein molecule (mol. wt. 140,000) were found with an average dissociation constant (K) of $5\times10^{-5}M$. Similar results were obtained with 2 different preparations of GAPD ($n$ varied between 2 and 4, K – between $4\times10^{-5}$ and $6\times10^{-5}M$).

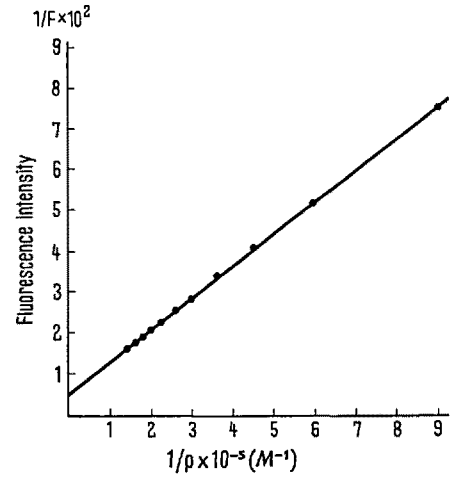


Fig. 1. Double-reciprocal plot of ANS fluorescence against GAPD concentration. $0.1\,M$ glycine buffer pH 8.5. ANS — $1.05\times10^{-5}M$. GAPD — $0.56\times10^{-6}$ — $6.16\times10^{-6}M$. F, fluorescence intensity at 490 nm.

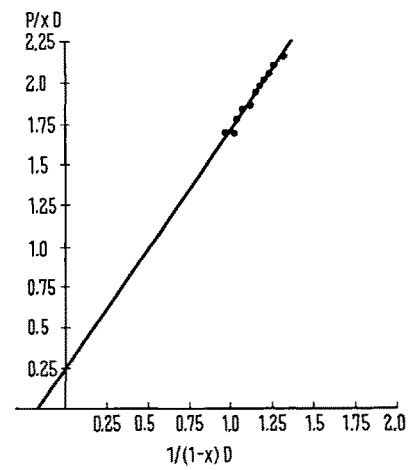


Fig. 2. Determination of N and K according to Eq.[2] (see text). The data of Figure 1 were used for the plot.

[1] Shih Tzy Yang and W. C. Deal, jr., Biochemistry *8*, 2806 (1969).
[2] N. K. Nagradova, M. K. Vorona and R. A. Asryants, Biokhimiya *34*, 631 (1969).
[3] G. M. Edelman and W. O. McClure, Acc. chem. Res. *1*, 65 (1968).
[4] L. Brand, J. R. Cohlke and D. S. Rao, Biochemistry *6*, 3510 (1967).
[5] G. H. Dodd and G. K. Radda, Biochem. J. *114*, 407 (1969).
[6] E. G. Krebs, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan; Academ. Press New York, 1955), vol. 1, p. 407.
[7] G. A. Parker and W. T. Rees, Analyst *85*, 587 (1960).
[8] G. Weber and L. B. Young, J. biol. Chem. *230*, 1415 (1964).

This finding prompts the suggestion that ANS binding sites might be related to the active site regions of the enzyme molecule. Yeast GAPD is known to consist of 4 identical subunits[9] and to bind 4 equivalents of $NAD^+$ per mole of protein[10]. In order to determine whether ANS is capable of binding at the active site of GAPD, the dye was tested as an inhibitor of the enzyme activity. The effect of ANS was studied with varying concentrations of $NAD^+$. The data in Figure 3 show that ANS is a competitive inhibitor with respect to the coenzyme, suggesting an interaction at a common site. The inhibitor dissociation constant found in these studies ranged from $5 \times 10^{-5} M$ to $6 \times 10^{-5} M$. These results are in good agreement with the fluorescence titration data.

A conclusion may be drawn from these results that ANS bound to GAPD is located at or very near the coenzyme binding site, suggesting the existence of some nonpolar region in this site. However, since ANS is an anion the possible role of ionic interactions between ANS and protein molecule must also be considered. We found, in fact, that inorganic phosphate was rather effective in displacing ANS from its complex with GAPD, presumably due to a competition for a common positively charged group on the protein surface. Such a group must be located in close proximity with the non-polar region in the active site of GAPD which also participates in ANS binding.

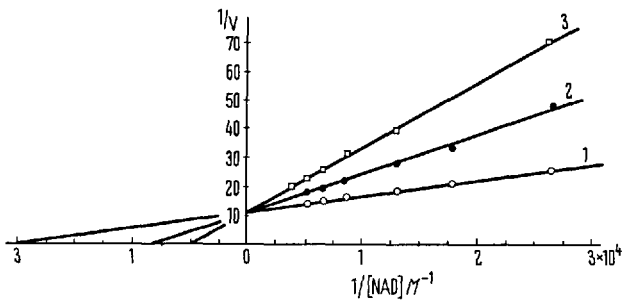


Fig. 3. Inhibition of GAPD activity by ANS with respect to varying NAD concentrations. The reaction mixture (3 ml) contained $0.1 M$ glycine-NaOH buffer pH 8.2, 5 m$M$ EDTA, 5 m$M$ disodium arsenate, $3 \times 10^{-4} M$ glyceraldehyde-3-phosphate, $1.2 \times 10^{-8} M$ GAPD and $0.38 \times 10^{-4} - 2.66 \times 10^{-4} M$ NAD. 1 — no ANS, 2 and 3 — $9.0 \times 10^{-5} M$ and $15.6 \times 10^{-5} M$ ANS respectively, 20 °C. Velocity is expressed in arbitrary units.

Выводы. Связывание 1-анилино-8-нафталин сульфоната (АНС) с дрожжевой глицеральдегид-3-фосфатдегидрогеназой (ГАФД) приводит к возрастанию квантового выхода флуоресценции АНС и сдвигу максимума эмиссии на 30 нм в сторону коротких длин волн. При молярном отношении [АНС]:[ГАФД] < 10:1 на 140000 г белка связывается $3 \pm 1$ моля АНС с константой диссоциации комплекса $5 \times 10^{-5} M$. АНС угнетает энзиматическую активность конкурентно с НАД, величина Ki при этом соответствует $5 \times 10^{-5} M - 6 \times 10^{-5} M$. Предполагается, что связывание краски происходит в области активного центра ГАФД.

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[9] G. M. T. JONES and J. I. HARRIS, Fedn Europ. Biochem. Soc., Abstr. 5th Meeting, Prague, No. 740.

[10] K. KIRSCHNER and B. VOIGHT, Hoppe-Seyler's Z. physiol. Chem. *349*, 632 (1968).

[11] Acknowledgement. We wish to thank Professor Dr. S. E. SEVERIN for encouragement and helpful discussions.

## The ATPases of the Sarcolemma from Skeletal Muscle in Experimental Myotonia

By treating rats with 20,25-diazacholesterol[1,2] or with 2,4-dichlorophenoxyacetate[3] the symptoms of myotonia are induced, i.e. a delayed relaxation of muscle after a contraction and repetitive firings in electromyogram.

It is in general accepted that myotonia is a phenomenon of the muscle membranes. In earlier studies we were able to demonstrate that in rats treated with 20,25-diazacholesterol the calcium pump of the sarcoplasmic reticulum is markedly affected[4]. Rats with induced myotonia also showed a significantly altered fatty acid pattern of the phospholipids and of the cholesterol esters of the sarcoplasmic vesicles[5].

However, these findings cannot explain the repetitive firings in electromyogram, which presumably are due to changes in the external membrane of muscle fibers, the sarcolemma. This membrane is very important for the transmission of electrical activity from the neuromuscular junction over the exterior of the muscle fibers. In the sarcolemma, too, from 20,25-diazacholesterol-treated rats, there are changes in the fatty acid composition of phospholipids and cholesterol ester[5].

The sarcolemma from skeletal muscle of rats contains a $Mg^{++}$ stimulated ATPase and an ATPase stimulated by $Na^+$ and $K^+$ in the presence of $Mg^{++}$[6]. We investigated the activities of these ATPases in the sarcolemma from the skeletal muscles of rats with 20,25-diazacholesterol induced myotonia, and we further determined the inhibition of the ATPases by 2,4-dichlorophenoxyacetate.

Female Wistar rats were given 10 mg 20,25-diazacholesteroldihydrochloride daily for a period of 6 weeks by oesophageal tube. The induced myotonia was demonstrated by electromyogram. The rats – always a myotonic and a normal one at the same time – were decapitated and the sarcolemma was isolated from the muscles of the hind legs according to the method of MCCOLLESTER[7,8] and ROSENTHAL et al.[9] as modified by PETER[6].

[1] N. WINER, D. M. KLACHKO, R. D. BAER, P. L. LANGLEY and T. W. BURNS, Science *153*, 312 (1966).

[2] E. KUHN, W. DOROW, W. KAHLKE and H. PFISTERER, Klin. Wschr. *46*, 1043 (1968).

[3] N. L. R. BUCHER, Proc. Soc. exp. Biol. Med. *63*, 204 (1946).

[4] D. SEILER, E. KUHN, W. FIEHN and W. HASSELBACH, Eur. J. Biochem. *12*, 375 (1970).

[5] D. SEILER and E. KUHN, Z. klin. Chemie, *9*, 245 (1971).

[6] J. B. PETER, Biochem. Biophys. Res. Commun. *40*, 1362 (1970).

[7] D. L. MCCOLLESTER, Biochim. biophys. Acta *57*, 427 (1962).

[8] D. L. MCCOLLESTER and G. SEMENTE, Biochim. biophys. Acta *90*, 146 (1964).

[9] S. L. ROSENTHAL, P. M. EDELMAN and I. L. SCHWARTZ, Biochim. biophys. Acta *109*, 512 (1965).