TABLE II

ACTION OF ERYTHROCYTE-MEMBRANE ATPASE ON ATP AND ATP ANALOGUES

Complete assay system was the same as employed by POST *et al.*[3]. The concentration of sodium was 80 mM and of potassium 32 mM. The activity is expressed as μmoles P_i liberated during 1-h incubation at 40° per mg dry wt. of the enzyme. Ouabain was added in final concentration of $8 \cdot 10^{-5}$ M.

Substrate	*Complete*	*Without Na^+ and K^+*	*With addition of ouabain*
ATP	0.96	0.59	0.55
2,6-Diaminopurine riboside triphosphate	0.14	0.18	0.14
ATP	2.06	1.28	1.26
2-Azaadenosine triphosphate	1.86	1.20	1.27

both sodium and potassium and was inhibited by ouabain the enzyme activity with the ATP analogues as substrate was investigated. As seen in the Table II, 2-aza-adenosine triphosphate was hydrolysed as rapidly as ATP in the presence of membrane ATPase and the activity was dependent upon Na^+ and K^+ and inhibited by ouabain. The enzyme action on 2,6-diaminopurine riboside triphosphate was much less, and a dependency on Na^+ and K^+ and an ouabain sensitivity were not seen.

These findings offer further evidence for the view that membrane ATPase is a part of a system for active transport of cations in erythrocyte.

It has been observed that when the ATP level in the aged erythrocytes is restored by incubation with both adenine and inosine, the shape of the cells changes from a smooth sphere to a shallow-bowl form[4]. The same transformation took place when 2-azaadenosine triphosphate, but not 2,6-diaminopurine riboside triphosphate, was accumulated in the cells. It is suggested that the principal functions of the erythrocyte membrane, active transport of cations and maintainance of the characteristic shape, are determined by a common structural element involving ATPase activity.

Department of Physiological Chemistry and Nutrition, Faculty of Medicine, The University of Tokyo, Tokyo (Japan)

MASAMITI TATIBANA
TAKASHI HASHIMOTO
HARUHISA YOSHIKAWA

[1] M. TATIBANA AND H. YOSHIKAWA, *Biochim. Biophys. Acta*, 57 (1962) 613.
[2] W. E. COHN AND C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 4273.
[3] R. L. POST, C. R. MERRIT, C. R. KINSOLVING AND C. D. ALBRIGHT, *J. Biol. Chem.*, 235 (1960) 1796.
[4] M. NAKAO, T. NAKAO, M. TATIBANA AND H. YOSHIKAWA, *J. Biochem. Tokyo*, 47 (1960) 661.

Received October 1st, 1962

SC 2235

Interference by menadiol in the colorimetric estimation of nitrite

During the course of our investigations on the properties of a menadione-dependent nitrate reductase in *Agrobacterium tumefaciens* (unpublished work) we observed that the enzyme activity as measured by nitrite formation was drastically decreased as

the concentration of the electron donor, menadiol (2-methyl-1,4-naphthohydroquinone) was increased in the assay mixture. Closer examination, however, revealed that this was due primarily to the interference of menadiol with the nitrite test. Our observations on some aspects of this interference are reported in this communication.

Nitrite (in 3 ml solution) was estimated by diazotisation with 1 % sulphanilamide in 3 N HCl (1 ml) and coupling with 0.02 % aq. *N*-(1-naphthyl)ethylenediamine hydrochloride (1 ml). The red azo-dye formed was read in a Klett-Summerson photoelectric colorimeter after 10 min using a 54 filter[1].

The appreciable influence exerted by menadiol on the colour intensity, especially at acid pH, is evident from Table I. As the pH of the medium was increased the nitrite was able to tolerate a considerably higher proportion of menadiol. That the

TABLE I

EFFECT OF MENADIOL CONCENTRATION ON NITRITE COLOUR TEST

Klett readings with 0.05 μmole of nitrite. Menadiol was dissolved in a drop of ethanol, made up with water and added to nitrite before diazotisation. 3 ml of the nitrite solution contained 1 ml of buffer. Values given are as % of nitrite added.

Medium	*Menadiol added (μmoles)*						
	0	*0.05*	*0.10*	*0.20*	*0.60*	*0.80*	*1.40*
Water	100	99	94	58	55	23	16
0.2 M phosphate buffer (pH 6.0)	100	75	53	53	36	28	22
0.2 M phosphate buffer (pH 6.4)	100	75	63	58	36	31	22
0.2 M phosphate buffer (pH 7.0)	100	78	69	61	38	31	20
0.2 M phosphate buffer (pH 7.4)	100	81	75	66	52	30	20
0.2 M phosphate buffer (pH 8.0)	100	100	100	100	100	100	20

factor involved is the stability of menadiol as influenced by pH is seen from Table II. In the alkaline range the reduced quinone is rapidly re-oxidised while at neutral and acid pH it is much more stable. This would account for the decrease in inhibitory effect with increase in pH. Menadione (the oxidised form) in amounts up to 5 μmoles had no effect on the colour at any pH.

It is evident from Table III that even relatively smaller concentrations of menadiol can completely suppress the colour development if added after diazotisation. This contrasts with the effect of NADH and NADPH whose inhibitory effect was completely annulled when added after diazotisation[3]. It may be noted that the reduced quinone in larger concentrations could still decrease the colour intensity when added even after the dye has been formed.

We have observed that the red colour of the dye can be completely discharged by treatment with reducing agents such as $NaBH_4$. Larger concentrations of menadiol can also bring about a considerable decrease in the colour intensity. Under conditions in which menadiol inhibits the colour intensity it is oxidised to the quinone. This would indicate that the mechanism of inhibition may be a reduction of the azo-dye to the leuco-dye. The higher potency on addition after diazotisation may be due to the greater susceptibility of the aryl diazonium chloride to reduction. A reductive cleavage of the diazonium chloride or azo-dye, though a posibility, is unlikely in the light of our observation that the colour is partially regenerated on prolonged aeration.

It is also unlikely that the menadiol enters into any complex formation with the diazonium chloride since it has been found that the quinone is fully extractable from the mixture by ether. On subsequent addition of the coupling agent no colour develops.

TABLE II

STABILITY OF MENADIOL AT DIFFERENT pH

To the quinol solution in 5 ml were added 0.5 ml of 0.5% α,α'-dipyridyl and 0.5 ml of 0.2% $FeCl_3$ and colour measured with a 54 filter. In the presence of phosphate the colour development is slow and takes 1 h (ref. 2). It is possible that there will be considerable oxidation of menadiol during this period, especially at alkaline pH. Klett readings are given.

Medium	*Time of reading after reagent adaition (min)*	*Menadiol added (μmoles)*				
		0.5	*1.0*	*1.5*	*2.0*	*2.5*
Water	15	90	165	—	340	450
	60	160	280	—	375	475
0.2 M phosphate buffer (pH 6.0)	15	50	105	204	310	370
	60	50	120	210	325	425
0.2 M phosphate buffer (pH 8.0)	15	0	0	0	0	0
	60	0	0	0	0	0

TABLE III

EFFECT OF ORDER OF ADDITION OF MENADIOL ON COLOUR INTENSITY

Colour developed in aqueous medium. 0.05 μmole of nitrite were used. Values are given as % of nitrite added.

Addition of menadiol	*Menadiol added (μmoles)*						
	0.00	*0.05*	*0.10*	*0.20*	*0.60*	*0.80*	*3.00*
Before diazotisation	100	99	94	58	55	23	13
After diazotisation	100	42	22	5	0	0	0
After coupling*	100	99	99	91	91	83	66

* Menadiol added 10 min after the addition of the coupling agent.

That the inhibitory effect is primarily due to the reducing property is also evident from the fact that menadione and also NAD^+ and $NADP^+$ exhibit no inhibitory properties. However, the mode of inhibition by NADH and NADPH appears to be different as they were ineffective when added after diazotisation. Competition with nitrite for the same site on the diazo compound has been suggested as a possibility in this case[3].

Department of Biochemistry, Indian Institute of Science, Bangalore (India)

C. K. RAMAKRISHNA KURUP
C. S. VAIDYANATHAN

1 F. D. SNELL AND C. T. SNELL, *Colorimetric Methods of Analysis*, Vol. 2, Van Nostrand, New York, 1959, p. 804.
2 T. RAMASARMA, N. G. SREENIVASAN, C. E. SRIPATHI AND V. M. SIVARAMAKRISHNAN, *Enzymologia*, 21 (1959) 133
3 A. MEDINA AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 23 (1957) 440.

Received November 26th, 1962