therefore markedly stimulated by the further addition of an uncoupler (Table II) (as is well known for the ferricyanide system[11–13]). The DCIP system is also more stimulated by an uncoupler than by the phosphorylating system at pH 7.4 indicating that the ATP-forming system is limiting the electron transport (Table I). In the diaminodurol system, however, NH_4Cl and the phosphorylating system give the same stimulation of the basal electron transport (Tables I and II) and further addition of NH_4Cl to the phosphorylating system has only a slight effect (Table II), indicating that the ATP-forming system is not or less limiting. NADPH formation with water or with diaminodurol as the electron donor is coupled to a stoichiometric ATP formation. The absolute rate of ATP formation during $NADP^+$ reduction at pH 7.4 with diaminodurol as the donor is therefore higher than with water as the electron donor. This might be taken as an indication that in the diaminodurol system at pH 7.4 another phosphorylating site is participating which has an optimum at a lower pH.

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On the possible role of structural protein in the binding and translocation of adenine nucleotides in mitochondria

The permeability studies of PFAFF, KLINGENBERG AND HELDT[1] as well as direct evidence obtained by M. KLINGENBERG AND F. PALMIERI (unpublished results) with mitochondria devoid of outer membranes (prepared according to PARSONS[2]) show that the exchange between exogenous and endogenous adenine nucleotides[1,3] occurs at

the level of the inner membrane. This reaction is highly specific, highly temperature-dependent and atractyloside-sensitive and has, therefore, been referred to as an enzyme activity called "exchange enzyme" or "translocase". What protein, if any, in the inner membrane is responsible for the adenine nucleotide translocation is still under discussion*.

Recently MORET *et al.*[5] and SILIPRANDI[6] have demonstrated that the binding of adenine nucleotides to structural protein is prevented or reversed by atractyloside. They concluded that this finding could be useful in understanding the effect of atractyloside either on the proposed binding sites of adenine nucleotides in mitochondria[7] or on their translocation[8–10]. In this study the binding of adenine nucleotides to structural protein has been investigated to determine whether it can participate in the translocation or in the binding of the adenine nucleotides in mitochondria.

In agreement with HULTIN AND RICHARDSON[11], the binding of adenine nucleotides to structural protein was found in our studies to be concentration-dependent and increased as the pH was lowered. Furthermore, in agreement with the finding of SILIPRANDI's group[5,6], atractyloside was found to inhibit the binding of ATP and ADP to structural protein or to remove them, if they were already bound. The non-specificity of the binding to structural protein is demonstrated in Table I. It is seen that in addition to ATP, there is also a binding of DPNH, orthophosphate and even anions such as succinate, isocitrate and carboxy-polyglucose (not shown). In contrast, positively charged compounds (Ca^{2+} or choline) or uncharged molecules, such as glucose, are not bound. Atractyloside non-specifically inhibits the binding of all anions. The inhibitory effect appears to be greater with the less negatively charged molecules, such as succinate and orthophosphate, than with ATP. These results show

TABLE I

BINDING OF DIFFERENT COMPOUNDS TO STRUCTURAL PROTEIN AND EFFECT OF ATRACTYLOSIDE

The reaction mixture contained 40 mM Tris acetate buffer (pH 7.0) and 1–3 mg structural protein, prepared from beef-heart mitochondria as described by RICHARDSON, HULTIN AND FLEISCHER[12], in 1 ml. Temperature: 21°. Incubation time: 10 min. Specific activity of added compounds: 40–150 counts/min per 10^{-9} mole. [^{14}C]ATP, [^{14}C]DPN (enzymatically reduced) from Schwarz BioResearch (Mount Vernon, N.Y.), ^{45}Ca from The Radiochemical Centre (Amersham), all the other compounds from New England Nuclear Corp. (Boston, Mass.). The insoluble protein was removed by centrifugation and washed twice before counting.

Additions (*1 mM*)	*Atractyloside* (*200 μM*)	*Compound bound* (*10^{-9} moles/mg protein*)	*% Inhibition by atractyloside*
[^{14}C]ATP^{4-}	—	21.4	—
	+	14.7	31
[^{14}C]$DPNH^{2-}$	—	11.7	—
	+	2.1	82
[^{14}C]$Succinate^{2-}$	—	11.0	—
	+	1.9	83
[^{14}C]$Isocitrate^{3-}$	—	16.2	—
	+	5.3	67
[^{32}P]HPO_4^{2-}	—	8.4	—
	+	1.0	88
$^{45}Ca^{2+}$	—	0.4	—
[^{14}C]$Choline^{+}$	—	0.1	—
[^{14}C]Glucose	—	0.2	—

* VIGNAIS *et al.*[4] have postulated a "carrier" of a steroid type.

that the competition between adenine nucleotides and atractyloside is non-specific and due simply to their negative charges.

If the different anions listed in Table I are bound to structural protein at the same site, they should all compete with the adenine nucleotides in the binding to structural protein. Accordingly, it was found that besides unlabelled ADP, atractyloside, isocitrate, succinate, orthophosphate and DPNH decrease the amount of [^{14}C]-ADP bound to structural protein. The dependence of the inhibition of ATP binding on atractyloside concentration shows (Fig. 1) that even at 1 mM atractyloside the inhibition is not complete. In contrast, much lower concentrations of atractyloside, such as 3–5 μM, are sufficient to inhibit 95–100 % of the adenine nucleotide exchange in mitochondria.

The functional similarity between structural protein and F_4, described by ZALKIN AND RACKER[13], prompted an examination of the binding properties of F_4 for adenine nucleotides. However, ATP was found to bind neither to F_4 nor to the F_4–phospholipid complex at pH 7.0. Only at low pH (4.5), as expected, was the adenine nucleotide bound and this binding was sensitive to atractyloside. This supports the idea that the binding to "structural protein" is non-specific and artificially induced by the preparation procedure, which differs from that of F_4.

It could also be shown that the adenine nucleotides contained in mitochondria appear not to be bound to structural protein (Fig. 2). In exclusion chromatography with Bio-Gel of cholate- and deoxycholate-solubilized mitochondria the adenine nucleotides are separated from the peak of mitochondrial proteins; under the same conditions ATP bound to isolated structural protein remains associated with the protein peak.

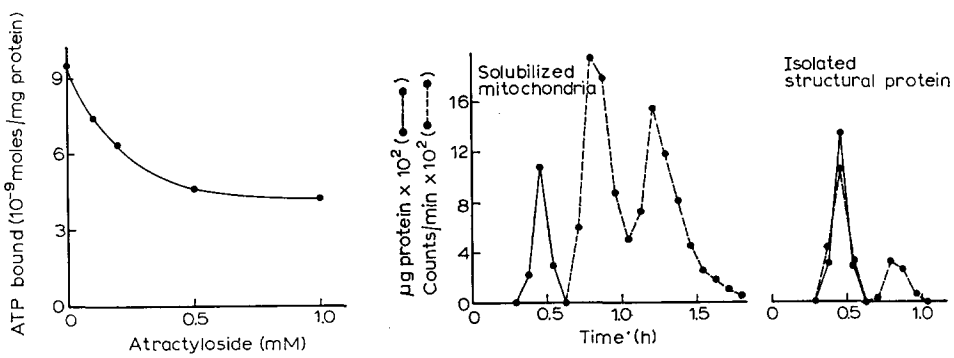

Fig. 1. The dependence of the ATP binding to structural protein on atractyloside concentration. The incubation mixture contained: structural protein, 1.15 mg; 40 mM Tris acetate buffer (pH 7.5); 1 mM [^{3}H]ATP and atractyloside at the concentrations indicated. Other details are given in the legend to Table I.

Fig. 2. Column chromatography of cholate-solubilized, [^{14}C]ADP-labelled rat-liver mitochondria on Bio-Gel P-2. Column: 1.2 cm × 21.5 cm, elution buffer: 20 mM Tris acetate (pH 7) containing 2.5 mg/ml cholate and 5 mg/ml deoxycholate, flow speed: 13.2 ml/h. Mitochondria were labelled with 0.25 mM [^{14}C]ADP for 45 min at 0° and washed twice. After this treatment practically all the endogenous adenine nucleotides are labelled by exchange[3]. The suspended mitochondria (specific activity = 490 counts/min per 10^{-9} mole AXP) were solubilized by the addition of 1.0 mg cholate and 2.0 mg deoxycholate per mg mitochondrial protein. Structural protein was labelled with 5 mM [^{3}H]ATP, pH 7.5 (specific activity = 33 counts/min per 10^{-9} mole). ●—●, proteins; ●---●, counts/min.

The results show that the properties of structural protein in binding adenine nucleotides and atractyloside are not adequate to correlate this protein with the adenine nucleotide translocase or with the binding of adenine nucleotides in mitochondria. The non-specificity of the binding sites of structural protein, the non-specificity of the effect of atractyloside and the very high concentrations required, strongly contrast with the well-known properties of the translocation of adenine nucleotides in mitochondria. Furthermore, the intramitochondrial adenine nucleotides appear not to be bound to mitochondrial structural protein *in situ*. They appear rather to be free in the matrix, where they are enclosed by the cristae membrane. This interpretation is also supported by recent electron micrographs of [^{3}H]ATP-labelled mitochondria (A. REIDT, W. VOGELL AND M. KLINGENBERG, unpublished results).

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