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Selective Labeling and Rotational Diffusion of the ADP/ATP Translocator in the Inner Mitochondrial Membrane*

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Submitochondrial particles were labeled with the triplet probe eosin-5-maleimide (EMA) after pretreatment with *N*-ethylmaleimide. On sodium dodecyl sulfate-polyacrylamide gels, eosin fluorescence occurred in a single band of M_r ~30,000. The labeled band was identified as the ADP/ATP translocator, since EMA binding was completely inhibited by carboxyatractylate. Furthermore, the EMA-labeled polypeptide had the same molecular weight as the purified carboxyatractylate-bound translocator and the purified EMA-labeled translocator.

Rotational diffusion of the translocator around the membrane normal in submitochondrial particles was measured by observing flash-induced absorption anisotropy of EMA. The translocator rotates with a time constant which varied from ~240 μs at 5 °C to ~100 μs at 37 °C. However, it is likely that only a fraction of the translocator rotates, the remainder being immobile over the measurement time of 500 μs. The mobile fraction of the translocator decreased with decrease in temperature. The observed fluorescence anisotropy of 0.24 indicates that EMA undergoes subnanosecond rapid wobbling in the binding site of the ADP/ATP translocator.

The ADP/ATP translocator is the most abundant integral protein in the inner membrane of beef heart mitochondria (for review, see Refs. 1 and 2). The translocator has been isolated as a carboxyatractylate·protein complex using extraction with Triton X-100. From CAT[1]-binding and hydrodynamic studies it was found that this complex is a dimer, each subunit having M_r ~ 30,000 (3). The ADP/ATP translocator catalyzes the translocation of 1 ADP against 1 ATP across the inner mitochondrial membrane. Masking or modification of sulfhydryl groups, essential for the nucleotide translocation, causes the inhibition of the translocation activity (1). CAT is one of the specific inhibitors of the translocator with a dissociation constant K_d ~ 10 nM. At least one sulfhydryl group is masked when the translocator is liganded by CAT. ADP/ATP translocation is an important step in the phosphorylation of ADP, which is catalyzed by the ATP-synthase in the inner mitochondrial membrane. For this process, a molecular interaction betwen the translocator and the ATP-synthase has been postulated (4).

Recent investigation showed that integral proteins in the inner membrane of mitochondria are able to undergo rotational and lateral diffusion in the plane of the membrane (5–7). Rotational diffusion measurements provide a powerful method for directly investigating protein-protein interactions in the membrane. In the case of cytochrome oxidase, such measurements were performed by observing flash-induced absorption anisotropy of the heme a_3·CO complex. However, since the ADP/ATP translocator does not have an intrinsic chromophore, it is necessary to label the translocator selectively with a triplet probe in order to study rotational motion. Such a selective labeling by eosin derivatives has been previously achieved for band 3 protein in erythrocyte membranes and (Ca^{2+}-Mg^{2+})-ATPase in sarcoplasmic reticulum (8, 9).

Here we present a method for specific labeling of the ADP/ATP translocator with EMA together with initial studies of its rotational mobility in beef heart SMP.

EXPERIMENTAL PROCEDURES

Membrane and Protein Preparation—Beef heart mitochondria were prepared according to Hatefi and Lester (10) as modified by Bock and Fleischer (11), and stored frozen at −80 °C. SMP were prepared from freshly thawed mitochondria by one passage through a French pressure cell at 16,000 p.s.i. Unbroken mitochondria were removed by centrifugation at 27,000 × *g* for 10 min. The SMP were sedimented by centrifugation at 100,000 × *g* for 60 min and resuspended in a buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and KOH to pH 7.4 (buffer A), at a protein concentration of 10–15 mg/ml.

Preparation of CAT (Boehringer Mannheim GmbH)-loaded mitochondria, SMP, and isolation of the ADP/ATP translocator were performed by following the procedure described by Klingenberg *et al.* (12). The EMA-labeled translocator was isolated using the same procedure.

Selective Labeling of the SMP—The SMP were labeled with EMA (Molecular Probes, Inc., Plano, TX) as follows: 10–15 μg of NEM/mg of protein were added to SMP and incubated for 5 min at 0 °C; then 15 μg of EMA /mg of protein were added to the SMP and incubated for 30 min in the dark at room temperature. The reaction was stopped by the addition of 1 mg of dithiothreitol/mg of protein. After a 30-min incubation at 0 °C, the sample was washed several times with buffer A to remove any free EMA. The amount of bound EMA was determined as described previously (13).

Rotational Diffusion Measurements and Analysis of Absorption Anisotropy—For transient dichroism experiments, EMA-labeled SMP were suspended in 80% (w/w) glycerol, in order to eliminate vesicle tumbling and reduce light scattering. The final concentration of EMA was 4 to 9 μM. The flash photolysis apparatus is described in detail elsewhere (13). Briefly, the sample was excited at 532 nm by a vertically polarized flash of 20-ns duration from a Nd/YAG laser (second harmonic). Absorbance changes due to ground state depletion were measured at 520 nm. The signals were analyzed by calculating the absorption anisotropy, $r_r(t)$, given by

$$r_r(t) = \{A_V(t) - A_H(t)\}/\{A_V(t) + 2A_H(t)\} \quad (1)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at the time t after the flash. In each experiment, 1024 signals were averaged using a Datalab DL 102 A signal averager. A further improvement of the signal-to-noise ratio was achieved by averaging data from several experiments.

The ADP/ATP translocator maintains a fixed orientation with respect to the plane of the membrane (14), implying that rotation occurs about the normal to the plane of the membrane. Therefore, decays in absorption anisotropy, $r_r(t)$, were analyzed based on a "rotation-about-membrane normal" model (13, 15). A theoretical treatment of this case for EMA-labeled proteins shows that the expected form of $r_r(t)$ is given by

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[1] The abbreviations used are: CAT, carboxyatractylate; EMA, eosin-5-maleimide; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles; NEM, *N*-ethylmaleimide.

$$r_r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-4t/\phi_1) + r_3 \quad (2)$$

where θ_1 is the mean rotational time and r_1, r_2, r_3 are constants (13, 15). However, since the accuracy of the data does not justify use of the full Equation 2, we employ the following simplified equation:

$$r_r(t) = A_1 \exp(-t/\phi_r) + A_2 \quad (3)$$

where θ_r is the rotational time constant. Curve fitting of the data by Equation 3 was accomplished by a Hewlett-Packard HP 9825 A desk top computer.

Fluorescence Polarization Measurements—The steady state fluorescence anisotropy was measured with an Aminco SPF 500 spectrofluorometer. The exciting light at 520 nm was vertically polarized and the sample fluorescence at 570 nm was analyzed into vertically and horizontally polarized components I_V and I_H. The steady state anisotropy, r^s, is obtained as

$$r^s = (I_V - I_H)/(I_V + 2I_H) \quad (4)$$

where I_V and I_H are corrected for light scattering and instrumental factors.

SDS-Polyacrylamide Gel Electrophoresis—A slightly modified method of Laemmli (16) was used to perform SDS-polyacrylamide gel electrophoresis. The samples were dissolved for 3 min at 95 °C in a buffer containing 10 mM sodium phosphate, pH 7, 10% (v/v) glycerol, 2.5% (w/v) SDS, 3.25 mM dithiothreitol. The sample was applied on a two-step (12 and 15%) polyacrylamide slab gel which was polymerized in a buffer containing 325 mM Tris, 2 mM EDTA, 0.1% (w/v) SDS, and HCl to pH 8.8. The electrode buffer was composed of 50 mM Tris, 380 mM glycin, 0.1% (w/v) SDS, 1.8 mM EDTA. After electrophoresis, fluorographs were obtained by illuminating the slab gel with UV light and photographing the fluorescent emission through cut-off filters. Subsequently, the gel was fixed and stained by conventional procedures using Coomassie brilliant blue R-250.

Protein Determination—The protein concentration was determined using the method of Lowry *et al.* (17), when required in the presence of 0.5% (w/v) SDS, or the Biuret procedure according to Gornall *et al.* (18). In both cases, bovine serum albumin was used as standard.

RESULTS AND DISCUSSION

Selective Labeling of SMP with EMA—Selective labeling of the ADP/ATP translocator with EMA was accomplished by preincubating the membranes with NEM (10–15 μg/mg protein) and subsequently incubating with EMA. A typical example is shown in Fig. 1 (*left*). The identification of the EMA-labeled band was achieved by a direct comparison with purified EMA-labeled ADP/ATP translocator and the CAT-bound translocator, both co-electrophoresed on the slab gel (Fig. 1, *left* and *right*). A good agreement of the $M_r \sim 30{,}000$ was achieved among the three samples (*i.e.* the EMA-labeled band in SMP, purified EMA-labeled translocator, and purified CAT-bound translocator). As a further control, CAT, a specific inhibitor for the ADP/ATP translocator, was used for competitive binding studies. When the labeling was performed with SMP prepared from mitochondria preloaded with CAT, almost no labeling of the translocator was observed (Fig. 1 (*left*)). Therefore, even if the band of $M_r \sim 30{,}000$ in SMP consists of more than one species of polypeptide, only the ADP/ATP translocator reacts with EMA. Also, no labeling by EMA was observed in mitochondria preincubated with CAT and NEM.[2] The competitive effect of CAT is presumably due to masking of a sulfhydryl group of the translocator. Since CAT does not penetrate into the inner mitochondrial membrane due to its amphiphilic properties (1), CAT should react with the translocator only from the cytoplasmic side in mitochondria. The inhibition of the EMA binding by CAT, therefore, implies that EMA binds to the CAT binding site of the translocator on the cytoplasmic side of the inner mitochondrial membrane. The ratio of bound EMA to the ADP/ATP translocator monomer ($M_r \sim 30{,}000$) was determined spectrophotometrically to be ~1 (mol/mol) in SMP using the value of 3.5 nmol of translocator/mg of protein in SMP (2, 19).

When the EMA-labeling was performed in the absence of NEM (Fig. 1 (*left*)), several other proteins were labeled including the phosphate carrier which is very reactive with NEM (20). The optimal EMA-labeling conditions were investigated with different concentrations of EMA ranging from 0.5 to 50 μg/mg protein without NEM. The maximal fluorescence of the translocator was obtained by incubating the SMP with 15 μg of EMA/mg of protein. Increasing the concentration of EMA above this value resulted in heavier labeling of other proteins, while the fluorescence intensity of the translocator remained unchanged.

The inhibitory effect of NEM on EMA binding to the translocator was also investigated by changing the concentration from 5 to 50 μg/mg protein with a constant EMA concentration of 15 μg/mg protein. Even at low NEM concentration of 5 μg/mg protein, there was a remarkable decrease of the multiple labeling. Only the ADP/ATP translocator was labeled when NEM was more than 10 μg/mg protein. However, above 15 μg of NEM/mg of protein, the fluorescence of the translocator was decreased and at high NEM concentration (50 μg/mg protein) the labeling was completely abolished. Thus, it appeared that 10–15 μg of NEM/mg of protein were optimal for spectroscopic measurements. As judged by fluorescence intensity, the amount of free EMA observed around the gel front was lower than 5% of that bound to the translocator in SMP when NEM was 10–15 μg/mg protein.

Isolation of the EMA-labeled Translocator—The translocator was isolated from SMP which were treated with 15 μg of NEM/mg of protein and then labeled with 15 μg of EMA/mg of protein. The procedure described by Klingenberg *et al.* (12) was used. The purity of the pass-through fraction of the hydroxylapatite column was about 70–80%, as reported elsewhere (21, 22). The slight amount of contaminants was mainly due to a protein with $M_r \sim 34{,}000$, which was identified as the phosphate carrier (22, 23). In contrast to the above preparation, the CAT-bound translocator is practically pure (Fig. 1 (*right*)). No free EMA was detected around the gel front in the purified EMA-labeled translocator preparation.

Rotation of the Translocator about the Membrane Normal—Rotation of the ADP/ATP translocator in SMP was measured at different temperatures. Fig. 2 shows representative $r_r(t)$ curves. In all cases examined, $r_r(t)$ decayed within 500 μs to a time-independent value. Data were analyzed by Equation 3 and are summarized in Table I. The rotational mobility of the translocator was temperature-dependent. The rotational time constant ϕ_r was 100 μs and the normalized time-independent anisotropy $A_2/r_r(0)$ was 0.78 at 37 °C. ϕ_r increased about 2.4 times and $A_2/r_r(0)$ increased by about 13% when the temperature decreased from 37 to 5 °C.

Most of the present experiments were made with French press SMP. SMP can also be prepared with sonication procedures, which may disturb the structure and organization of proteins in the membrane. The effect of sonication on rotational mobility of the translocator was examined. EMA-labeled French press SMP were sonified mildly for 4 min using a Branson B 30-type sonifier with pulsed mode (50%) at 50-watt output at 0 °C under a N_2 stream. This resulted in an increased decay in $r_r(t)$ with $A_r/r_r(0) \simeq 0.64$ and $\phi_r \simeq 190$ μs at 37 °C (see Fig. 2). Rotational diffusion of the EMA-labeled translocator was also investigated in bovine heart mitoplasts[2] prepared with digitonin essentially as described by Krebs *et al.* (19). A large decay in $r_r(t)$ was observed with $A_2/r_r(0) \simeq 0.57$ and $\phi_r \simeq 140$ μs at 37 °C.

The normalized residual anisotropy $A_2/r_r(0)$ is related to the fraction of immobile ADP/ATP translocator (15). When

[2] M. Müller, unpublished results.

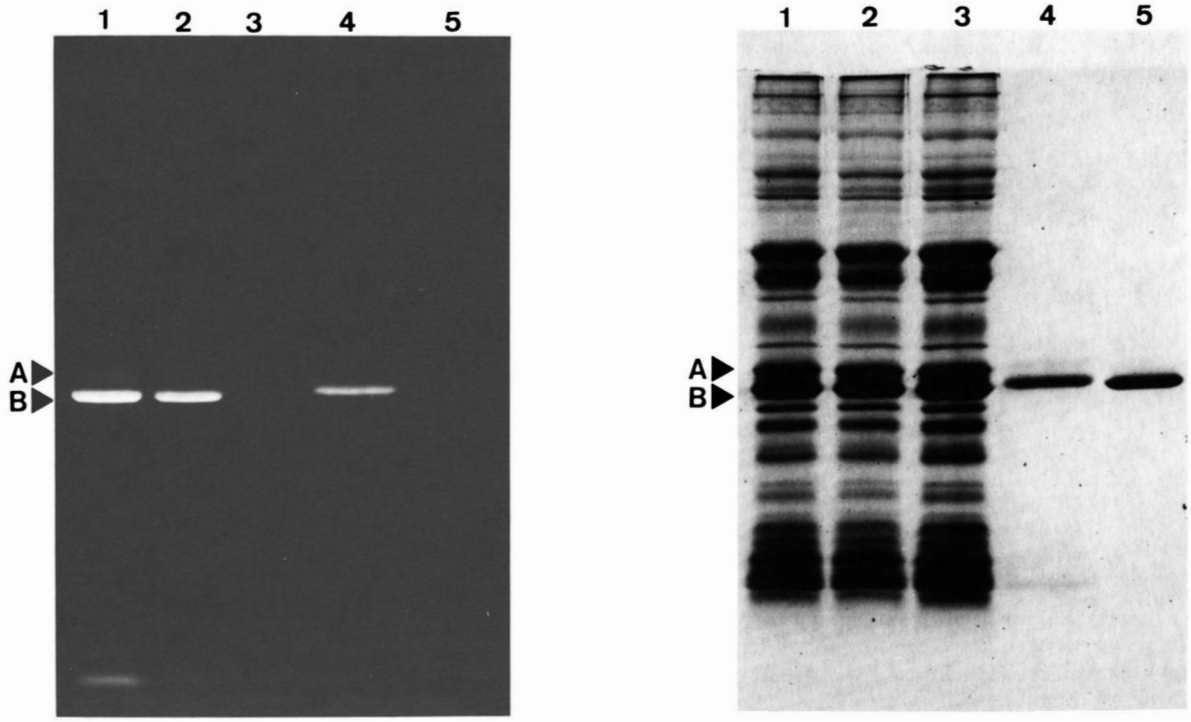

FIG. 1. **SDS-polyacrylamide gel electrophoresis of SMP and purified ADP/ATP translocator after labeling with EMA.** *Left,* fluorograph of the gel before staining with Coomassie blue. Labeling of SMP was performed with 15 μg of EMA/mg of protein for 30 min at 5 °C, resulting in a multiple labeling (*lane 1*). By treatment of the SMP with 10–15 μg of NEM/mg of protein before EMA-labeling, selective labeling of the ADP/ATP translocator (*band B*) was achieved (*lane 2*), while labeling of the NEM-sensitive phosphate carrier (*band A*) was eliminated. No fluorescence of the ADP/ATP translocator was observed when SMP were pretreated with 3.6 μg of CAT/mg of protein and 15 μg of NEM/mg of protein prior to labeling with 15 μg of EMA/mg of protein (*lane 3*). The EMA-labeled translocator was isolated from SMP treated with NEM (15 μg/mg protein) and EMA (15 μg/mg protein) (*lane 4*). *Right,* Coomassie blue stain of the same gel. For a direct identification of *band B* in SMP and of the isolated EMA-labeled translocator, purified CAT-bound translocator was co-electrophoresed (*lane 5*) showing a good agreement in M_r. The preparation of the EMA-labeled translocator (*lane 4*) showed a contamination by the phosphate carrier (*band A*), which, however, was not fluorescent (see *left*).

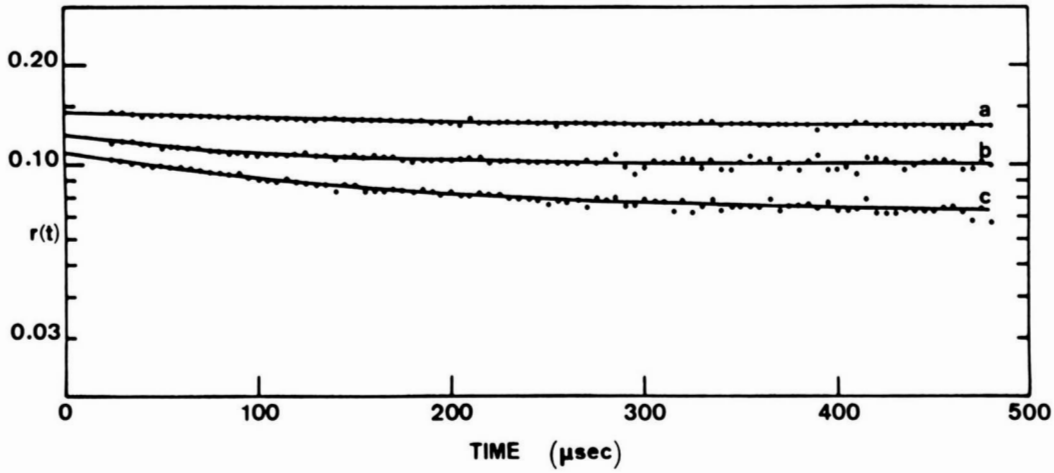

FIG. 2. **Time-dependent absorption anisotropy of the ADP/ATP translocator in SMP.** The ADP/ATP translocator in French press SMP was selectively labeled with EMA after pretreatment with NEM. Samples (4–9 μM in EMA) were photolyzed by a vertically polarized laser flash at 532 nm, and $r_r(t)$ was recorded at 520 nm. All measurements were performed in 80% (w/w) glycerol. *Curve a,* SMP at 5 °C; *curve b,* SMP at 37 °C; *curve c,* sonicated SMP at 37 °C. For this, French press SMP were mildly sonicated for 4 min using a Branson B 30-type sonifier with pulsed mode at 50-watt output at 0 °C under a N_2 stream. *Solid lines* were obtained by fitting the data to Equation 3. $r_r(0)$ values were different among these measurements because of different excitation intensities (15). When $r_r(0)$ was carefully determined with a weak excitation flash, we obtained $r_r(0) = 0.16 \pm 0.01$ independent of temperature. It should be noted that $r_r(t)/r_r(0)$ is independent of the flash intensity (15). Possible contribution of vesicle tumbling of SMP to the observed decay in $r_r(t)$ is excluded, as reported by Kawato *et al.* (28).

all the translocator is mobile, $A_2/r_r(0)$ has a minimum value $[A_2/r_r(0)]_{min}$. The fraction of immobile translocator, f_{im}, can be calculated by

$$A_2/r_r(0) = (1 - f_{im}) \cdot [A_2/r_r(0)]_{min} + f_{im} \quad (5)$$

The value of $[A_2/r_r(0)]_{min}$ is a function of the orientation of the eosin probe, which is not known. However, from the results obtained with mitoplasts, $A_2/r_r(0)_{min} \leq 0.57$. Assuming that the probe orientation is the same in the different preparations, the higher value of $A_2/r_r(0)$ obtained with French

TABLE I

Decay parameters of the time-dependent absorption anisotropy of the EMA-labeled ADP/ATP translocator in SMP at different temperatures analyzed by Equation 3

Experimental conditions are the same as those in Fig. 2.

Membranes	ϕ_r	A_2/r_r (0)	Temperature
	μs		°C
SMP	239 ± 35[a]	0.87 ± 0.03	5
SMP	213 ± 12	0.80 ± 0.02	20
SMP	99 ± 20	0.78 ± 0.05	37
SMP sonicated	190 ± 29	0.64 ± 0.02	37

[a] Standard deviation.

press SMP shows that an immobile fraction (over a time of 500 μs) is present in this preparation. The temperature-dependent increase in $A_2/r_r(0)$ in French press SMP indicates a decrease in the mobile fraction upon going from 37 to 5 °C. Sonication of SMP increased the mobile fraction of the translocator, suggesting that sonication disturbs the organization of proteins in the membrane. The present studies may be compared with previous measurements of the rotational mobility of cytochrome oxidase in the inner mitochondrial membrane (7). It was also found that both mobile and immobile populations are present, the mobile fraction having a rotational relaxation time of about 400 μs.

A possible alternative interpretation of the observed $r(t)$ curves is that the decay in $r(t)$ is due to a wobbling motion of the whole translocator molecule about axes in the plane of the membrane. Increase in temperature, sonication, and digitonin treatment might increase the angular amplitude of wobbling motion and hence decrease $r(\infty)/r(0)$ (15). This appears, however, to be unlikely because such a wobbling motion has not been observed for other integral proteins, such as cytochrome oxidase and bacteriorhodopsin (15, 24).

Independent Probe Wobbling—In order to obtain good signal-to-noise ratio in protein rotation measurements, we used a flash excitation of sufficient intensity, which decreases $r_r(0)$ (15). An accurate initial anisotropy $r_r(0)$ was therefore determined with a weak excitation flash, when we obtained $r_r(0) = 0.16 \pm 0.01$ independent of temperature. As compared with the limiting anisotropy for EMA of $r_0 = 0.380$,[3] the observed initial anisotropy $r_r(0) = 0.16$ is significantly lower, suggesting an additional rapid intramolecular motion which decreases the anisotropy from r_0 to the observed $r_r(0)$. This could be due to independent wobbling of EMA about the attached site in the translocator. The probe wobbling was investigated with fluorescence polarization measurements. The time-averaged fluorescence anisotropy r^s of EMA was 0.240 ± 0.005 for SMP in 80% (w/w) glycerol solution independent of temperature over 5–37 °C. Since the fluorescence lifetime of eosin is about 2 ns (25), the observed $r^s = 0.240$ suggests a significant motional freedom of EMA about the binding site within a few nanoseconds.

A more detailed picture of independent probe wobbling can be obtained with time-resolved fluorescence polarization. The time-dependent anisotropy $r_p(t)$ of EMA bound to the translocator decayed with a subnanosecond time constant to a time-independent residual anisotropy $r_p(\infty) \simeq 0.18$ within the time range of several tens of nanoseconds.[4] The reasonable agreement between $r_r(0) = 0.16$ and $r_p(\infty) \simeq 0.18$ indicates that the observed low initial anisotropy $r_r(0)$ is due to loss of anisotropy by independent wobbling of EMA about the binding site of the translocator. A similar decay in $r_p(t)$ by probe wobbling about the attached site has also been shown for *N*-(1-anilinonaphthyl-4)maleimide bound to cytochrome oxidase (26) and for eosin-5-thiosemicarbazide bound to oligosaccharides in erythrocytes (27).

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