the process enhancing the cell growth rate by increasing fidelity through kinetic proofreading of codon–anticodon interactions and chirality of the aminoacyl group and consequently producing a better chance for the species survival. It may also have eliminated D-amino acids from proteins at the same time.

Acknowledgments

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Uptake of Ornithine by Rat Liver Mitochondria†

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ABSTRACT: Uptake of [^{14}C]-L-ornithine by rat liver mitochondria has been measured by using the silicone sampling technique. The uptake of ornithine measured after 20–45 s of incubation exhibits stereospecificity, pH dependence, and a lack of dependence on respiratory energy. A slower subsequent increase in [^{14}C]-L-ornithine counts associated with the mitochondria, which is blocked by the transaminase inhibitor aminooxyacetate, is attributed to metabolism of the labeled ornithine. Each of the reagents *N*-ethylmaleimide, Tris (HCl) buffer, $Tl_2^+SO_4^{2-}$, $Mg^{2+}SO_4^{2-}$, and choline chloride inhibits ornithine accumulation. A lack of inhibition by mersalyl is interpreted as indicating that ornithine uptake does not require transmembrane P_i flux. Uptake of ornithine to levels in excess of the concentration in the medium can largely be accounted for by an osmotically insensitive fraction of the ornithine taken up, which is assumed to be adsorbed to solid structures of the mitochondria.

Mechanisms of uptake by mitochondria of various acidic and neutral amino acids have been extensively studied [e.g., see LaNoue & Schoolwerth (1979)]. Less is known about the transport of basic amino acids across mitochondrial membranes. Measurements of uptake of arginine by dog kidney mitochondria (Keller, 1968) and of lysine (King & Diwan, 1973; Diwan & Aram, 1974) and ornithine (Gamble & Lehninger, 1973; Bryla & Harris, 1976; McGivan et al., 1977)

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by rat liver mitochondria have been reported. Rat liver mitochondria appear to be permeable also to the ornithine analogue N^{δ}-(phosphonacetyl)-L-ornithine (Hoogenraad et al., 1979).

The existence of a mechanism for transport of ornithine into mammalian liver mitochondria is important because of the localization within the mitochondrial matrix of that portion of the urea cycle in which ornithine is carbamoylated to citrulline and the localization outside of the mitochondria of the reactions leading from citrulline to production of ornithine (Ratner, 1973; Gamble & Lehninger, 1973; Tatibana et al., 1976). Ornithine uptake by rat liver mitochondria was initially reported to be dependent on respiration and dependent on availability of a proton-yielding anion such as phosphate (Gamble & Lehninger, 1973). More recent studies have show a lack of dependence of ornithine uptake on respiratory energy (Bryla & Harris, 1976; McGivan et al., 1977). A stimulation of metabolism of exogenous ornithine by external P_i has been interpreted by McGivan et al. (1977) as indicating a role of P_i/OH^- exchange in neutralizing pH shifts associated with ornithine/H^+ exchange. An inhibitory effect of the mercurial mersalyl on metabolism of externally added ornithine was similarly attributed to inhibition by mersalyl of P_i/OH^- exchange (McGivan et al., 1977). In contrast, Bryla & Harris (1976) report no effect of added P_i on ornithine uptake.

Apparent accumulation of ornithine against a concentration gradient has been reported (Gamble & Lehninger, 1973; Bryla & Harris, 1976). Inhibition of ornithine aminotransferase activity by addition of aminooxyacetate was found to largely abolish accumulation of [^{14}C]ornithine (McGivan et al., 1977). Addition of 10 mM lysine further decreased the uptake of low concentrations of labeled ornithine (McGivan et al., 1977). Thus, McGivan and co-workers attribute the apparent accumulation in the absence of aminooxyacetate or lysine to metabolism of the labeled ornithine and to surface adsorption. In the experiments of Bryla & Harris (1976), 1 mM lysine was found not to affect uptake of [^{14}C]ornithine, while 1 mM arginine was inhibitory.

The present studies have focused on the uptake of [^{14}C]-L-ornithine by isolated rat liver mitochondria in the presence and absence of various metabolic inhibitors.

Experimental Procedures

Rat liver mitochondria were isolated by essentially standard procedures (Johnson & Lardy, 1967). The 0.25 M sucrose isolation medium was supplemented with 0.4 mM Tris–EDTA in the initial stages of preparation. The mitochondria were washed twice by centrifugation and resuspension in 0.25 M sucrose. Mitochondrial protein was assayed by the biuret procedure (Layne, 1957).

Mitochondria (5–6 mg of protein/mL) were incubated at 20 °C in the media which contained, unless otherwise specified (see the figure and table legends), 200 mM sucrose, 8 mM Tris, various concentrations of ornithine, ^3H_2O (ca. 2.6 μCi/mL), and [1-^{14}C]-L-ornithine (ca. 80 nCi/mL), with the pH adjusted to 7.5 with HCl. For some samples [^{14}C]sucrose (ca. 0.4 μCi/mL) was substituted for the labeled ornithine. At timed intervals mitochondrial samples were separated from incubation media by rapid centrifugation through silicone (Harris & VanDam, 1968). Radioisotopes were assayed by liquid scintillation counting.

Some ornithine uptake measurements are expressed as the ratio of the distribution spaces of [^{14}C]-L-ornithine and ^3H_2O in the sedimented mitochondria (ornithine space/H_2O space). It should be realized that such ratios are lower than the ratios of internal to external ornithine concentrations, since under most of the conditions studied approximately 70% of the water volume of the sedimented mitochondria is penetrated by [^{14}C]sucrose and thus is assumed to be external to the matrix compartment (Harris & VanDam, 1968). The intramitochondrial ornithine concentrations depicted are calculated from the amount of labeled ornithine associated with the mitochondria, corrected for contaminating extramitochondrial ornithine which is estimated to be the product of the [^{14}C]-sucrose distribution space and the ornithine concentration in the medium. The sucrose distribution space either was measured in parallel or, in some experiments utilizing the standard incubation medium, was assumed to be 70% of the water space, an average value determined in many experiments using such incubation conditions. The corrected estimate of ornithine content is divided by the matrix volume (^3H_2O distribution space minus [^{14}C]sucrose distribution space) to yield the apparent intramitochondrial ornithine concentration.

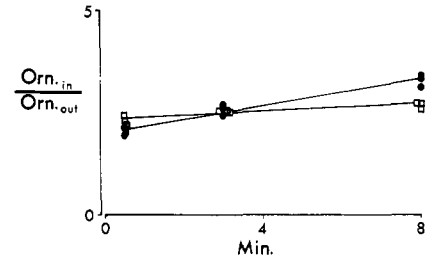

FIGURE 1: Time course of uptake of [^{14}C]-L-ornithine in the presence and absence of aminooxyacetate. The medium contained 0.5 mM L-ornithine. The ratio of the intramitochondrial ornithine concentration (calculated as indicated under Experimental Procedures) to the ornithine concentration in the medium is plotted against the incubation time in minutes. The lines drawn are calculated by the method of least squares. Symbols: (●) control samples; (□) the medium included 0.75 mM aminooxyacetate.

All radioisotopes were obtained from New England Nuclear. L and D isomers of ornithine and aminooxyacetate were obtained from Sigma Chemical Co. Silicone SF1154 was a gift of the General Electric Co.

Results and Discussion

The time course of [^{14}C]-L-ornithine uptake is depicted in Figure 1. The uptake is biphasic. Within 0.5 min of incubation, the apparent ornithine concentration within the mitochondria exceeds the ornithine concentration in the medium. The initial rapid uptake is followed by a slower increase in the amount of labeled ornithine associated with the mitochondria. The presence in the medium of aminooxyacetate, at a concentration sufficient to block the mitochondrial ornithine aminotransferase (McGivan et al., 1977), causes a very slight, but reproducible, increase in the initial accumulation of labeled ornithine. The aminooxyacetate then largely eliminates the slower phase of [^{14}C]-L-ornithine uptake. These results suggest that the slower uptake of label may reflect metabolism of the [^{14}C]-L-ornithine via transamination. Thus, the remaining experiments to be described were all carried out in the presence of aminooxyacetate, and the values reported are for early samples, e.g., for 20–45-s incubations. Experiments similar to those to be described have been carried out also in the absence of aminooxyacetate (data not shown). The results obtained are generally similar, provided that the incubation time is short. In addition, data were obtained in the presence of aminooxyacetate at longer time intervals (e.g., 7-min incubations), for most experiments described. Generally results in the presence of aminooxyacetate are similar with 20–45-s or 7-min incubations, except for differences arising from slow rates of change of [^{14}C]-L-ornithine uptake which persist in the presence of the transaminase inhibitor.

Table I: Lack of Dependence of Ornithine Uptake on Respiration[a]

additions	ornithine space/H_2O space	
	0.5 min	8 min
none	1.40 ± 0.03	1.51 ± 0.03
succinate	1.37 ± 0.03	1.56 ± 0.01
succinate + antimycin A	1.34 ± 0.03	1.46 ± 0.02
succinate + Dnp	1.30 ± 0.04	1.51 ± 0.06

[a] The medium included 0.5 mM L-ornithine and 0.75 mM aminooxyacetate. When present, succinate was at 2.5 mM, antimycin A at 40 ng/mg of protein, and 2,4-dinitrophenol (Dnp) at 100 μM.

McGivan et al. (1977) observed a substantial reduction of steady-state (2-min incubations) [^{14}C]ornithine accumulation by 0.5 mM aminooxyacetate. In the present experiments a similar or higher concentration of the transaminase inhibitor is observed to block only the slow progressive accumulation of the label. In the experiments of McGivan et al., ornithine was taken up in the presence of aminooxyacetate to levels only slightly exceeding the concentration in the medium, while in the present studies significant ornithine accumulation has been observed even in the presence of the transaminase inhibitor. We cannot fully account for these discrepancies. However, one difference in experimental design which may account for lower ornithine uptakes in the experiments of McGivan et al. is the presence of high (e.g., isosmolar) concentrations of choline chloride in their incubation media.

Experiments have shown that choline chloride, as well as other ionic solutes, inhibits ornithine uptake. For example, in one experiment in which the L-ornithine concentration was 0.5 mM and aminooxyacetate was present at 0.7 mM, the ratio of ornithine space/water space after 0.75 min was 1.85 ± 0.06 for control samples, 1.22 ± 0.03 in the presence of 20 mM choline chloride, and 1.14 ± 0.04 in the presence of 40 mM choline chloride (means of three determinations ± standard deviations). The effect of choline chloride is not attributable to changes in osmolarity, since the measured matrix volume (^3H_2O space minus [^{14}C]sucrose space) showed little change and the decreases in the distribution ratio were paralleled by decreases in the estimated intramitochondrial ornithine concentration from 1.91 ± 0.14 mM for control samples to 0.87 ± 0.05 mM in the presence of 20 mM choline chloride and 0.77 ± 0.03 mM in the presence of 40 mM choline chloride. Thus accumulation of the labeled ornithine to levels in excess of the medium concentration is greatly diminished by choline chloride.

In another experiment in which the L-ornithine concentration was 0.2 mM and aminooxyacetate was present at 0.5 mM, the 0.75-min ratio of ornithine space/water space was 1.61 ± 0.01 for control samples, 1.20 ± 0.03 with addition of 5 mM $Tl_2^+SO_4^{2-}$, 1.23 ± 0.02 with addition of 5 mM $Mg^{2+}SO_4^{2-}$, and 1.29 ± 0.01 with increase of the Tris (SO_4^{2-}) buffer concentration from 8 to 32 mM (means of three determinations ± standard deviations). Other experiments have shown that the presence of succinate, which would support uptake of the inorganic cations (Skulskii et al., 1978; Diwan et al., 1979), has little effect on the inhibitory action of Tl^+ or Mg^{2+}.

The lack of dependence of ornithine uptake on respiratory energy is shown in Table I. Addition of the respiratory substrate succinate has no effect on uptake of the labeled ornithine. Further addition of the respiratory inhibitor antimycin A or the uncoupler dinitrophenol also fails to significantly affect ornithine accumulation. The observed lack of energy dependence is consistent with the observations of Bryla & Harris (1976) and of McGivan et al. (1977) but inconsistent with the report of Gamble & Lehninger (1973). The lack of effects of dinitrophenol and antimycin A also indicates that ATP-dependent carbamoylation of ornithine does not contribute to uptake of the label under the conditions studied.

Table II: Effect of *N*-Ethylmaleimide, Mersalyl, and pH on Ornithine Uptake[a]

additions	ornithine space/H_2O space	
	pH 7	pH 8
none	1.28	2.09
	1.29	2.10
	1.34	2.23
MalNEt	1.22	1.44
	1.21	1.50
mersalyl	1.44	2.35
	1.56	2.51
MalNEt + mersalyl	1.44	1.68
	1.37	1.65

[a] The media, which were titrated to pH 7.0 or 8.0 with HCl, included 4 mM Tris and 0.2 mM L-ornithine. Mitochondria were pretreated with 7 mM aminooxyacetate in the cold, yielding a concentration of 1.1 mM aminooxyacetate following dilution into incubation media. When present, *N*-ethylmaleimide (MalNEt) was at 500 μM and mersalyl was at 165 μM (35 nmol/mg of protein). The incubation time was 20 s.

Effects of pH and the sulfhydryl reagents mersalyl and *N*-ethylmaleimide (MalNEt) are shown in Table II. Ornithine uptake is greater at pH 8 than at pH 7. McGivan et al. (1977) found that enhanced ornithine uptake resulted from increased acidity of the matrix relative to that of the external medium, induced by addition of dinitrophenol to mitochondria respiring in the presence of valinomycin. Those results were interpreted as evidence for an ornithine/H^+ antiport mechanism of transport. However, such evidence for dependence on the pH gradient is difficult to reconcile with the lack of effect of respiration in the absence of added ionophores, since transmembrane pH shifts are known to accompany activation or uncoupling of respiration. One possible explanation of the effect of external pH on ornithine uptake might be the titration of acidic membrane groups capable of binding ornithine.

The data in Table II show that mersalyl has a slight stimulatory effect which is diminished in the presence of MalNEt. Other experiments (data not shown) have indicated that the stimulatory effect of mersalyl on uptake of labeled ornithine is not enhanced by availability of respiratory energy. Thus, it does not appear to be analogous to the stimulation of K^+ uptake by mersalyl, which is dependent on respiration (Brierley et al., 1968; Diwan et al., 1977). The lack of inhibitory effect of mersalyl, at a concentration sufficient to block mitochondrial transmembrane P_i exchange (Meijer et al., 1970), suggests that P_i translocation is not essential to uptake of at least low (e.g., 0.2 mM) concentrations of ornithine. In the earlier report of McGivan et al. (1977), inhibition of ornithine catabolism by mersalyl was interpreted as resulting from inhibition of ornithine uptake secondary to inhibition of P_i/OH^- exchange. However, no measurements of ornithine uptake in the presence of mersalyl were presented.

MalNEt inhibits ornithine uptake, especially at pH 8, as shown in Table II. The effect may be attributable to a slowing of the transport process, since the ratio of ornithine space/water space in the presence of MalNEt more closely approaches control values after 7-min incubations (data not shown). If there is a direct effect on the transport mechanism, it appears to be only a partial inactivation, since significant ornithine accumulation occurs within 20 s even in the presence of MalNEt. The inhibitory effect of MalNEt on ornithine uptake cannot be attributed to the known blockage of P_i/OH^-

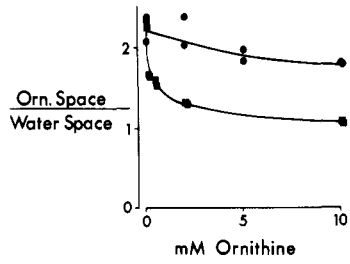

FIGURE 2: Effect of D and L isomers of ornithine on uptake of [^{14}C]-L-ornithine. The incubation time was 0.75 min. The medium included 0.7 mM aminooxyacetate. The ratio of the distribution space of [^{14}C]-L-ornithine to the ^3H_2O distribution space is plotted against the millimolar ornithine concentration in the medium. Symbols: (■) the values on the abscissa represent concentrations of L-ornithine; (●) the values on the abscissa represent concentrations of D-ornithine in the presence of a constant level of 50 μM L-ornithine.

exchange by MalNEt (Meijer et al., 1970) since mersalyl, which also blocks P_i/OH^- exchange, has an opposite effect.

The stereospecificity of ornithine uptake is shown in Figure 2. Increasing the concentration of unlabeled L-ornithine decreases the [^{14}C]-L-ornithine distribution space. At 10 mM external L-ornithine, the ratio of ornithine space/water space nearly approaches the value 1, which would indicate transmembrane ornithine equilibration. In other experiments in which effects of higher concentrations of L-ornithine have been tested, ratios of ornithine space/water space of less than 1 have been observed (e.g., ratio of 0.8 in the presence of 40 mM L-ornithine). Such data suggest saturability of the process responsible for ornithine uptake and accumulation. In contrast, D-ornithine only slightly diminishes the uptake of [^{14}C]-L-ornithine. This evidence for stereospecificity of ornithine uptake is consistent with the earlier report (Gamble & Lehninger, 1973) that mitochondria swell in isosmotic solutions of L-ornithine, but not D- although it should be noted that in those experiments the swelling was found to be respiration dependent.

Because of the rapidity and apparent lack of energy dependence of ornithine accumulation, the possibility that much or all of the uptake is attributable to adsorption must be considered. In the experiment depicted in Figure 3, the dependence of ornithine uptake on matrix volume was examined. Matrix volume was manipulated osmotically by varying the sucrose concentration in the medium. The rise in total water space at high osmolalities shown in Figure 3B is not predicted from osmotic relationships, but it has been observed before when the siilicone sampling technique (Harris & VanDam, 1968) was used. The matrix volume (total water space minus sucrose-penetrable space) exhibits a linear dependence on the reciprocal of the osmolality, as predicted from the known osmotic behavior of mitochondria (Tedeschi & Harris, 1958).

The variation in ornithine content with changing matrix volume shown in Figure 3A is consistent with the interpretation that a portion of the ornithine is dissolved in the aqueous matrix compartment in equilibrium with the external medium. As the matrix volume is extrapolated to zero, the ornithine uptake declines, extrapolating to a value which may be interpreted as representing the amount of ornithine adsorbed to solid structures of the mitochondria. If one estimates the amount of ornithine taken up at some osmolality, subtracting from this the amount of ornithine uptake at extrapolated zero matrix volume, and divides by the matrix volume at the osmolality of interest, the corrected internal ornithine concentration is estimated to be close to, although still slightly in excess of, the concentration in the medium. For example, in the experiment shown in Figure 3, at an osmolality of 0.26

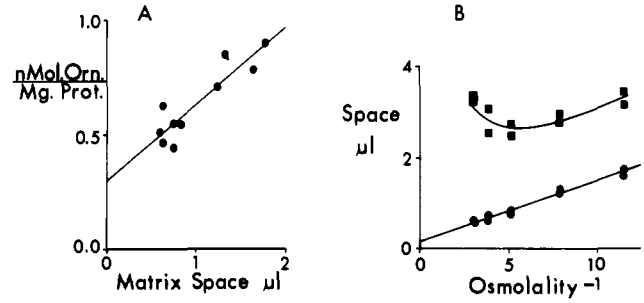

FIGURE 3: Effect of matrix volume on [^{14}C]-L-ornithine uptake. The media, which included 4 mM Tris (adjusted to pH 7.5 with HCl) and 0.21 mM L-ornithine, contained variable amounts of sucrose (from 85 to 303 mM). The mitochondria were pretreated with 6.3 mM aminooxyacetate in the cold, yielding a concentration of 0.9 mM aminooxyacetate following dilution into incubation media. Osmolalities were obtained from tables in the *Handbook of Chemistry and Physics* (1978), assuming negligible contribution of the Tris buffer, ornithine, and aminooxyacetate. Incubations with [^{14}C]sucrose and [^{14}C]-L-ornithine were run in parallel. The incubation time was 20 s. In (A), the amount of labeled ornithine taken up, corrected for ornithine in the sucrose-penetrable space, in units of nanomoles per milligram of protein, is plotted against the volume of the matrix space in microliters, calculated as the ^3H_2O distribution space minus the [^{14}C]sucrose distribution space. In (B), distribution spaces were plotted against the reciprocal of the osmolality. The distribution spaces reported are for mitochondria sedimented from 200-μL samples of the reaction mixture containing ~1 mg of protein. Symbols: (■) total ^3H_2O space; (●) matrix volume calculated as ^3H_2O space minus [^{14}C]sucrose space.

the average matrix volume is estimated to be 0.68 μL/sample containing 1 mg of protein; the total apparent ornithine uptake averages 0.51 nmol/mg of protein, while the extrapolated uptake at zero matrix volume averages 0.31 nmol/mg of protein. The uncorrected intramitochondrial ornithine concentration at 0.26 osmolality is thus estimated to be 0.76 mM in this experiment. The estimate of internal ornithine concentration corrected for osmotically insensitive ornithine is 0.30 mM, which is closer to, although still slightly greater than, the external concentration of 0.21 mM ornithine. These values must of course be considered approximate, since changes in ornithine adsorption with changing matrix volume cannot be ruled out.

It should be noted that in the report of McGivan et al. (1977) an osmotic study indicated no ornithine uptake at zero matrix volume, while the present data show that a significant portion of accumulated ornithine is osmotically insensitive. As pointed out earlier, in the experiments of McGivan et al. the major osmotic support in incubation media was provided by choline chloride, which inhibits ornithine uptake, perhaps by blocking adsorption of ornithine to anionic membrane sites.

The fact that D-ornithine has only a slight effect on uptake of L-ornithine, coupled with the evidence that L-ornithine distributes in part into the osmotically active matrix compartment and in part is adsorbed to solid structures, suggests that the transport of ornithine is stereospecific and adsorption sites are at least partially internal. Alternatively, one would have to postulate that binding sites on the external surface are stereospecific.

In summary, the data are consistent with the conclusion that ornithine rapidly enters mitochondria via a stereospecific mechanism, distributing in the matrix water and additionally binding to solid structures of the organelle.

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Purification and Characteristics of Photoreceptor Light-Activated Guanosinetriphosphatase†

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ABSTRACT: We describe a reconstitution of light-activated vertebrate photoreceptor GTPase and a purification of the GTP-binding protein (G protein), which is a component of the GTPase and modulates the light-activated phosphodiesterase (PDE) enzyme system. Rod outer segments (ROS) of bull frogs were treated with ethylenediaminetetraacetic acid (EDTA), and the GTPase and PDE fractions were solubilized (EDTA supernatant). When the EDTA supernatant and EDTA-treated membrane fraction (EDTA-washed membranes) were recombined, light-dependent GTPase activity appeared. In the reconstituted system, the K_m for GTP as substrate was 0.5 μM; the optimum pH was 7.5-8.0. The isoelectric point of GTPase in EDTA supernatant was 4.8. G protein was purified 400-fold from ROS, and the molecular weight of G protein was determined to be 40 000 by polyacrylamide gel electrophoresis. The amount of G protein in ROS was calculated as at least 1 molecule per 400 rhodopsin molecules. By recombining (in the presence or absence of GTP) purified G protein, PDE, H fraction (an additional component of GTPase), and illuminated or unilluminated EDTA-washed membranes (as a source of rhodopsin), we showed that illuminated rhodopsin, G protein, PDE, and GTP are the minimum requirements for light-dependent PDE activity. We discuss the significance of these findings in the regulation of the light-activated GTPase and PDE activities, especially with regard to the mechanism of activation.

The capacity of GTP to serve as an activator for a variety of multienzyme complexes is receiving increasing attention. Examples of this phenomenon include the hormone-activated adenylate cyclase (Londos et al., 1974), light-activated phoshpodiesterase (PDE) (Wheeler et al., 1977), the involvement of GTP in the messenger RNA translation system (Nomura et al., 1974), and the polymerization of tubulin (Roberts & Hyams, 1979). In the hormone-activated adenylate cyclase and light-activated PDE systems, a model has emerged which depicts an oscillating control mechanism that depends upon the binding and hydrolysis of GTP. In this model, the regulated enzyme (adenylate cyclase or PDE) is active when the GTP is bound to an independent activator locus. The bound GTP is hydrolyzed by a GTPase,[1] which appears to be contiguous with the activator locus (Wheeler & Bitensky, 1977; Cassel & Selinger, 1976; Wheeler et al., 1977; Shinozawa et al., 1979). The light-activated photoreceptor PDE bears a striking resemblance to the epinephrine-activated adenylate cyclase found in turkey erythrocytes. Both systems are activated by nonhydrolyzable GTP analogues such as guanylyl imidodiphosphate [Gpp(NH)p] (Wheeler & Bitensky, 1977; Schramm & Rodbell, 1975), and they exhibit similar GTP affinities, which are in the micromolar range (Wheeler & Bitensky, 1977; Cassel & Selinger, 1976). The rate constants for the activation and inactivation steps for the epinephrine-activated adenylate cyclase of turkey red cells have been recently described (Cassel et al., 1977).

The light-activated GTPase has an action spectrum which is indistinguishable from that of the light-activated PDE (λ_{max} is close to 500 nm). Maximal activation of both the GTPase and PDE is observed following the bleaching of 1 molecule in 1000 rhodopsin molecules (Wheeler & Bitensky, 1977;

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[1] Abbreviations used: GTPase, guanosinetriphosphatase; PDE, phosphodiesterase; ROS, rod outer segments; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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