which determine not only how the phospholipid is structured with fatty acid (Sinensky, 1971) but subsequently what fatty acids are synthesized. To the extent that an exogenous fatty acid is recognized by these enzymes and placed into one or both positions of the phospholipid, those endogenously derived fatty acids normally put into these sites are kept out and in unknown fashion must shut down their own synthesis.

In the course of these studies we have observed that there is more unsupplemented AB1623-1 cultures grown at 37° than cis unsaturated fatty acids in the phospholipid derived from in the phospholipid from other *E. coli* K-12 strains (see also Silbert, 1970) grown under similar conditions. This difference is associated with a greater incorporation of *cis*-monoenoic fatty acids into position 1. These findings suggest that there may be strain differences at 37° in the specificity of the acyl transferase(s) participating in phospholipid biosynthesis or in the relative rates of saturated *vs.* unsaturated fatty acid synthesis.

Although extensive replacement of those fatty acids present in phospholipid (especially 16:0, 16:1, and 18:1) can be achieved by exogenous supplementation, the same is not true for those acyl groups found in lipid A (12:0, 14:0, and βOH-14:0). The presence of β-hydroxymyristate in the cultures has not been effective in reducing synthesis of that fatty acid although the cell can grow on it as carbon source (Dr. Robert Bell, personal communication) and, therefore, can transport and activate this fatty acid. The failure to incorporate exogenous acyl groups into lipid A but not into phospholipid suggests that the acyl transferase activity involved in synthesis of the former complex lipid may show a greater specificity for the acyl-ACP which can be derived possibly only from *de novo* synthesis.

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Role of Sulfhydryl Groups in Erythrocyte Membrane Structure†

James R. Carter, Jr.

ABSTRACT: The sulfhydryl binding reagent *p*-chloromercuribenzenesulfonate extracts up to 40% of the protein present in hypotonically lysed, extensively washed red cell "ghost" membranes. The process is rapid, shows no temperature dependence in the range of 15–37°, and is not reversed by dithiothreitol added after extraction. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate demonstrates that all major peptides are extracted in part except for the predominant species which remains entirely in the membrane. Only one minor glycoprotein is solubilized. Coincident with the elution of protein, there is breakdown of the "ghosts" into smaller vesicles. The extracted membranes are more soluble than untreated "ghosts" in Triton X-100 and aggregate spontaneously in the presence of 0.1–0.4 M NaCl. *N*-Ethylmaleimide-treated membranes show the same physical changes although there is no significant release of protein. It is suggested that sulfhydryl groups play a role in hydrophobic bonding of proteins to membranes and that many of the effects attributed to sulfhydryl binding agents may be related to nonspecific structural alterations in the membrane.

Many functions associated with cellular membranes have been shown to be inhibited by reagents that bind free sulfhydryl groups (Garrahan and Rega, 1967; Makinose, 1969; Scott *et al.*, 1970; Sutherland *et al.*, 1967; van Steveninck *et al.*, 1965; Janacek, 1962; Carter and Martin, 1969a; Rothstein, 1970). Generally, this has been interpreted as indicating that a given class of sulfhydryl groups is intimately involved in the process under study, either a part of the critical membrane protein (enzyme, transport protein, antigen, etc.) or closely associated with it in the membrane. In at least one case, that involving the β-galactoside "permease" of *Escherichia coli*, this specificity has been confirmed by the radioactive

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TABLE I: Extraction of Red Cell Membrane Proteins with pCMBS.[a]

	Protein Extracted (mg)			
	Control		pCMBS	
Temp (°C)	60 min	120 min	60 min	120 min
15	0.093	0.115	0.640	0.693
23	0.112	0.165	0.673	0.606
27	0.112	0.231	0.653	0.640
32	0.256	0.326	0.713	0.642

[a] Aliquots (1 ml) of packed, washed red cell ghosts containing 2.55 mg of membrane protein were diluted to a final volume of 4.0 ml with 5P(8) with or without pCMBS at a final concentration of 1 mM. Incubations were carried out for 60 and 120 min at the indicated temperatures and the tubes were rapidly chilled to 4° and centrifuged at 40,000*g* for 20 min. Supernates were recovered by careful aspiration and protein determined on an appropriate aliquot.

labeling of the transport protein with NEM[1] (Fox and Kennedy, 1965; Fox *et al.*, 1967).

Much information has been accumulated on the role of sulfhydryl groups in red cell membranes (Rothstein, 1971), and distinct "classes" of sulfhydryl groups have been defined on the basis of their rates of reaction and accessibility to different sulfhydryl reagents. Godin and Schrier (1972) have shown that a variety of red cell membrane-associated enzymes demonstrate marked variability to inhibition by different sulfhydryl binding agents. These authors suggested that many of the effects seen could relate to general changes in membrane structure and not to specific enzyme inhibition. The possibility that free sulfhydryl groups play a more general role in membrane structure has been previously suggested (Robinson, 1966). The demonstration that certain sulfhydryl blocking agents release a fraction of red cell ghost proteins in soluble form (Smith and Verpoorte, 1970; Godin and Schrier, 1972) lends further support to this idea. The significance of inhibition of any particular function by sulfhydryl blockade thus becomes difficult to interpret. We decided to examine this more general effect of a sulfhydryl reagent on ghost membranes in greater detail.

We report here the selective extraction of certain classes of red cell membrane proteins by the sulfhydryl binding agent pCMBS.

Materials and Methods

Outdated human blood in anticoagulant acid citrate dextrose (ACD) solution obtained from a blood bank was used for all studies; preliminary experiments showed no significant differences between freshly drawn and aged human red cells in these experiments. Red cell ghosts, grossly free of hemoglobin, were prepared by hypotonic lysis in 5P(8) by the method of Dodge *et al.* (1963) as modified by Fairbanks *et al.* (1971). Final washed suspensions contained 2–3 mg of membrane protein/ml.

Polyacrylamide gel electrophoresis of membrane and solubilized proteins in 1% sodium dodecyl sulfate, protein staining with Coomassie brilliant Blue, and glycoprotein staining with periodic acid–Schiff reagent were carried out as described by Fairbanks *et al.* (1971). Numbering of major peptide and glycoprotein bands follows their assignment (Fairbanks *et al.*, 1971) and is indicated in Figures 1 and 3.

For studies of glucose uptake, packed red cell ghosts were suspended in 5P(8) containing 0.25 M sucrose and were digested with trypsin (10 μg/ml) for 1 hr at 23°. The reaction was stopped by adding a fivefold excess of soybean trypsin inhibitor and fragmentation of the ghosts completed by rapid passage through a fine-bore hypodermic needle (no. 27). The resulting suspension of membrane vesicles was diluted with 5P(8)–0.25 M sucrose to a final protein concentration of 0.2–0.3 mg/ml.

Glucose uptake was measured at 23° as follows: to a 225-μl reaction mixture containing 35–40 μg of membrane protein, with or without pCMBS and DTT,[1] was added 25 μl of a mixture of L-[^{14}C]glucose and D-[^{3}H]glucose, each at 50 mM (final concentration in reaction mixture 5mM each). At the indicated times, 50-μl aliquots were rapidly filtered over prechilled Millipore filters (0.45-μ pore size) and the pellets were washed with 7 drops of ice-cold Krebs–Ringer–phosphate buffer. The filters were dried and added directly to counting vials, and radioactivity was determined in 10 ml of a toluene–ethanol scintillation solution (Buhler, 1962) after dispersion in 0.2 ml of water. Only the difference in uptake between D- and L-glucose is reported. A detailed description of the above procedure, including the demonstration that this provides a measure of glucose transport into closed membrane vesicles, has been given (Avruch *et al.*, 1972) and is based on earlier work demonstrating glucose transport in isolated adipose cell membranes (Carter and Martin, 1969b).

Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine albumin (Pentex, Inc.) as standard. Phospholipids were determined by a modification (Newman *et al.*, 1961) of the method of Bartlett (1959).

Results

Extraction of Ghost Proteins with pCMBS. As demonstrated in Table I, between 24 and 28% of red cell ghost protein was extracted by 1 mM pCMBS. In other experiments as much as 40% of the total protein was eluted. As shown (Table I), prolonging the incubation or increasing the temperature did not significantly increase the extraction with pCMBS, but there was a progressive rise in the amount eluted by buffer alone. At 37°, with prolonged incubation, virtually as much protein was extracted by 5P(8) alone as by pCMBS. Extraction with NEM, at concentrations as high as 5 mM, on the other hand, led to release of no more protein than extraction with buffer alone at the same temperature.

Treatment of red cell membranes with concentrations of pCMBS as low as 5×10^{-5} M led to release of small amounts of protein (Table II). There was a progressive increase in the amount of protein extracted with increasing concentrations of the sulfhydryl reagent up to 1 mM; beyond this there was little further release of membrane protein.

Polyacrylamide Gel Electrophoresis of pCMBS-Treated Red Cell Ghosts. Figure 1 shows the gel patterns of extracts and remaining membranes after exposure of red cell ghosts to 1 mM pCMBS at 15° compared to untreated membranes. There was extensive release of all the major peptides in the ghost membrane except for the one present in the greatest amount. This peptide (band III), which always appeared in electrophoresis as a broad band with a sharp leading and

[1] Abbreviations used are: NEM, *N*-ethylmaleimide; pCMBS, *p*-chloromercuribenzenesulfonic acid; 5P(8), 5 mM sodium phosphate buffer, pH 8.0; DTT, dithiothreitol (Cleland's reagent).

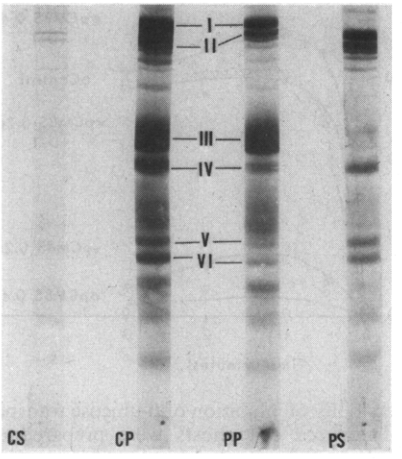

FIGURE 1: Polyacrylamide gel patterns of membranes and extracted proteins. Red cell ghosts were extracted for 60 min at 15° as described in Table I. Electrophoresis was done on 100 μl of a 4:10 dilution of the supernates and on 50 μl of a 4:10 dilution of the packed, extracted membranes, and the gels were stained with Coomassie Blue: CS, control supernate; CP, control pellet; PP, pCMBS-extracted pellet; PS, pCMBS supernate. The roman numerals indicate the six major peptide bands.

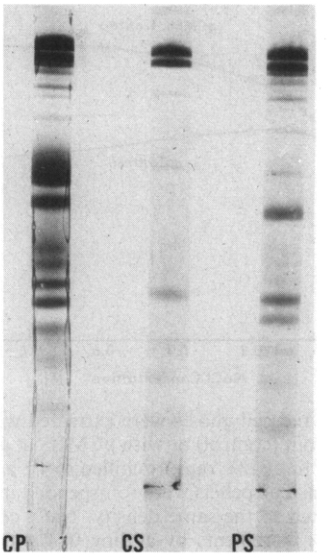

FIGURE 2: Effect of temperature on extraction of ghost membranes. Conditions were the same as Figure 1 except extraction was done at 37° for 60 min: CP, control pellet; CS, control supernate; PS, pCMBS extract.

diffuse trailing edge, was never extracted from the membrane under any of the conditions of temperature or pCMBS concentration used.

As noted above, at higher temperatures a considerable amount of membrane protein was released by buffer extraction alone. Gel patterns of these solubilized proteins were somewhat different from those obtained with pCMBS extraction; the buffer extracts (Figure 2) consisted almost entirely of the two highest molecular weight components (bands I and II) and the larger of the two major low molecular weight bands (band V). Only with pCMBS extraction did significant amounts of bands IV and VI appear in the eluate. We never succeeded in obtaining quantitative elution of any of the fractions, although in some experiments only traces of band V were left in the pellet.

The glycoproteins of human red cell membranes appear as one major and two minor fractions on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Fairbanks *et al.*, 1971). Of these, only the component present in the smallest amount was affected by pCMBS extraction. As shown in Figure 3, this band disappeared from the pellet following extraction but was not detected in the supernate, possibly because of its very low concentration. The bulk of the glycoproteins remained in the ghost membranes during all extractions.

Characteristics of pCMBS-Treated Membranes. As observed under phase contrast microscopy, hypotonically lysed ghosts retained the characteristic dumb-bell shape of the parent red cell despite repeated washing and sedimentation. Treatment with the sulfhydryl reagent led to marked disruption of the ghost morphology. After extraction with 1 mM pCMBS for 30 min at 15°, followed by centrifugation and resuspension of the pellet, essentially no intact forms were observed. The membranes appeared as smooth, spherical, closed vesicles of varying sizes, none as large as the original ghosts.

There was no detectable release of phospholipid from the membrane during pCMBS extraction; as a result the treated

TABLE II: Extraction of Ghost Membrane Proteins with Varying Concentrations of pCMBS.[a]

pCMBS Concn (M)	Protein (mg)	Extracted (%)
0	0.060	1.4
5×10^{-5}	0.165	3.8
10^{-4}	0.345	8.0
2×10^{-4}	0.705	16.4
5×10^{-4}	1.020	23.7
10^{-3}	1.180	27.6

[a] Membrane protein (4.3 mg) in 4.0 ml of 5P(8) was treated with the indicated concentration of pCMBS for 30 min at 15°. The amount of protein extracted was determined as in Figure 1.

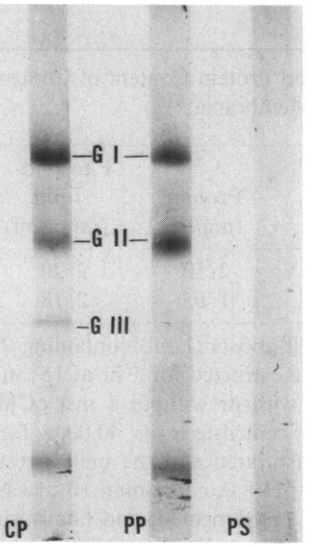

FIGURE 3: The glycoproteins of red cell ghosts before and after pCMBS extraction. Experimental conditions were the same as Figure 1 except twice as much membrane protein was applied to the gel and staining was with periodic acid–Schiff reagent: CP, control pellet; PP, pCMBS-extracted pellet; PS, pCMBS supernate.

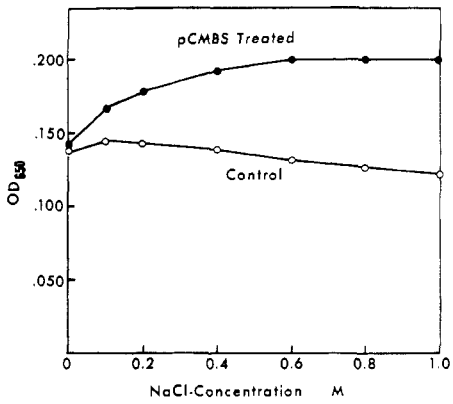

FIGURE 4: Packed red cell ghosts were extracted with an equal volume of 5P(8) without (control) or with pCMBS at a final concentration of 1 mM for 1 hr at 23°, rapidly chilled to 4°, and centrifuged at 40,000*g* for 30 min. The pellets were resuspended in 5P(8), placed in cuvets, and adjusted to the same density. NaCl concentration was increased in 0.1 M increments by adding 0.02 vol of 5.0 M NaCl; the tubes were mixed by inversion and OD_{650} was determined in a Coleman Jr. spectrophotometer after 60 sec.

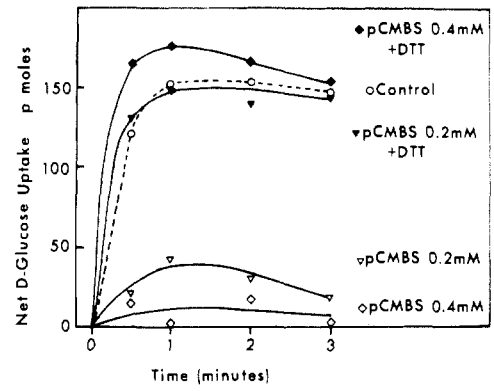

FIGURE 5: Reversibility of inhibition of D-glucose transport. Trypsin-treated, vesiculated red cell ghosts were prepared as described (Methods). Aliquots (0.2 ml) were incubated in duplicate with the indicated concentration of pCMBS. After 30 min at 23°, one of each pair was assayed for glucose uptake (Methods). To the other tube a tenfold excess of DTT was added and incubation was continued for another 30 min, following which glucose uptake was determined.

membranes had a considerably higher phospholipid-to-protein ratio than the controls (Table III).

After treatment with pCMBS, membrane vesicles were markedly affected by the addition of concentrated salt solutions. As observed under phase contrast microscopy, the vesicles became somewhat shrunken and showed a pronounced tendency to aggregate; untreated ghosts showed essentially no change. The effect of increasing concentrations of salt could be followed by changes in the optical density of the membrane suspension (Figure 4).

Following pCMBS extraction, the residual membranes were readily solubilized by increasing concentrations of the nonionic detergent Triton X-100 (Table IV). The untreated ghosts showed considerably more resistance to the effect of the detergent.

Of considerable interest was the fact that NEM-treated membranes showed the same spontaneous vesiculation, salt-induced aggregation, and detergent solubility, despite the fact that this sulfhydryl reagent did not lead to the release of more membrane protein than buffer alone (see above).

Reversible and Irreversible Changes in Ghost Membranes Induced by pCMBS Treatment. Addition of the potent reducing agent DTT to the reaction prior to pCMBS completely prevented the extraction of ghost membrane protein. Addition of DTT to membranes after pCMBS treatment had no effect on the extraction of membrane proteins. When pCMBS-treated ghosts were exposed to a tenfold excess of DTT for 1 hr at 23° prior to sedimentation, polyacrylamide gel electrophoresis of the separated membranes and extracts showed that all of the solubilized proteins remained in the supernate. These proteins thus showed no tendency to reassociate spontaneously with the residual membanes after extraction despite reduction of the mercaptides.

Alkylation of the membranes with 5 mM NEM for 1 hr before treatment with pCMBS did not prevent release of the membrane proteins. As noted above, NEM treatment by itself brought about the same changes in the physical state of the membrane as pCMBS but without the selective release of membrane protein.

TABLE III: Lipid and Protein Content of Control and pCMBS-Extracted Ghost Membranes.[a]

Membranes	Protein (mg/ml)	Phospholipid (μmol/ml)	Phospholipid/Protein (μmol/mg)
Control	2.50	2.20	0.88
pCMBS-treated	1.65	2.18	1.32

[a] Packed red cell ghosts (1 ml) containing 2.7 mg of membrane protein were extracted for 1 hr at 15° in a total volume of 4 ml of 5P(8) with or without 1 mM pCMBS. The tubes were chilled and centrifuged at 40,000*g* for 20 min. The supernates were aspirated and the pellets resuspended in 10 ml of 1 mM Tris, pH 8.0, containing 10 mM NaCl to remove phosphate buffer, resedimented, and finally suspended in 1.0 ml of the Tris-NaCl medium for determination of protein and phospholipid content. In this experiment, 0.2 mg of protein was eluted from the control membranes and 0.95 mg from the pCMBS-treated membranes.

TABLE IV: Effect of pCMBS Treatment on Membrane Solubility in Detergents.[a]

Triton X-100 (mg/ml)	Soluble Protein (mg/ml)	
	Control	pCMBS
0.1	0.06	0.23
0.2	0.09	0.29
0.5	0.25	0.65
1.0	0.46	0.86

[a] Ghosts were treated with or without 1 mM pCMBS for 30 min at 15°, chilled, and centrifuged at 40,000*g* for 15 min. After discarding the supernates the pellets were taken up in twice their original volume of 5P(8) and further adjusted to contain approximately equal protein concentrations. Aliquots (2.0 ml) of control (1.03 mg of protein/ml) and pCMBS-treated membranes (0.88 mg of protein/ml) were added to tubes containing appropriate volumes of a concentrated (10 mg/ml) solution of Triton X-100 to give the final indicated concentrations, mixed gently at room temperature, and sedimented at 40,000*g* for 30 min. Aliquots of the clear supernates were carefully aspirated and assayed for protein content.

Not all of the effects of pCMBS on red cell membranes were irreversible. We have shown previously (Avruch *et al.*, 1972) that vesicles prepared from trypsin-treated red cell ghost membranes show transport of D-glucose and that this transport is inhibited by NEM. Similar inhibition of transport was observed when membrane vesicles were exposed to pCMBS (0.2–0.4 mM) prior to glucose uptake, but this inhibition was completely reversed by the addition of DTT (Figure 5). At the higher concentrations of pCMBS used for the extraction experiments (1–2 mM), inhibition of glucose transport in trypsin-treated membrane vesicles was only partially reversed by DTT.

Discussion

The present experiments clearly demonstrate that sulfhydryl reagents can have profound effects on red cell membrane structure. Grossly, the ghost exposed to pCMBS or NEM is observed to lose its characteristic shape and vesiculate spontaneously. This is accompanied by an increase in susceptibility to salt-induced aggregation and to solution in detergents. The effects of salt are probably due to a reduction in the net negative charge on the external membrane surface following pCMBS treatment, perhaps by the formation of "inside-out" vesicles (Steck *et al.*, 1970); it has been clearly shown that the major negative charge of the membrane resides with the sialic acid residues exposed to the external surface of the intact cell (Eylar *et al.*, 1962). The increased solubility of the treated membranes in detergent implies a decrease in the strength of hydrophobic bonds. Cecil and Thomas (1965) have postulated that free sulfhydryl groups are involved in hydrophobic interactions, and Heitmann (1968) has provided direct evidence for this view. Cysteine side chains may thus be important in apolar regions of the protein that are intimately associated with the lipid core of the membrane (Korn, 1966; Wallach and Gordon, 1968). Whatever the mechanism, it is clear that free –SH groups are important in maintaining the structural integrity of the red cell membrane. The changes in physical characteristics are not due simply to the introduction of a net negative charge into the membrane by pCMBS, as suggested by Godin and Schrier (1972), since they occur following treatment with the uncharged reagent NEM.

In addition, pCMBS but not NEM leads to the selective release of certain classes of membrane peptides. It is less certain that this action involves SH groups. Pretreatment with NEM does not prevent this effect, and it has been reported (Rothstein, 1970) that NEM reacts with more SH groups in red cell ghosts than does pCMBS. However, since both agents react only with a fraction of the total membrane sulfhydryls (van Steveninck *et al.*, 1965; Rothstein, 1970), it is possible that pCMBS attacks certain classes of SH groups not available to NEM. The ability of pretreatment with a competing thiol, DTT, to prevent the protein release also suggests that membrane sulfhydryls are involved.

The eluted peptides are not released as membrane fragments, since no phospholipid release is observed following pCMBS treatment. Recent elegant studies by Steck *et al.* (1970, 1971) and Steck (1972) have shown that only one major peptide (band III) and one major glycoprotein (band GI) appear to traverse the entire membrane; these are the only residues not extracted by pCMBS. The peptides released by pCMBS are those that can also be eluted by simple changes in ionic strength (Mitchell and Hanahan, 1966; Rosenberg and Guidotti, 1969; Fairbanks *et al.*, 1971) or by dilute alkali with β-mercaptoethanol (Steck, 1972) and are probably superficially attached to the inner membrane surface (Steck, 1972).

Since NEM does not lead to specific loss of membrane proteins, the effect of pCMBS here may be partly related to the introduction of a net negative charge into the surface portions of the membrane.

The present results confirm the reports of Smith and Verpoorte (1970) and Godin and Schrier (1972) on the elution of red cell membrane proteins with sulfhydryl reagents. Free SH groups appear to play an important role in maintaining membrane structure. In view of the extensive changes induced by sulfhydryl reagents, interpretation of specific inhibitory effects on membrane function remains speculative.

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Optimal Conditions for Amino Acid Incorporation by Isolated Rat Liver Mitochondria. Stimulation by Valinomycin and Other Agents†

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ABSTRACT: Amino acid incorporation by isolated rat liver mitochondria proceeded at the same rate when either succinate plus ATP or an ATP generating system containing phosphoenolpyruvate was used as an energy source. Antimycin A caused a 90% inhibition of the incorporation rate when succinate was present suggesting that ATP was largely synthesized by respiratory chain-linked phosphorylations. In contrast, atractyloside caused a 70% inhibition of the incorporation rate, when ATP and phosphoenolpyruvate were present, suggesting that the ATP necessary for protein synthesis under these conditions had been previously transported across the mitochondrial membrane by the atractyloside-sensitive adenine translocase. The inhibitory effects of atractyloside in the ATP–P-enolpyruvate system could be completely reversed by the addition of glutamate or succinate. Substances which affect the adenine translocase such that the transport of ATP into the mitochondria is increased also stimulated the rates of leucine incorporation. Addition of low concentrations of the ionophore valinomycin (1 μg/mg of protein) stimulated amino acid incorporation two- to threefold provided that KCl was present. Phosphate was not required for the valinomycin stimulation. Valinomycin caused identical or even greater stimulations of the incorporation rate when antimycin A or the uncoupler, carbonyl cyanide phenylhydrazone (CCP), was also present. Low concentrations of CCP doubled the rate of amino acid incorporation provided that oligomycin was also present. Calcium ions also stimulated the incorporation rate when the ATP concentration was lowered to 0.2 mM. Atractyloside, the inhibitor of the adenine translocase, prevented the stimulation of amino acid incorporation by all these substances. These results suggest that ATP present in the matrix of the mitochondria whether generated by respiration or previously transported across the membrane is necessary for optimal rates of amino acid incorporation in rat liver mitochondria. Any substances which affect the intramitochondrial ATP level will increase the incorporation rate.

Investigators in many laboratories have established the conditions necessary to study amino acid incorporation into protein by isolated mitochondria *in vitro* (Beattie, 1971). The many different, and often, conflicting factors which have been reported to influence the rate of incorporation may be a reflection of the different mitochondrial preparations used by the various groups rather than any intrinsic differences in the incorporation mechanism. The recent publications (Hamberger *et al.*, 1969; Coote and Work, 1971; Williams and Birt, 1971a, 1971b) of incorporation media which varied significantly from that defined in our previous studies (Beattie *et al.*, 1967a) prompted us to reinvestigate the conditions necessary to achieve optimal rates of amino acid incorporation with isolated mitochondria.

The results obtained suggest that the ionic composition of the medium is critical for optimal rates of amino acid incorporation by either intact mitochondria or the isolated inner membrane–matrix fraction. The substitution of sodium or ammonium ions for potassium ion in the medium resulted in greatly lowered rates of incorporation. A need for a source of energy to support amino acid incorporation has been generally accepted by workers in this field (Beattie, 1971). In the present study, we observed that nearly identical rates of incorporation could be obtained if the necessary ATP was either generated by respiratory chain-linked phosphorylations or by the addition of an external ATP-generating system. In the latter case the exogenous ATP had to be first transported across the inner mitochondrial membrane by the adenine translocase (Klingenberg, 1970). The addition of substances such as valinomycin (plus potassium), low concentrations of calcium, or uncouplers of oxidative phosphorylation which affect the adenine translocase such that a higher intramitochondrial concentration of ATP results caused a large increase in the rate of amino acid incorporation.

Methods

Preparation of Mitochondria. Liver mitochondria were prepared under sterile conditions in a medium containing 0.25 M sucrose, 0.01 M Tris-chloride, pH 7.8, and 0.001 M EDTA (sodium salt) by previously described methods (Beattie, 1968) which yield a mitochondrial pellet which is 3% contaminated

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