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Effects of Optically Active 1-(α-Methylbenzyl)-3-(3,4-dichlorophenyl)urea on Reactions of Mitochondria and Chloroplasts[1]

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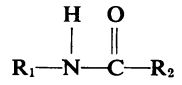
ABSTRACT

Effects of the R- and S-isomers and racemate of 1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea (MBPU) were measured on phosphorylation and electron transport in mung bean (*Phaseolus aureus* L.) mitochondria and spinach (*Spinacia oleracea* L.) chloroplasts.

In chloroplasts, S-MBPU inhibited basal and methylamine-uncoupled electron transport with ferricyanide as the oxidant, both photoreduction and coupled photophosphorylation with water as the electron donor and with ferricyanide and nicotinamide adenine dinucleotide phosphate (NADP) as oxidants, and cyclic photophosphorylation with phenazine methosulfate as the electron mediator under an argon gas phase. With ascorbate 2,6-dichlorophenolindophenol as the electron donor, phosphorylation coupled to NADP reduction was inhibited, but the reduction of NADP was not inhibited. The R-isomer of MBPU, like the S-isomer, inhibited all of the photophosphorylation reactions studied. However, unlike the S-isomer, the R-isomer either did not inhibit or was a very weak inhibitor of all photoreduction reactions. The effects of the MBPUs on the chloroplast reactions can be explained by action at two different sites: an optically specific site near photosystem II and the oxygen evolution pathway, and a second optically nonspecific site associated with the generation of ATP.

In mitochondria, both the R- and S-isomers stimulated state 4 respiration, inhibited state 3 respiration, and released oligomycin-inhibited respiration with malate, succinate, and NADH as substrates. Both enantiomers were equally active in all studies with malate and succinate as substrates. However, with NADH as substrate, R-MBPU was a stronger inhibitor of state 3 respiration and a weaker stimulator of state 4 respiration than S-MBPU.

Interference with photochemically induced electron transport in chloroplasts at a site closely associated with light reaction II and the oxygen evolution pathway has been demonstrated for a large number of structurally diverse chemicals. Some of the strongest and most extensively studied inhibitors of the Hill reaction are substituted amides which have the following general formula



In the strongest inhibitors, R_1 is an aromatic ring dichlorinated in positions 3 and 4, and R_2 is an alkylamino or alkyl group with dialkylamino derivatives being the more inhibitory (10, 22). Inhibitory activity is associated also with cyclic moieties such as cyclopropyl, furyl, morpholino, benzyl, piperidino, and chlorinated phenyl radicals substituted at R_2 (10, 22). From the diverse chemistry represented among the inhibitors of the Hill reaction, it is unlikely that all interfere at a common site through a similar mechanism. However, at this time, there is very little information available concerning the differential action of the diversely structured Hill inhibitors. Structure-activity studies should provide information on the molecular architecture of the active sites in the electron transport pathways and may assist in elucidation of the mechanisms through which inhibition is expressed.

In conjunction with continuing efforts to describe the geometry of sensitive sites in the electron transport and phosphorylation sequences of chloroplasts and mitochondria, a model system possessing an asymmetric carbon atom was selected for consideration. Hence, the R- and S-enantiomers and RS-racemate of 1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea were synthesized. Interferences imposed by the two enantiomers and racemate of MBPU[2] on various electron transport and phosphorylation reactions mediated by isolated chloroplasts and mitochondria were compared. Specifically, in chloroplasts, effects were measured on nonphosphorylating and uncoupled electron transport, on coupled photoreduction and photophosphorylation, and on cyclic

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[2] Abbreviations: MBPU: 1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)-urea; diuron: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS: phenazine methosulfate; DPIP: 2,6-dichlorophenolindophenol; DNP: 2,4-dinitrophenol; HOQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; and HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

photophosphorylation. In mitochondria, effects on state 3, state 4, and oligomycin-inhibited respiration were measured.

The effect of MBPU on the Hill reaction has not been reported previously. Good (10), however, synthesized and reported that 1-benzyl-3-(3,4-dichlorophenyl)urea was 100 times less active than diuron, based on a comparison of pI_{50} values.

MATERIALS AND METHODS

The R- and S-isomers and RS-racemate of MBPU were synthesized by allowing equimolar quantities of 3,4-dichlorophenylisocyanate to react with the appropriate α-methylbenzylamine in dry benzene at room temperature for 120 hr. Subsequently, the urea precipitates were removed by filtration and recrystallized from ethyl acetate. The starting R- and S-amines produced optical rotations of $[\alpha]_D^{25}$ +39.2° and −40.1° (2 g/100 ml benzene), respectively, as compared to literature values of +41.5° and −42.3° (14). Uncorrected melting points, as determined with a Thomas-Hoover[3] capillary apparatus, were 149 to 150, 171 to 172, and 171 to 172 C for the racemate, and R- and S-isomers, respectively. Optical rotations of $[\alpha]_D^{25}$ +65.5° and −64.7° (1.2 g/100 ml dimethylsulfoxide) were measured for the R- and S-isomers, respectively, in a Perkin-Elmer 141 Polarimeter. Elemental analyses for C, H, and N were within ±0.2% of the theoretical values.

Once-washed chloroplasts were prepared from commercially obtained spinach leaves (*Spinacia oleracea* L.) essentially as described by Avron (2). With the exception of the chloroplasts used to measure cyclic photophosphorylation, ascorbate was omitted from the washing and resuspension medium. Chlorophyll concentration was determined by the method of Arnon (1). Reaction mixtures, in a final volume of 5.0 ml, contained in μmoles: tris (pH 8.0), 80; NaCl, 50; $MgCl_2$, 10; Na_2HPO_4 (pH 8.0), 2; ADP, 5; chloroplasts containing approximately 75 μg of chlorophyll; MBPU in the amounts identified in the text; electron mediator where used (PMS, 0.2; NADP, 2; potassium ferricyanide, 2; or ascorbate, 20, + DPIP, 0.3, + diuron, 0.005). Saturating amounts of ferredoxin were also added when NADP reduction was being followed. Phosphorylation reagents (ADP, Mg^{2+}, and P_i) were omitted from the reaction mixture when basal electron transport was studied. Methylamine (30 μmoles) was added to the basal reaction mixture to measure effects on uncoupled electron transport.

Reactions were conducted in small beakers which were illuminated from below at an intensity of 30,000 lux. Temperature of the reaction mixture during illumination was maintained at 25 C. Cyclic photophosphorylation reactions were conducted in 50-ml Erlenmeyer flasks modified to permit purging with argon. Flasks were flushed for 2 min with argon prior to illumination and continuously during the illumination period.

Reactions were terminated by the addition of 1.0 ml of 10% trichloroacetic acid, and the precipitated protein was removed by centrifugation. Ferricyanide reduction in the supernatants was measured as a decrease in absorbance at 420 nm, and P_i uptake was determined colorimetrically (26) to provide an estimation of the esterification of P_i. Reduction of NADP was measured at 340 nm prior to the addition of trichloroacetic acid.

Mitochondria were isolated from 3- to 4-day-old dark-grown mung bean (*Phaseolus aureus* L. var. Jumbo) hypocotyls essentially as described by Ikuma and Bonner (16). Both the grinding and washing media, however, were supplemented with 0.02 M HEPES buffer (pH 7.1). The 2.0-ml reaction mixture contained: 0.3 M mannitol; 0.01 M KCl; 0.01 M potassium phosphate buffer (pH 7.1); 0.5 mM $MgCl_2$; 16 μmoles of succinate, 0.2 mmole of malate or 2.2 μmoles of NADH; mitochondria containing approximately 0.8 mg of protein; and ADP and test chemicals as identified in the text. Oxygen uptake was measured polarographically with a Clark electrode in a 2-ml, water-thermostated glass reaction cell maintained at 25 C. Oxygen content of the air-saturated media was calculated to be 236 mμmoles/ml. Protein was estimated, after solubilization of the mitochondria with NaOH, by the Lowry procedure (21).

In the presentation of data on oxygen utilization by mitochondria, state 3 will indicate respiration that occurred in the presence of excess ADP and state 4 will refer to ADP-limited respiration, in accordance with the nomenclature of Chance and Williams (6). In experiments in which uncoupling activity was measured, a limited amount of ADP was added to the reaction medium to obtain a state 3 to state 4 transition upon the depletion of ADP. The chemical under study was added approximately 2 min after the commencement of state 4 respiration. In calculating the degree of uncoupling obtained (% stimulation), the effectiveness of the test chemical was compared to that of ADP in the promotion of oxygen utilization; hence, 100% stimulation equals the increase obtained with ADP. Effects on state 3 respiration were measured by addition of the MBPU approximately 1 min after the introduction of excess ADP and the establishment of linearity. Percentage inhibition was calculated by relating the inhibited rate of oxygen utilization to the state 3 control rate.

Stock solutions of MBPU were prepared in acetone for the mitochondrial studies and in ethanol for the chloroplast studies. The final concentration of the solvents in all assays, including the controls, was held constant at 1% by volume. This concentration of acetone had no detectable effect on the mitochondrial responses. However, in the chloroplast studies, 1% ethanol produced from 5 to 8% inhibition, compared to the water controls, with the phosphorylation reactions being slightly more sensitive than the reduction reactions. Sensitivity of photophosphorylation reactions to organic solvents has also been noted by other investigators (4, 30).

Data reported for the mitochondrial studies were averaged from determinations made with a minimum of three separate isolations. Data for the chloroplast studies were arithmetic averages of duplicate determinations obtained with each of three separate chloroplast extractions.

RESULTS

Chloroplasts. Effects expressed by the R- and S-isomers and RS-racemate of MBPU on several photoreactions mediated by chloroplasts are presented in Table I. Data are presented as I_{50} values (molar concentrations required to inhibit the reactions by 50%) which were obtained from concentration-response curves, developed for the several reactions, or as percentage inhibition (in parentheses) measured at 30 μM for those reactions for which I_{50} values could not be obtained. Averaged specific activities (μmoles of product formed per mg of chloroplast chlorophyll per hr) obtained in the presence of 1% ethanol are also presented in Table I.

With water as the electron donor, and ferricyanide or NADP as oxidants, the S-isomer was a much stronger inhibitor of electron transport than the R-isomer, under nonphosphorylating, uncoupled, and coupled conditions (Table I; reactions A, B, C, and E). Except for the methylamine-uncoupled reaction (reaction B), the R-isomer either expressed no effect or was a relatively weak inhibitor. The R-isomer did not inhibit basal electron transport with ferricyanide as the electron acceptor (reaction A) and only marginally inhibited coupled NADP photoreduction (reaction E). The inhibitory action of the racemate, which is a 1:1 mixture of

[3] Mention of trade products, equipment, or commercial companies does not imply endorsement by the United States Department of Agriculture, the North Carolina Agricultural Experiment Station, or the Virginia Commonwealth University, over similar products or companies not named.

Table I. *Effects of R-, S-, and RS-1-(α-Methylbenzyl)-3-(3,4-dichlorophenyl)urea on Photoreactions of Spinach Chloroplasts*

Reaction Code	Electron Mediator—Measurement	Specific Activity	Isomer or Racemate[1]		
			R	S	RS
		μmoles/mg chlorophyll·hr			
A	Ferricyanide—reduction (basal)	300	(0)	2.0	4.2
B	Ferricyanide (with methylamine)—reduction	1000	8.1	0.6	1.1
C	Ferricyanide—reduction	523	30.0	1.2	3.1
D	Ferricyanide—phosphorylation	220	2.5	0.5	1.7
E	NADP—reduction	79	(10)	6.2	14.0
F	NADP—phosphorylation	76	2.9	1.6	3.0
G	NADP (with ascorbate + DPIP)—reduction	20	[2]	[2]	[2]
H	NADP (with ascorbate + DPIP)—phosphorylation	28	5.5	5.5	5.5
I	PMS (argon)—phosphorylation	201	4.0	4.0	4.0

[1] Data are presented as I_{50} molarities (μM) or as % inhibition (in parentheses) measured at 30 μM.

[2] No inhibition of NADP reduction at 15 μM, approximately 10% inhibition at 0.15 mM.

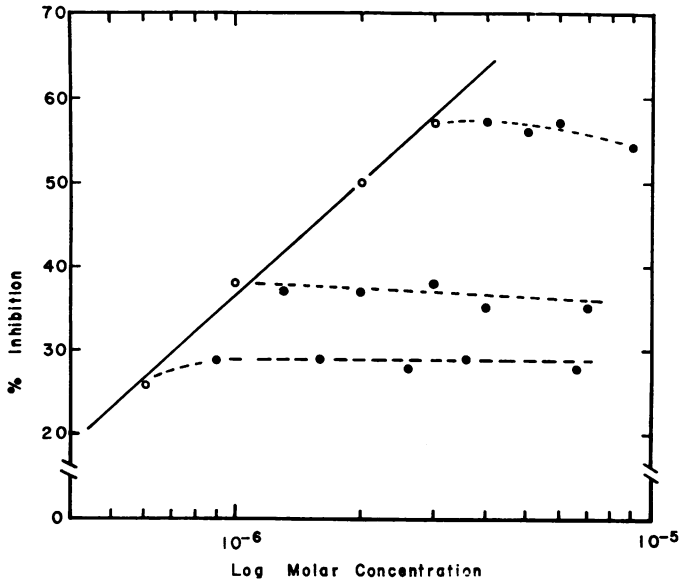

FIG. 1. Effects of the S-isomer of 1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea alone and in combination with the R-isomer on the photoreduction of ferricyanide by spinach chloroplasts under nonphosphorylating conditions. See text for details. ○: Inhibition response curve for the S-isomer alone; ●: inhibition measured for different concentrations of the R-isomer added to 0.6, 1.0, and 3.0 μM concentrations of the S-isomer, plotted as the total molar concentration of MBPU.

the R- and S-isomers, can be explained by the concentration of the S-isomer. Theoretically, I_{50} values obtained with the racemate should be double those of the active S-isomer if it is assumed that the inactive R-isomer does not compete or interfere with the approach to, and action at, the reactive site in the chloroplasts of the inhibitory S-isomer. Reasonably close agreement with the expected value was obtained for the photoreduction reactions in which water served as the electron donor.

The influence on ferricyanide reduction under nonphosphorylating conditions of the inactive R-isomer on the behavior of the active S-isomer was studied also with physical mixtures of the two isomers (Fig. 1). The R-isomer by itself did not inhibit ferricyanide reduction at concentrations up to 10 μM. With the S-isomer present at concentrations of 0.6, 1.0, and 3.0 μM, physical mixtures were prepared with increasing concentrations of the R-isomer, so that the ratios of the active to inactive isomer ranged from 1:0.5 to 1:10. In Figure 1, inhibition is plotted as a logarithmic function of the molar concentration for the S-isomer by itself (solid line), and for the total molar concentration of the combined R- and S-isomer (dashed lines). Inhibitions produced by the mixtures did not exceed ±4% of the inhibition induced by the respective concentration of the S-isomer. Hence, only the S-isomer appears to be inhibitory to basal electron transport, and the R-isomer apparently does not compete or interfere with the S-isomer for the reactive site.

Neither of the two isomers nor the racemate, at 15 μM, inhibited the photoreduction of NADP with ascorbate-DPIP serving as the electron donor when diuron (1.0 μM) was used to block light reaction II (Table I; reaction G). However, a negligible but reproducible amount of inhibition (10%) was produced by all three MBPUs at 0.15 mM.

Photophosphorylation did not show the strong isomeric specificity that was manifested by the photoreduction reactions. Potent inhibition of coupled photophosphorylation was obtained with the R- and S-isomers and the racemate with water as electron donor and ferricyanide and NADP as electron acceptors (Table I; reactions D and F). As indicated by the lower I_{50} values, the photophosphorylation reactions were considerably more sensitive than the coupled photoreduction reactions. Whereas the R-isomer was a relatively poor inhibitor of the photoreduction reactions, it did inhibit the coupled photophosphorylation reactions. However, the S-isomer was a slightly better inhibitor than the R-isomer as evidenced by the lower I_{50} values (Table I; reactions D and F). The I_{50} values for the RS-racemate approximated the average of the I_{50} values obtained for the R- and S-isomers (reactions D and F). Hence, the R-isomer in the racemate contributed to the inhibition of the coupled photophosphorylations, whereas it did not contribute to the inhibition of the photoreduction reactions.

When light reaction II was inhibited with diuron, and the ascorbate-DPIP couple served as the electron donor, and NADP was the electron acceptor, the R- and S-isomers and the racemate inhibited photophosphorylation to the same extent (Table I, reaction H). Cyclic photophosphorylation was also inhibited equally by all three compounds (reaction I). No stereospecific requirement was shown by these photophosphorylation reactions.

Mitochondria. Representative recorder tracings which reflect the utilization of oxygen by mung bean mitochondria under various conditions are presented for illustrative purposes in Figure 2. The pattern of succinate oxidation by mung bean mitochondria showing a stimulation of respiration upon the addition of ADP (state 3) followed by a decrease in respiration when the added ADP (state 4) is exhausted, is shown in trace A. With malate, NADH, and succinate as substrates, respiratory control (R/C) ratios (state 3 rate divided by state 4 rate) of the mitochondria used in this study averaged 4.3, 4.0, and 2.5; and calculated ADP/O ratios averaged 2.3, 1.4, and 1.6, respectively. These values are in reasonably close agreement with data reported previously by Ikuma and Bonner (16) for mung bean mitochondria.

R-, S-, and RS-MBPU stimulated state 4 respiration at a low molar concentration, inhibited state 3 respiration at a higher concentration, and circumvented oligomycin-inhibited state 3 respiration with all three substrates (succinate, malate, and NADH). Representative tracings, which reflect the above responses, ob-

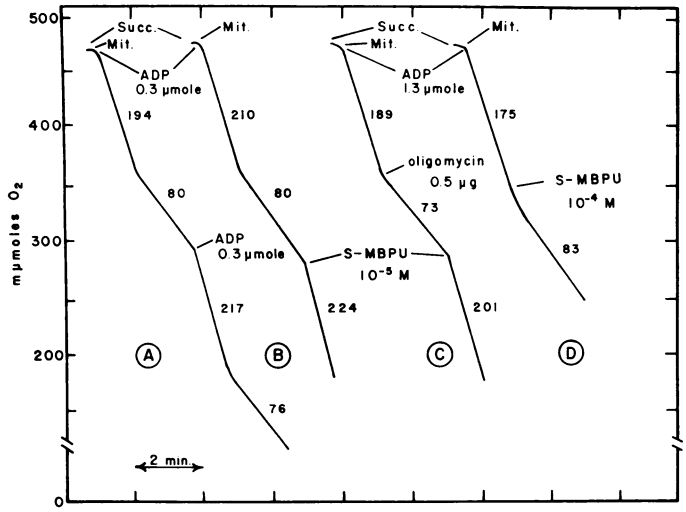

FIG. 2. Representative polarographic traces depicting oxygen utilization obtained with mung bean mitochondria for succinate oxidation. A: Respiratory control obtained in the absence of an inhibitor; B: stimulation of state 4 respiration by S-MBPU (10^{-5} M); C: circumvention of oligomycin-inhibited respiration by S-MBPU (10^{-5} M); D: inhibition of state 3 respiration by S-MBPU (10^{-4} M). Rates of oxygen utilization (mμmoles O_2 per min per 2 ml) are indicated above the traces. Mitochondria (Mit) containing 0.8 mg of protein, succinate (16 μmoles), ADP, oligomycin, and S-MBPU were added at the points indicated. Concentrations of components are shown as μmoles (succinate and ADP), or μg (oligomycin) supplied to, or as the final molarity (S-MBPU) in, the 2-ml reaction medium.

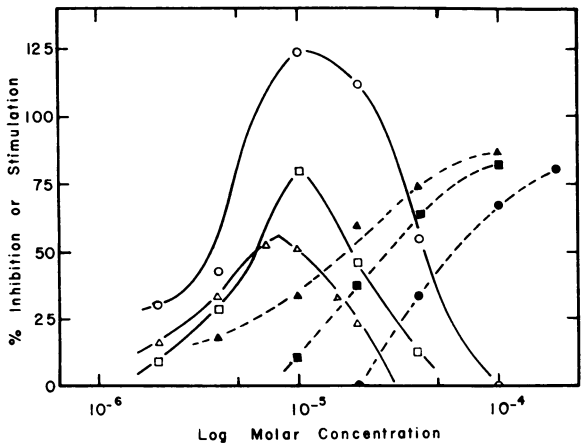

FIG. 3. Effects of R-, S-, and RS-1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea on the oxidation of NADH by mung bean mitochondria. Stimulation of state 4 respiration by the R-isomer (△), S-isomer (○), and RS-racemate (□); inhibition of state 3 respiration by the R-isomer (▲), S-isomer (●), and RS-racemate (■).

tained with S-MBPU for the oxidation of succinate are shown in Figure 2 (traces B, C, and D). Marked isomeric differences were apparent only for the oxidation of NADH (Fig. 3). Data used to develop the curves in Figure 3 were obtained from traces such as shown in Figure 2 and were derived as described under "Materials and Methods." Stimulation of state 4 respiration was initiated at a low concentration of the compound, increased with the concentration of the compound, and then declined as the concentration was further increased to provide a parabolic or bell-shaped curve (Fig. 3). Within the stimulatory range, inhibition of state 3 respiration could be detected. Hence, the higher concentrations of the enantiomers and racemate both stimulated state 4 and inhibited state 3 respiration. The S-isomer was a stronger stimulator of state 4 respiration (125 *versus* 55%) and a weaker inhibitor of state 3 respiration (I_{50} = 60 *versus* 17 μM) than the R-isomer. The action of the racemate (Fig. 3) was intermediate between that of the R- and S-isomers with respect to the maximal amount of stimulation produced (80%), and the I_{50} value for inhibition of state 3 respiration (28 μM). Inhibition of state 3 respiration is generally considered to reflect interference with electron transport, whereas the state 4 response is considered to measure an effect imposed on a component in the ATP-generating sequence. The occurrence of "overlapping" effects, *i.e.*, inhibition of state 3 and stimulation of state 4 respiration at the same molar concentration, can be expected to reduce the actual or apparent uncoupling capabilities of a compound because the capacity for utilizing oxygen has been reduced by a competing reaction.

Table II. *Circumvention of Oligomycin-inhibited Respiration of Mung Bean Mitochondria by Enantiomers and Racemate of 1-(α-Methylbenzyl)-3-(3,4-dichlorophenyl)urea*

Substrate	Isomer or Racemate[1]	State 3 Respiration		
		Uninhibited control	Oligomycin[2]	Oligomycin + isomer or racemate
		mμmoles O_2 per min per 2 ml		
Malate	R=S=RS	208	38	192
Succinate	R=S=RS	154	63	170
NADH	R	199	45	113
	S	201	46	166
	RS	204	46	140

[1] Concentration = 10 μM.
[2] Oligomycin concentration = 0.5 μg.

The R- and S-isomers and racemate of MBPU also stimulated state 4 and inhibited state 3 respiration during the oxidation of both malate and succinate. No marked differences were present in the inhibitory patterns; hence, optical specificity was not correlated with response as it was for the oxidation of NADH. Response curves developed for the three MBPUs for stimulation of state 4 and inhibition of state 3 respiration for the oxidation of malate and succinate approximated those of the S-isomer for the oxidation of NADH (Fig. 3) with respect to the shape of the state 4 stimulation curve, the maximal amount of stimulation obtained, and the I_{50} value for inhibition of state 3 respiration.

The enantiomers and racemate of MBPU were tested for their ability to circumvent oligomycin-imposed inhibition of state 3 respiration when added as shown in Figure 2 (trace C) for the oxidation of malate, succinate, and NADH. Shown in Table II are the initial state 3 respiration rates measured prior to the introduction of oligomycin, the decreased rate following the addition of 0.5 μg of oligomycin, and the rate of oxygen utilization obtained after 10 μM MBPU was added to the oligomycin-inhibited mitochondria. None of the MBPUs at 10 μM inhibited state 3 respiration with malate and succinate as substrates. For the oxidation of NADH, slight inhibition of state 3 respiration was obtained with R-MBPU (30%) and RS-MBPU (10%) (Fig. 3). R-, S-, and RS-MBPU circumvented oligomycin-inhibited respiration of malate and succinate (Table II), and no differences in their behavior were evident. However, some slight optically related differences were obtained with NADH as substrate. The circumvention of oligomycin-inhibited respiration was greatest for the S-isomer, least for the R-isomer, and intermediate for the racemate (Table II). The failure of the R-isomer and racemate to circumvent the oligomycin-imposed block as fully as the S-isomer correlates with their inhibition of state 3 respiration at 10 μM. The use of lower concentrations of the MBPUs in the circumven-

tion experiments, which did not inhibit state 3 respiration with NADH as substrate, eliminated the optically related differences.

DISCUSSION

S-MBPU inhibited basal electron transport with ferricyanide as the oxidant, both photoreduction and coupled photophosphorylation with water as the electron donor and ferricyanide and NADP as oxidants, and cyclic photophosphorylation. With ascorbate-DPIP as the electron donor, phosphorylation coupled to NADP reduction was inhibited, but the reduction of NADP was not inhibited. The R-isomer of MBPU, like the S-isomer, inhibited all of the photophosphorylation reactions studied. However, unlike the S-isomer, the R-isomer either did not inhibit or was a very weak inhibitor of all photoreduction reactions. Methylamine-uncoupled electron transport was the most sensitive of the photoreduction reactions to R-MBPU. However, treatment of chloroplasts with methylamine has been reported to produce irreversible structural alterations (17). Even under these conditions, the S-isomer was still approximately 16 times more active than the R-isomer based on a comparison of the I_{50} values (Table I). The action of the racemate could be explained, for the most part, by the concentration of the S-isomer present in the mixture. No evidence was obtained for interference by the inactive R-isomer with inhibition of basal electron transport by the active S-isomer (Fig. 1).

Effects of the MBPUs on the chloroplast reactions can be explained by action at two different sites: one near photosystem II and the oxygen evolution pathway and the second associated with the generation of ATP. Evidence for this suggestion was contributed by the increased sensitivity of photophosphorylations over those of the coupled photoreductions when water served as the electron donor; by the inhibition of photophosphorylation mediated by PMS in argon; and by the inhibition of photophosphorylation when light reaction II was blocked with diuron and ascorbate-DPIP was the electron donor. The second site of inhibition appears not to be located on the electron transport chain between ascorbate-DPIP and NADP but on a pathway unique to the cyclic system, because NADP photoreduction was not inhibited when ascorbate-DPIP served as the electron donor. Consequently, a site associated with the generation of ATP is implicated. Both enantiomers appeared to be equally inhibitory at the phosphorylation site, but the site associated with light reaction II showed optical specificity for the S-isomer. Chemical reactivity or partitioning characteristics would be identical for the two enantiomers; hence, the most probable explanation for the differences in inhibitory behavior must be related to steric relations between the two isomers and their approach to the reactive site associated with light reaction II and the oxygen evolution sequence. The receptor apparently can discriminate between the two isomers, and the R-isomer is repulsed sterically.

Compounds which, like S-MBPU, inhibit photoreduction and photophosphorylation with water as the electron donor, cyclic photophosphorylation, and photophosphorylation but not photoreduction with ascorbate-DPIP as the electron donor and NADP as the oxidant, all within a relatively narrow range of concentrations, include the halogenated 2-trifluoromethylbenzimidazoles (5, 11), salicylaldoxime (19, 24, 27), and 3,5-dibromo-4-hydroxybenzaldehyde *O*-(2,4-dinitrophenyl)oxime (23). The action of S-MBPU differs from that of the energy transfer inhibitors Dio-9 and phlorizin (11), and chlorotributyltin (18), in that inhibition of electron transport was not released by the uncoupler methylamine. On the contrary, electron transport became more sensitive and a lower I_{50} value was obtained with uncoupler-treated chloroplasts (Table I).

Interference by the MBPUs with the conservation of energy during oxidative phosphorylation was also demonstrated in this study. At low molar concentrations, the MBPUs stimulated ADP-limited oxygen utilization (state 4 respiration) and circumvented oligomycin-inhibited state 3 respiration. Hence, they expressed two of the actions characteristic of uncouplers (25). Oligomycin is considered to inhibit respiration by blocking the formation of a high energy intermediate required for ATP production (20, 25). Therefore, the site of interference of the MBPUs appears to be located prior to the oligomycin block, on the energy transfer pathway, at or near the site identified with the action of DNP. At higher molar concentrations, interference of the MBPUs with components of the electron transport chain was also implicated.

No differences in responses elicited by the R- and S-isomers were obtained for the oxidation of malate and succinate. However, optical specificity was manifested for the oxidation of NADH. The action of R-MBPU differed from S-MBPU in that state 4 respiration was not stimulated as strongly, and state 3 respiration was inhibited more strongly (Fig. 3). For R-MBPU, marked inhibition of state 3 respiration occurred at the molar concentration which maximally stimulated state 4 respiration. The inhibition of state 3 respiration probably masked the full expression of uncoupling action expressed on state 4 respiration. Only minimal overlapping was shown by S-MBPU (Fig. 3).

Oxidation of exogenously supplied NADH by the mung bean mitochondrial preparations used herein showed sensitivity to HOQNO, antimycin A, azide, and cyanide, and insensitivity to rotenone, which is in agreement with the observations of other investigators (3, 8, 12, 15). The P/O ratio of 1.4 also agrees with previously published values (3, 8, 12, 16) and implicates the involvement of only two coupling sites. The response to standard inhibitors and the P/O ratio suggest that oxidation through the electron-transport chain occurs subsequent to energy coupling site 1. However, cognizance should be extended to the availability of alternate pathways for NADH oxidation (29).

Bell-shaped response curves, such as shown in Figure 3, are produced characteristically by uncouplers of oxidative phosphorylation when oxygen utilization is measured polarographically (7, 9, 23). Chance *et al.* (7) postulated a mechanism involving a single site of action that would account for the observed stimulation and inhibition of oxygen uptake. In this hypothesis, inhibitor association and dissociation with high energy intermediates are considered to release a respiration-activating component. At low uncoupler concentrations, dissociation occurs readily and oxygen utilization is not inhibited; but at higher concentrations, dissociation becomes rate-limiting and respiration is inhibited. Chance *et al.* (7) envisioned a ternary complex being formed between carriers or intermediates and uncouplers such as DNP ($C \sim I \cdot DNP$ or $X \sim I \cdot DNP$). Other investigators have considered the formation of only a binary complex ($X \cdot DNP$) (13). Accumulation of the complexes would limit the availability of C, X, or I for recycling; hence, the rate of oxygen uptake would be decreased.

An alternate explanation can be proposed for the bell-shaped curves from the observations summarized by Weinbach and Garbus (28). The MBPUs, like the phenolic types of uncouplers (28), may bind to mitochondrial proteins. Proteins undergo conformational transitions as a consequence of this binding, which could result in structural disorganization and an altered function of the enzymes which catalyze the coupling of phosphorylation to electron transport. A conformational change could disrupt contact between the energy conservation sequence and the electron transport system. If this should happen, oxygen uptake would be increased because of the removal of the rate-limiting control exerted by ADP. At higher concentrations of the uncoupler, binding to additional proteins located on the electron transport pathway could be expected to cause conformational shifts which would prevent contact between components of the

electron transport sequence. Consequently, the rate of oxygen utilization would be decreased.

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