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1 : 1 : 3-TRICYANO-2-AMINO-1-PROPENE (U-9189), A NEW UNCOUPLING AGENT OF OXIDATIVE PHOSPHORYLATION*

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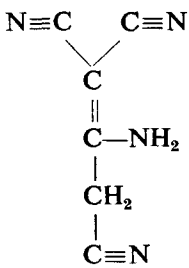
Abstract—1 : 1 : 3-Tricyano-2-amino-1-propene (U-9189) has been shown to uncouple phosphorylation associated with the oxidation of various substrates by rabbit liver mitochondria. It was similar to 2 : 4-dinitrophenol on the basis of its ability to stimulate oxidation in media deficient in inorganic phosphate or phosphate acceptors, to stimulate adenosine triphosphatase activity, or to prevent mitochondrial swelling. Although U-9189 contains both the nitrile and enamine functions, uncoupling was not found to be a general property of either class of compounds.

THE characterization of 1 : 1 : 3-tricyano-2-amino-1-propene (U-9198)† as a component of aqueous solutions of malononitrile recently has been described.[1, 2]



(U-9189)

Preliminary toxicological studies showed that U-9189 administered orally or intraperitoneally to rats produced a rapid and marked hyperthermia.[3] Since DNP also produces a similar hyperthermia, it was of interest to determine whether U-9189 would affect the oxidative–phosphorylation mechanism of isolated mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated by the method of Schneider[4] from livers of New Zealand albino rabbits (from 3 to 5 kg), unselected for sex, or from livers of male Sprague–

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† Abbreviations used: U-9189: 1 : 1 : 3-tricyano-2-amino-1-propene; ATP: adenosine triphosphate; ATPase: adenosine triphosphatase; DNP: 2 : 4-dinitrophenol; Tris: tris-(hydroxymethyl) aminomethane; RNA: ribonucleic acid.

Dawley (Stoner) rats (from 250 to 400 g). All animals were fed *ad libitum*. For measurement of P/O ratios or oxidation of substrates in deficient media, livers were homogenized in 0·25 M sucrose in a Waring blender for 5 sec at high speed and 10 sec at low speed. For measurement of ATPase activity, livers were homogenized in 0·25 M sucrose with a Potter–Elvehjem type homogenizer with smooth glass tube and Kel-F pestle. For mitochondrial-swelling studies, homogenates were prepared as for the ATPase experiments except that 0·33 M sucrose was used as a homogenizing and suspending medium.

P/O measurements were carried out at 30 °C by the method of Hunter.[5] ATPase measurements were made at 30 °C by the method of Lardy and Wellman.[6] Mitochondrial swelling was determined by following the change in absorbance at 520 mμ at 24 °C in a medium containing 0·33 M sucrose and 0·025 M Tris, pH 7·4.[7] Inorganic phosphate was determined by the method of Lowry and Lopez,[8] and protein was measured by a modification[9] of the procedure of Lowry *et al.*[10]

RESULTS

Uncoupling of oxidative phosphorylation

The curves illustrated in Fig. 1 show that U-9189 was an uncoupling agent of mitochondrial oxidative phosphorylation. Approximately 50 per cent uncoupling was

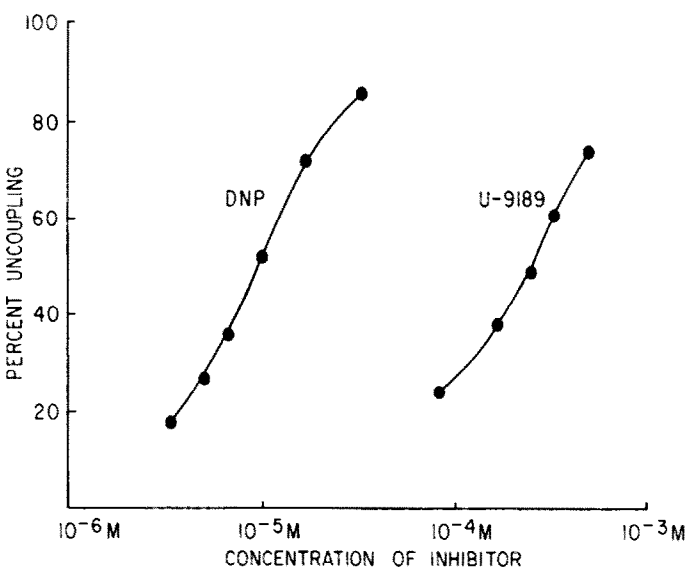


FIG. 1. Uncoupling of oxidative phosphorylation by DNP and U-9189. Complete system[5] plus malonate with α-ketoglutarate as substrate and rabbit liver mitochondria.

produced by $2{\cdot}5 \times 10^{-4}$ M U-9189 and 1×10^{-5} M DNP. Although DNP was twenty-five times more effective than U-9189 in decreasing P/O ratios, the shapes and slopes of the curves were similar. This order of activity places U-9189 in a category with other interesting uncoupling agents such as thiopentone, chlorotetracycline, 2 : 4-dichlorophenoxyacetic acid, etc.[5]

The uncoupling observed in the oxidation of α-ketoglutarate was not a specific effect related to this substrate. When concentrations of U-9189 and DNP which

produced equivalent uncoupling with α-ketoglutarate were tested with other substrates, similar results were obtained (Table 1). These data suggested that U-9189 might be a DNP-like uncoupling agent, which differed only in the concentration required to produce an equivalent effect.

TABLE 1. UNCOUPLING BY U-9189 AND DNP WITH DIFFERENT SUBSTRATES

Substrate	ΔO (μA/mg prot.)	ΔP (μM)	P : O	% uncoupling
α-Ketoglutarate	0·565	23·9	2·7	—
α-Ketoglutarate + U-9189	0·564	12·1	1·4	50
α-Ketoglutarate	0·523	26·3	2·9	—
α-Ketoglutarate + DNP	0·518	10·5	1·2	59
L-Glutamate	0·765	30·7	2·4	—
L-Glutamate + U-9189	0·776	17·3	1·3	44
L-Glutamate	0·675	31·1	2·2	—
L-Glutamate + DNP	0·681	16·2	1·2	48
Succinate	1·49	17·4	1·3	—
Succinate + U-9189	1·03	5·58	·6	55
Succinate	1·78	25·6	1·4	—
Succinate + DNP	1·28	7·51	·6	61
L-Malate+pyruvate	1·18	28·6	1·9	—
L-Malate+pyruvate+U-9189	1·16	20·4	1·3	32
L-Malate+pyruvate	1·23	23·3	2·0	—
L-Malate+pyruvate+DNP	1·13	11·8	1·2	43

Complete system[5] plus malonate with rabbit liver mitochondria. Malonate omitted in flasks with succinate. [U-9189]$=2{\cdot}5\times10^{-4}$ M, [DNP]$=10^{-5}$ M.

TABLE 2. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN PHOSPHATE-DEFICIENT MEDIUM

Enzyme source	Substrate	ΔO (μA/mg prot.)	% stimulation
Rabbit	α-ketoglutarate	0·127	—
Rabbit	α-ketoglutarate + U-9189	0·303	139
Rabbit	α-ketoglutarate + DNP	0·293	131
Rat	α-ketoglutarate	0·060	—
Rat	α-ketoglutarate + U-9189	0·125	108
Rat	α-ketoglutarate + DNP	0·193	222

Complete system[5] plus malonate; phosphate replaced by 60 μM Tris Buffer. [U-9189]$=2{\cdot}5\times10^{-4}$ M, [DNP]$=10^{-5}$ M.

Stimulation of mitochondrial oxidation in deficient media

In an attempt to characterize further the action of U-9189, it was compared with DNP for its effect on oxidation of substrate by mitochondria in media deficient in inorganic phosphate or phosphate acceptor. In these experiments both U-9189 and DNP were tested at concentrations which produced equivalent uncoupling (50 per cent). Table 2 shows that when inorganic phosphate was omitted from the medium, the addition of either U-9189 or DNP elicited an increased rate of oxidation. Equal stimulation of α-ketoglutarate oxidation by rabbit liver mitochondria was produced

by $2{\cdot}5\times10^{-4}$ M U-9189 and 1×10^{-5} M DNP. In the case of rat liver mitochondria, the effect observed with DNP was much greater than that obtained with U-9189.

When the system was made deficient in phosphate acceptor by omission of ATP, equivalent stimulation of oxidation was produced by the above mentioned concentrations of U-9189 and DNP using mitochondria from either rabbit or rat liver, with either α-ketoglutarate or L-glutamate as substrate (Table 3). In the L-glutamate system essentially maximal stimulation was obtained with three times the concentrations of U-9189 or DNP which produced 50 per cent uncoupling.

TABLE 3. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN ATP-DEFICIENT MEDIUM*

Enzyme source	Substrate	Molarity uncoupler	ΔO (μA/mg prot.)	% stimulation
Rabbit	α-ketoglutarate		0·124	—
Rabbit	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	0·158	28
Rabbit	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	0·151	22
Rat	α-ketoglutarate		0·544	—
Rat	α-ketoglutarate + U-9189	$2{\cdot}5\times10^{-4}$	0·737	36
Rat	α-ketoglutarate + DNP	$1{\cdot}0\times10^{-5}$	0·762	40
Rabbit	L-glutamate†		0·182	—
Rabbit	L-glutamate† + U-9189	$2{\cdot}5\times10^{-4}$	0·232	27
Rabbit	L-glutamate† + U-9189	$7{\cdot}5\times10^{-4}$	0·314	72
Rabbit	L-glutamate† + U-9189	$1{\cdot}5\times10^{-3}$	0·256	41
Rabbit	L-glutamate† + DNP	$1{\cdot}0\times10^{-5}$	0·232	28
Rabbit	L-glutamate† + DNP	$3{\cdot}0\times10^{-5}$	0·339	86
Rabbit	L-glutamate† + DNP	$6{\cdot}0\times10^{-5}$	0·356	96

* Complete system[5] plus malonate; ATP omitted.
† Malonate omitted.

TABLE 4. STIMULATION OF LIVER MITOCHONDRIA OXIDATION BY U-9189 AND DNP IN HEXOKINASE : GLUCOSE-DEFICIENT MEDIUM

Enzyme source	Substrate	Molarity uncoupler	Malonate	ΔO (μA/ml prot.)	% stimulation
Rabbit	α-ketoglutarate	—	+	0·329	—
Rabbit	α-ketoglutarate+U-9189	$2{\cdot}5\times10^{-4}$	+	0·412	25
Rabbit	α-ketoglutarate+DNP	$1{\cdot}0\times10^{-5}$	+	0·473	44
Rabbit	α-ketoglutarate	—	—	0·377	—
Rabbit	α-ketoglutarate+U-9189	$2{\cdot}5\times10^{-4}$	—	1·015	169
Rabbit	α-ketoglutarate+DNP	$1{\cdot}0\times10^{-5}$	—	1·063	182
Rat	α-ketoglutarate	—	+	0·589	—
Rat	α-ketoglutarate+U-9189	$2{\cdot}5\times10^{-4}$	+	0·863	47
Rat	α-ketoglutarate+DNP	$1{\cdot}0\times10^{-5}$	+	0·904	53
Rabbit	L-glutamate	—	—	0·810	—
Rabbit	L-glutamate+U-9189	$2{\cdot}5\times10^{-4}$	—	1·315	62
Rabbit	L-glutamate+U-9189	$7{\cdot}5\times10^{-4}$	—	1·829	126
Rabbit	L-glutamate+U-9189	$1{\cdot}5\times10^{-3}$	—	1·778	120
Rabbit	L-glutamate+DNP	$1{\cdot}0\times10^{-5}$	—	1·331	64
Rabbit	L-glutamate+DNP	$3{\cdot}0\times10^{-5}$	—	1·885	133
Rabbit	L-glutamate+DNP	$6{\cdot}0\times10^{-5}$	—	1·885	133

Complete system[5] minus glucose and hexokinase.

When the hexokinase–glucose coupling system was omitted from the medium, again U-9189 and DNP appeared to have comparable effects (Table 4) on rabbit and rat liver mitochondria. This conclusion is reinforced by the finding that, at given concentrations of U-9189 and DNP with α-ketoglutarate as substrate, comparable effects were noted in the presence and absence of malonate. As was the case with ATP omission, maximal effect was achieved with three times the concentrations which produced 50 per cent uncoupling.

Stimulation of ATPase activity

Another well-known effect of DNP is its stimulation of mitochondrial ATPase activity. ATPase activity of rabbit or rat liver mitochondria was stimulated by both U-9189 and DNP as shown in Table 5. Although the endogenous ATPase activity of

TABLE 5. STIMULATION OF LIVER MITOCHONDRIAL ATPASE ACTIVITY BY U-9189 AND DNP

Addition	Inorganic phosphate formed (μmoles/10 min per mg protein)	
	Rat	Rabbit
None	0·14	0·57
U-9189, 10^{-3} M	1·53	0·95
U-9189, 10^{-4} M	0·58	0·70
U-9189, 10^{-5} M	0·26	0·65
DNP, 10^{-4} M	1·92	1·19
DNP, 10^{-5} M	1·09	0·80
DNP, 10^{-6} M	0·28	0·66

Reaction mixture contained per ml: 6 μmoles of ATP, 10 μmoles of Tris, 75 μmoles of KCl, and 0·3 ml of mitochondria in 0·25 M sucrose, pH 7·4, 30°C.

rabbit liver mitochondria was always quite high, it is clear that U-9189 treatment enhanced this activity. DNP would be estimated as about twenty-five times more active than U-9189.

Prevention of mitochondrial swelling

Another basis for comparison of uncoupling agents is their effect on mitochondrial swelling. As shown in Fig. 2, U-9189 was effective in preventing swelling induced in rat mitochondria either by 2×10^{-3} M inorganic phosphate or by 10^{-5} M L-thyroxine, as well as spontaneous swelling (induced by Tris buffer). Analogous results were obtained with rabbit liver mitochondria (Fig. 3). Also shown here is the effect of a low concentration of DNP (10^{-5} M). Again DNP was about twenty-five times more active than U-9189.

Effect of other nitriles on oxidative phosphorylation

In Table 6 are listed twelve mononitriles and six dinitriles which were tested for their ability to uncouple oxidative phosphorylation. All compounds were tested at a concentration of 10^{-3} M with a system containing rabbit liver mitochondria and α-ketoglutarate as substrate in the presence of malonate. In no case was there any

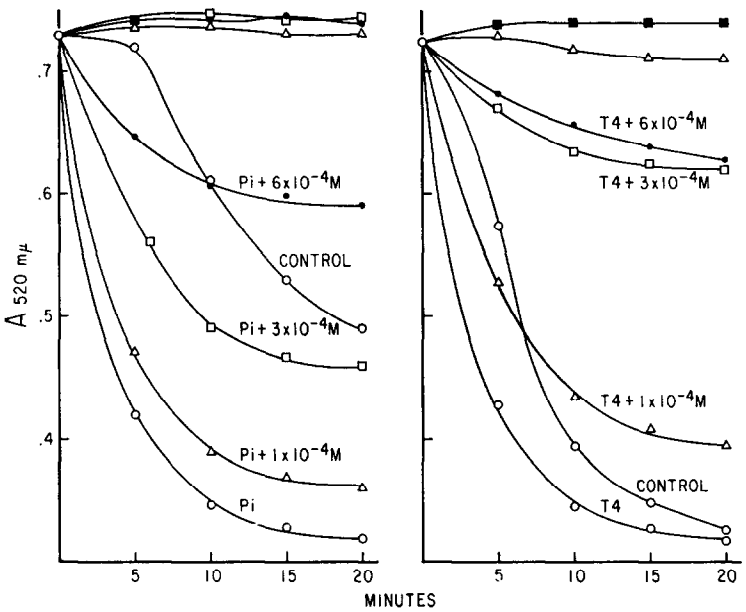


FIG. 2. Inhibition of phosphate- and L-thyroxine-induced swelling of rat liver mitochondria by U-9189. Medium: 0·33 M sucrose, 0·02 M Tris, and 0·002 M phosphate (Pi) or 10^{-5} M. L-Thyroxine (T_4), pH 7·4, 24°C. Unlabeled curves represent controls corresponding to labeled curves (medium plus inhibitor but minus swelling agent).

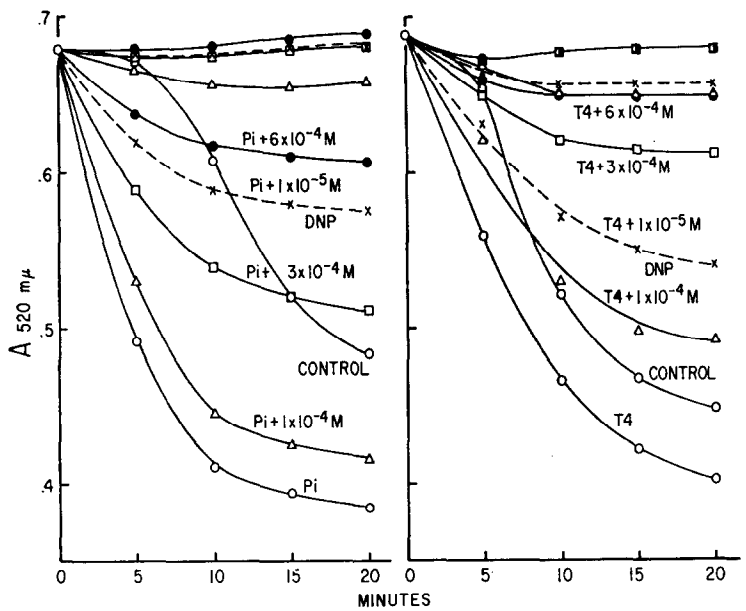


FIG. 3. Inhibition of phosphate- and L-thyroxine-induced swelling of rabbit liver mitochondria by U-9189. Medium: 0·33 M sucrose, 0·02 M Tris, and 0·002 M phosphate (Pi) or 10^{-5} M L-thyroxine (T_4), pH 7·4, 24°C. Unlabeled curves represent controls corresponding to labeled curves (medium plus inhibitor but minus swelling agent).

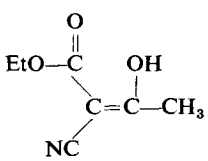
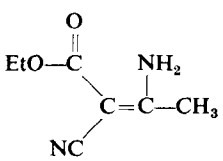
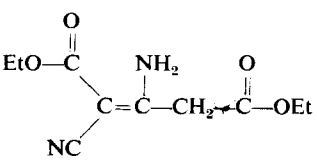
significant effect either on oxidation or phosphorylation. These results were somewhat surprising inasmuch as three of the compounds tested (U-13,758, U-14,509, U-13,761) bear rather close structural similarities to U-9189. Thus, uncoupling would not appear to be a general property of nitriles or enamines.

DISCUSSION

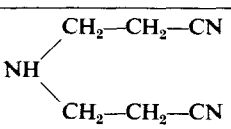
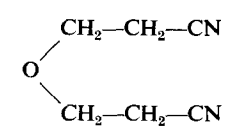
From the data presented in this paper it is clear that U-9189 is a DNP-like uncoupling agent, differing from DNP only quantitatively in effect. In addition, both agents can produce hyperthermia in experimental animals. Since the hyperthermia induced by DNP is considered to be a peripheral effect by some investigators and a central effect by others,[11] certain additional activities of U-9189 should be considered

TABLE 6. NITRILES HAVING NO EFFECT ON OXIDATIVE PHOSPHORYLATION

Monoitriles

$CH_3—CN$	$(CH_3)_2—CH—CN$	$CH_2OH—CH_2—CN$
$CH_3—CH_2—CN$	$CH_3—(CH_2)_3—CN$	$(CH_3)\ (CH_2OH)—CH—CN$
$CH_3—(CH_2)_2—CN$	$HO_2C—CH_2—CN$	$(CH_3)_2—CH—O—(CH_2)_2—CN$
		
U-13,761*	U-14,508*	U-13,758*

Dinitriles

$NC—CH_2—CN$	
$NC—(CH_2)_2—CN$	
$NC—(CH_2)_3—CN$	
$NC—(CH_2)_4—CN$	

* These compounds were synthesized by Dr. G. A. Youngdale, The Upjohn Company.

in this regard. Grenell and Hydén[12] recently demonstrated that U-9189 increased concentrations of protein and RNA in neurons of the rabbit with a concomitant decrease of these components in glial cells. This balance between the two cell populations may explain why we were unable to observe any gross change in total composition of various areas of the brain (unpublished observations). Concentrations of protein and RNA of single Deiter cells were as much as 25 per cent greater than those found in the controls, and the RNA showed altered base ratios.[12] Also, two enzyme activities were shown to be increased several fold, namely cytochrome oxidase and

succinoxidase. In view of these striking changes induced in the central nervous system by U-9189, it is tempting to speculate that the hyperthermia brought about by U-9189 might also be a result of direct action on the central nervous system. However, U-9189 inhibited succinate oxidation in liver mitochondria at concentrations which produced uncoupling (Table 1). Thus, if the U-9189 hyperthermia is centrally mediated it may be produced by some mechanism other than uncoupling of oxidative phosphorylation.

Recent studies by Ingbar[13] showed that in rats U-9189 induced acute inhibition of the organic-binding of iodine, suppressed the formation of thyroxine, and inhibited the conversion of mono- to di-iodotyrosine. Following prolonged administration, these actions were supplemented by inhibition of the thyroidal iodine-concentrating mechanism. Although U-9189 prevented the mitochondrial swelling induced by L-thyroxine, this effect alone does not explain its antithyroid activity. DNP prevents swelling at much lower concentrations and exerts its effects only on the iodide-concentrating mechanism, while U-9189, less potent in uncoupling and swelling-prevention properties, inhibits both the iodide-concentrating and organic-binding mechanisms.[13, 14]

That U-9189 should possess DNP-like activities is not apparent from its structure. Since uncoupling was shown not to be a general property of either nitriles or enamines (Table 6), the effect would appear to be attributed to the special stereochemical configuration of U-9189, or alternatively to a more highly activated enamine grouping. The wide diversity of activities shown by U-9189 suggests that the active sites affected in liver, thyroid, and brain may be different and that some of these are unaffected by DNP. Because of the similarities to and differences from DNP, U-9189 may prove to be a useful tool for the elucidation of the precise mechanism of oxidative phosphorylation and other tissue function.

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