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(+)-α-(*N*-1-Phenethyl)Urea Stereospecifically Inhibits Ca²⁺- but Not ADP-Stimulated Mitochondrial Respiration

Abstract. *The (+) isomer of α-(N-1-phenethyl)urea is a moderately potent inhibitor of Ca^{2+}-stimulated mitochondrial respiration of $^{45}Ca^{2+}$ uptake (50 percent inhibition at ~0.18 mM) while having no effect on adenosine diphosphate–stimulated respiration; the (−) isomer is without effect up to 4 mM. β-Phenethylurea does not inhibit with either stimulus. The data support the involvement of a Ca^{2+}-specific protein in energized mitochondrial Ca^{2+} uptake.*

The hypoglycemic agent phenethylbiguanide, an organic base that is positively charged at physiologic *p*H, competitively inhibits nonenergized mitochondrial Ca^{2+} binding and blocks energized Ca^{2+} uptake, exactly in parallel with inhibition of adenosine diphosphate (ADP)–stimulated respiration (*1*). We therefore explored the structure-function relationships of the phenethylbiguanide molecule, in particular to assess the role of the positive charge as compared to that of the hydrophobic moiety. In the course of our studies, we have found that (+)-α-(*N*-1-phenethyl)urea is a potent inhibitor of Ca^{2+}- but not ADP-stimulated mitochondrial respiration, while the (−) isomer is entirely without activity toward either stimulus. The high degree of structural specificity of this pair of compounds is further emphasized by the observation that β-phenethylurea is completely inactive, while *n*-alkylureas of medium chain length and the homologous monoguanidinium compounds are moderately effective inhibitors of both ADP- and Ca^{2+}-stimulated respiration.

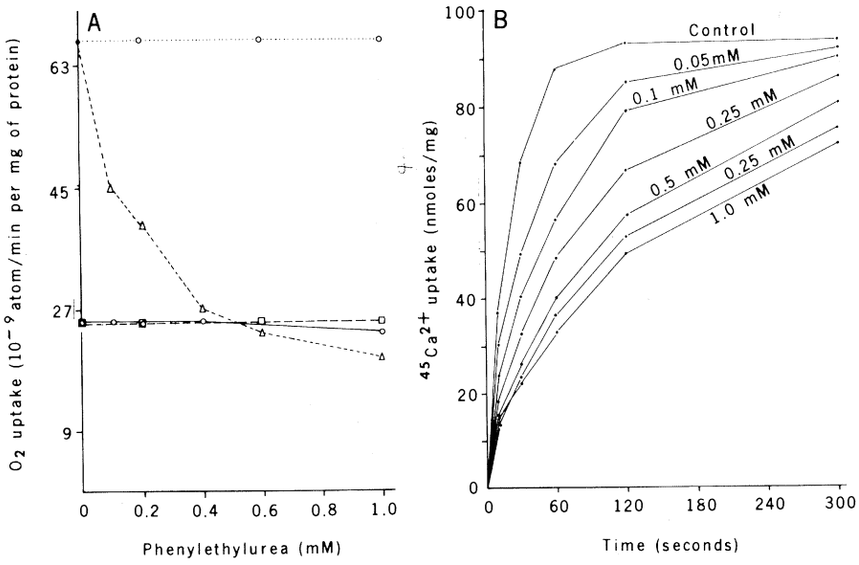

Fig. 1. Inhibition of mitochondrial Ca^{2+}-linked functions by α-(*N*-1-phenethyl)ureas. (A) Oxygen uptake rates. Incubation medium for studies of O_2 uptake contained 0.25*M* sucrose, 10 m*M* HEPES (sodium salt) buffer (*p*H 7.4), and 10 m*M* glutamate. Mitochondrial protein concentration was 4.5 mg/ml, and respiration was initiated with either 0.25 μmole of ADP plus 0.25 μmole sodium potassium phosphate, or 0.5 μmole of Ca^{2+}, in a final volume of 1.6 ml at 30°C. Uptake rate of O_2 was calculated as the stimulated rate minus the state 2 rate (*13*). (△---△), (+)-α-(*N*-1-phenethyl)urea, Ca^{2+}; (○···○), (−)-α-(*N*-1-phenethyl)urea, Ca^{2+}; (○——○), (+)-α-(*N*-1-phenethyl)urea, ADP; and (□---□), (−)-α-(*N*-1-phenethyl)urea, ADP. (B) Rate of $^{45}Ca^{2+}$ uptake. The suspension contained 1.0 mg of mitochondrial protein per milliliter, and uptake was initiated with 0.1 m*M* $^{45}Ca^{2+}$. Concentrations of (+)-α-(*N*-1-phenethyl)urea are indicated on curves.

Guinea pig liver mitochondria were prepared and O_2 uptake was measured as described (*1*); $^{45}Ca^{2+}$ uptake was analyzed by a rapid filtration method (*2*). The (+)- and (−)-α-(*N*-1-phenethyl)ureas (K and K Laboratories) were dissolved in a mixture of ethanol and water without heating and recrystallized twice. Both compounds moved as single components on thin-layer chromatography (TLC) in a butanol, acetic acid, H_2O system. Specific rotations $[\alpha]_D^{24°}$ of the two compounds were +43.6° and −44.6°, respectively. Gas-liquid chromatography revealed the presence of two minor peaks of low molecular weight that were identical in the two stereoisomers; these peaks constituted less than 6 percent of the total mass of each isomer. Mass spectroscopy of each major and minor component revealed no differences between the stereoisomers (*3*). *n*-Alkylureas were obtained from commercial sources. β-Phenethylurea was synthesized as described (*4*); the twice-crystallized product melted sharply at 112°C and moved as a single component on TLC. β-Phenethylguanidine was synthesized as described (*5*). The (+)- and (−)-α-(*N*-1-phenethyl)guanidines were provided by R. Fielden (*6*).

In an experiment showing the effect of (+)- and (−)-α-(*N*-1-phenethyl)urea on Ca^{2+}- and ADP-stimulated respiration (Fig. 1A), 50 percent inhibition of Ca^{2+}-stimulated respiration by the (+) isomer was seen at 0.28 m*M* Ca^{2+}, but the concentration required varied somewhat from experiment to experiment around a mean of about 0.175 m*M* (Table 1). The complete lack of effect of the (+) isomer on ADP-stimulated respiration and the total inactivity of the (−) isomer are also easily apparent. The maximal rate of O_2 uptake with saturating concentrations of ADP (more than 0.25 m*M*) was less than the rate obtained with Ca^{2+}; the (+) isomer differentially inhibited Ca^{2+} but not ADP-stimulated respiration at these maximally stimulated rates. β-Phenethylurea and *N*-benzylurea were entirely without effect on either Ca^{2+}- or ADP-stimulated respiration. In contrast, both the (+) and (−) isomers of α-(*N*-1-phenethyl)guanidine were moderately potent respiratory inhibitors, slightly more effective with ADP than with Ca^{2+} as the respiratory

Table 1. Inhibition of ADP- or Ca^{2+}-stimulated respiration and $^{45}Ca^{2+}$ uptake in guinea pig liver mitochondria. Incubation conditions are given in Fig. 1. Substrates were either 10 m*M* glutamate or 20 m*M* succinate.

Compound	Concentration (m*M*) required for inhibition					
	Succinate			Glutamate		
	Respiratory stimulation by		$^{45}Ca^{2+}$ uptake	Respiratory stimulation by		$^{45}Ca^{2+}$ uptake
	ADP	Ca^{2+}		ADP	Ca^{2+}	
Butylurea	>5*	>5*		>5*	>5*	
Heptylurea	0.9†	0–10% at 3‡		1.2†	2.2†	
Octylurea	0.8†	10–20% at 3‡		0.5†	1.2†	
Benzylurea	>5*	>5*		>5*	>5*	
β-Phenethylurea	>4*	>4*		>4*	>4*	
(+)-α-(*N*-1-phenethyl)urea	>3*	0.17†	0.08†	<10% at 3‡	0.070–0.28†	0.25†
(−)-α-(*N*-1-phenethyl)urea	>4*	>4*	>3*	>4*	>4*	>1*
β-Phenethylguanidine	~0.22†	~0.75†	~25% at 1‡	0.025†	0.055†	0.060†
(+)-α-(*N*-1-phenethyl)guanidine	~15% at 1‡	~15% at 1‡	>1*	0.4†	0.5†	0.7†
(−)-α-(*N*-1-phenethyl)guanidine	~15% at 1‡	~15% at 1‡	>1*	0.5†	0.6†	0.9†

*No inhibition up to the indicated concentration. †Fifty percent inhibition at the indicated concentration. ‡Percentage of inhibition shown at the indicated concentration.

stimulus (Table 1); β-phenethylguanidine was at least ten times more potent than the corresponding α isomers.

Whereas the inhibition of Ca^{2+}-stimulated respiration by (+)-α-(*N*-1-phenethyl)urea was equally effective with glutamate or succinate as substrate, the α- and β-phenethylguanidines showed definite site specificity; that is, inhibition was much more effective when the mitochondria were energized with glutamate (Table 1). Monoguanidinium derivatives show similar site specificity (*7*). *n*-Octylurea inhibited both Ca^{2+}- and ADP-stimulated respiration and was somewhat less active with succinate than with glutamate (Table 1); *n*-heptylurea was somewhat less potent than the octyl derivative, but had qualitatively similar actions.

In Fig. 1B is shown the effect of (+)-α-(*N*-1-phenethyl)urea on $^{45}Ca^{2+}$ uptake. Although the initial rate of Ca^{2+} uptake was inhibited, uptake continued at slower rates until a maximum was reached which closely approached the control by about 5 minutes. This result resembles the effect of La^{3+} and of biguanides on Ca^{2+} accumulation (*1*, *8*) and suggests interference with the kinetics of Ca^{2+} movement rather than interference with the steady-state level of energy generation or coupling. (−)-α-(*N*-1-phenethyl)urea was without effect on $^{45}Ca^{2+}$ uptake rates.

Kinetic studies have suggested the presence of both regulator and carrier sites for mitochondrial Ca^{2+} transport (*9*). The chemical nature of these sites is not known, although some data have favored phospholipid as the carrier, and a protein (or proteins) as the regulator (*1*, *10*). The exquisite structural specificity of the (+)- and (−)-α-phenethylureas shown in our work supports the concept that proteins are involved in either the carrier or regulator mechanisms, since phospholipids would be unlikely to distinguish between the stereoisomers, nor would phospholipid bilayers be likely to respond to a nonionic analog of phenethylguanidine more sensitively than to phenethylguanidine itself (see Table 1). This high degree of inhibitor specificity also rules out a simple chaotropic effect of the phenethylureas as the mechanism of interference with Ca^{2+} uptake.

The other major agents known to inhibit mitochondrial Ca^{2+} uptake without affecting ADP-linked functions are the lanthanides (*8*) and the dye ruthenium red (*11*), both of which are cations. Although the presence of the positive charge on these two compounds indicates that both probably seek the same mitochondrial binding site as Ca^{2+} itself (*8*), the structural requirement for hydrophobic residues on ruthenium red (*11*) also suggests that hydrophobic binding regions lie closely adjacent to the Ca^{2+} binding site. Inhibition of Ca^{2+} uptake by a nonionic agent such as (+)-α-(*N*-1-phenethyl)urea with considerable hydrophobicity is in keeping with this construct. Binding studies (*11*) and kinetic studies with an as yet uncharacterized cytoplasmic factor (*12*) of low molecular weight indicate that La^{3+} and ruthenium red probably operate at two different mitochondrial loci.

Our data do not directly address the question whether each phenethylurea binds to the same mitochondrial site as its guanidinium analog. A priori, the close structural similarities of each pair of compounds make such a common site appear likely, particularly in view of the importance of the hydrophobic residue for the binding of β-phenethylguanidinium analogs to the Ca^{2+}-specific site on pyruvate kinase (*5*). However, the identical inhibitory effects of the (+) and (−) isomers of α-(*N*-1-phenethyl)guanidine stand in such contrast to the distinct effects of the (+)- as compared to the (−)-α-phenethylureas that separate sites for guanidinium and for (+)-(*N*-1-phenethyl)urea seem very likely. The inhibition of Ca^{2+} uptake by (+)-α-(*N*-1-phenethyl)urea was not site-specific (Table 1); in this property the urea compound again resembled La^{3+} and ruthenium red, but differed from the substituted monoguanidines. The inhibitory activity of the β-phenethyl and (−)-α-(*N*-1-phenethyl)guanidinium compounds also distinguishes them from the inactivity of the corresponding ureas, in support of the existence of separate urea and guanidinium sites, and indicates further that addition of a positive charge to the molecule exerts a dominant effect in determining mitochondrial binding locus.

Recent work from this laboratory has suggested that the ability of guanidinium derivatives to affect transmembrane Ca^{2+} movement, rather than inhibition of mitochondrial energy production, may be the factor associated with hypoglycemic activity (*1*). If this were indeed the case, (+)-α-(*N*-1-phenethyl)urea might be expected to exhibit hypoglycemic properties similar to phenethylbiguanide. However, administration of (+)- and (−)-α-(*N*-1-phenethyl)ureas in doses of 45 mg/kg to separate groups of ten guinea pigs produced small rises in blood sugar (145 ± 4 and 151 ± 7 mg per 100 ml of blood, respectively), while β-phenethylbiguanide (30 mg/kg) significantly reduced blood sugar (108 ± 12 mg per 100 ml) compared to the control animals (130 ± 4 mg per 100 ml of blood) at 3 hours.

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Thermal Polyamino Acids: Synthesis at Less Than 100°C

Abstract. *Thermally prepared polyamino acids, regarded as models for prebiotic protein, typically have been synthesized at 120° to 200°C. In this study, three different sets of amino acid mixtures were found to yield material of relatively high molecular weight (molecular sieving and diffusion techniques) when heated for up to 81 days at 85°, 75°, and 65°C. These temperatures, which today are generated by radiation from the sun in some terrestrial locales, probably were more common on the prebiotic earth than those above the boiling point of water. The results thus suggest that prebiotic polyamino acids may have been relatively common and widespread.*

Polyamino acids are readily prepared under simulated prebiotic conditions by heating suitable proportions of dry amino acids (*1*, *2*). Such polymers, termed proteinoids, show many properties envisionable as being important in evolutionary processes (for example, catalytic activity, morphogenesis), and they are regarded as models for prebiotic protein (*1*).

Temperatures conducive to the removal of nascent water (120° to 200°C) typically have been used in the synthesis of proteinoids, although the polymerizations are known to occur at 60°C in the presence of dehydrating agents (*1*). It has been suggested (*3*), however, that polyamino acids formed at 120° to 200°C could not have played a major role in evolutionary processes, because such temperatures probably occurred only locally on the surface of the prebiotic earth (juxtaposed with much higher, detrimental, temperatures) and would have, on long contact, destroyed any resulting polyamino acids. These objections have been rebutted [(*1*, *4*); for example igneous areas are numerous today and were more plentiful in the past; rain is a common and effective vehicle for removing proteinoid from a heat source], but with little doubt temperatures below 100°C were (and are) more prevalent (*5*).

An understanding of whether amino acid mixtures would polymerize at relatively low temperatures would thus permit inferences concerning the abundance—or scarcity—of prebiotic protein (*6*). The temperatures tested in this study (65° to 85°C) are within the range of those that are generated by solar radiation today (*7*, *8*).

Three types of amino acid mixtures were used as reactants: aspartic acid, glutamic acid, and an equimolar mixture of 16 proteinous amino acids (2 : 2 : 1 by weight), yielding 2 : 2 : 1 proteinoid; lysine as the free base and an equimolar mixture of 16 amino acids (1 : 1 by weight), yielding lysine-rich proteinoid; and the proportions of amino acids, rich in glycine, reported by Fox and Windsor (*9*) as being formed in a simulated prebiotic synthesis, yielding "Fox-Windsor" preparation. Proteinoids made from the former two sets of reactants have been well characterized (*1*); the latter set was chosen because it is representative of several sets, known to polymerize at 175°C, that may reflect the kinds and proportions of amino acids that were available prebiotically (*10*).

Each set of reactant amino acids (10 g) was slurred in triplicate in 10 ml of water, placed on a watch glass, and heated in ovens at 85°, 75°, and 65°C (± 2°C). No attempt was made to control humidity. Samples of about 2 g were removed periodically, the remaining material being reslurried in 5 to 6 ml of water (*11*). With one exception, the maximum heating time was 81 days, in contrast to the few hours typically used at higher temperatures (*1*). Unless otherwise indicated, the samples were tested without purification.

The presence of material of relatively high molecular weight in the undialyzed products was evaluated by elution patterns from a Bio-Gel P-2 column (2.0 $cm^2 \times 25$ cm, exclusion limit 1600 daltons), with the use of 0.1*M* phosphate buffer (*p*H 7.0) containing 0.2 percent sodium dodecyl sulfate and 0.02 percent sodium azide; the void volume of the column (about 21 ml) was determined with Blue Dextran (2×10^6 daltons). Relative size was also estimated by the Craig diffusion technique (*12*) (Union Carbide dialysis tubing and diffusion apparatuses that, with a 0.5-ml sample, had an area to volume ratio of about 75). Diffusion in 0.1*M* tris buffer (*p*H 8.0) was monitored at 220 nm and in some cases via ninhydrin color.

Figure 1 shows Bio-Gel elution patterns of several of the undialyzed 2 : 2 : 1 proteinoids, as well as those of the reactant amino acids and Blue Dextran. As the time of heating at 85°C increases (Fig. 1, a to d), there is a progressive increase in the amount of material eluting at Ve/Vo = 1 (elution volume/void volume), indicative (*13*) of molecules having molecular weights exceeding the exclusion limit of the gel (1600 daltons). This is accompanied by a decrease in the amount of free amino

Table 1. Diffusion data for various undialyzed 2 : 2 : 1 proteinoids. T-50 is the time required for diffusion of 50 percent of the sample; see text.

Proteinoid (time and temperature of synthesis)	Ratio, T-50 of proteinoid/ T-50 of reactants*	*N*†	Significance level‡
20 days, 85°C	1.30 ± 0.14§	4	< 0.05
48 days, 85°C	1.85 ± 0.11	4	< 0.01
81 days, 85°C	2.31 ± 0.23	4	< 0.01
81 days, 75°C	1.56 ± 0.28	3	< 0.02
81 days, 65°C	0.90 ± 0.05	3	< 0.40
81 days, 65°C (dialyzed)	2.82, 2.35	2	

*The observed T-50 value for the reactant amino acid mixture was 6.88 ± 1.03 minutes (four determinations). †Number of determinations. ‡Probability that the difference between proteinoid and reactants is due to chance (*18*). §Mean and standard deviation.