Biochemical Pharmacology, 1962, Vol. 11, pp. 1115–1122. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECTS *IN VITRO* OF 1-(1-PHENYLCYCLOHEXYL) PIPERIDINE HYDROCHLORIDE (SERNYL) ON OXIDATION BY LIVER HOMOGENATES AND MITOCHONDRIA OF RAT*

HELEN LEES

The Lafayette Clinic, Detroit, Mich., U.S.A.

(*Received* 2 *July* 1962; *accepted* 13 *August* 1962)

Abstract—A study of the effects *in vitro* of 1-(1-phenylcyclohexyl)piperidine·HCl (Sernyl) has shown that with succinate, α-ketoglutarate, β-hydroxybutyrate, or citrate as the substrate, the oxygen uptake by homogenates of rat liver was stimulated by 0·2 and 0·5 mM concentrations of the drug. Experiments with mitochondria gave similar results with succinate and α-ketoglutarate. Using the first three substrates or ferrocytochrome *c*, oxidative phosphorylation was uncoupled slightly by 0·1 mM and more markedly by 0·5 mM Sernyl. Mitochondrial swelling in 0·125 M KCl-0·02 M Tris·HCl buffer, pH 7·4, was enhanced by 0·1 mM Sernyl.

A comparison of the effects of some analogs of the drug on the oxidation of succinate by liver homogenates showed that N-ethyl-1-cyclohexylcyclohexylamine·HCl and N-ethyl-1-phenylcyclohexylamine·HCl also had a stimulatory effect but less than that of Sernyl. No significant effect was shown by N-ethylcyclohexylamine·HCl, N-ethylpiperidine·HCl, or N-cyclohexylpiperidine·HCl.

RECENT reports indicate that Sernyl (1-(1-phenylcyclohexyl)piperidine monohydrochloride), an anesthetic,[1] has psychotomimetic effects in humans.[2–4] In conjunction with these studies, the biochemical effects of the drug were of interest. This investigation of the effects *in vitro* of the drug on respiration was prompted by the observation of Domino[5] that the injection of Sernyl into rats increased oxygen consumption.

MATERIALS AND METHODS

α-Ketoglutaric and L-malic acids and the sodium salt of DL-β-hydroxybutyrate were obtained from the California Corporation for Biochemical Research; ATP as the sodium salt, cytochrome *c* (type III), and hexokinase (type III) from the Sigma Chemical Company; and N-ethylpiperidine from Eastman Organic Chemicals. Parke, Davis and Company donated 1-(1-phenylcyclohexyl)piperidine·HCl, N-ethyl-1-cyclohexylcyclohexylamine·HCl, N-ethyl-1-phenylcyclohexylamine·HCl, and N-ethylcyclohexylamine·HCl. N-Cyclohexylpiperidine·HCl was kindly prepared by Irwin Klundt (Wayne State University).

Succinate and malate were neutralized with KOH to pH 7·4, citrate to pH 7·2; α-ketoglutarate was neutralized with equimolar K_2CO_3.

* A preliminary report of this work appeared in *Fed. Proc.* **20**, 306 (1961).

Male Holtzman rats, 2 to 3 months old, were fasted overnight and sacrificed by a blow on the head. The excised liver was homogenized in 9 volumes of ice-cold 0·25 M sucrose with a loosely-fitting Potter–Elvehjem glass homogenizer.

For studies on oxygen uptake, homogenates were centrifuged briefly to remove red cells and connective tissue, and 0·5 ml of supernatant was added to each flask. When mitochondria were used, they were prepared according to the procedure described by Schneider,[6] washed twice with 0·25 M sucrose and resuspended so that the amount obtained from 200 mg of liver in a volume of 0·5 ml was added to each flask.

Oxygen uptake was determined by conventional Warburg technique using duplicate reaction vessels. Flask contents are described in the tables. The center wells contained 0·2 ml of 3 N KOH and filter paper. Unless otherwise indicated, the temperature was 38° and the equilibration period was 10 min. All results reported here are typical of at least three experiments.

For P:O ratios, each flask contained the mitochondria obtained from 500 mg of liver suspended in 0·5 ml of 0·25 M sucrose. The side arms contained 0·5 ml of 20% trichloroacetic acid. After an equilibration period of 7 or 8 min, the measurement of oxygen uptake was begun and the initial phosphorus values were obtained. Final values were determined 15 min later. Phosphorus was measured by the method of Fiske and Subbarow,[7] slightly modified.

Mitochondrial swelling was studied essentially as described by Lehninger.[8] Mitochondria were prepared in 0·25 M sucrose and resuspended in this medium so that 1 ml contained the mitochondria obtained from 500 mg of liver. One-tenth ml of mitochondrial suspension was added to 3·1 ml of 0·125 M KCl–0·02 M Tris·HCl buffer, pH 7·4, both with and without 0·1 mM Sernyl added. Swelling was measured at room temperature in 1-cm Beckman cuvets by following the change in optical density at 520 mμ with a Beckman model DU spectrophotometer. In some experiments 0·016 M succinate was added to stimulate swelling.

TABLE 1. THE EFFECT OF SERNYL ON OXIDATION BY RAT LIVER HOMOGENATES

Substrate	Control	0·2 mM Sernyl	0·5 mM Sernyl
	(μl O_2 uptake in 20 min)		
Succinate	117	149	132
α-Ketoglutarate	73	81	91
β-Hydroxybutyrate	63	76	77
Citrate	67	74	88
Malate	64	61	56

Each flask contained 108 μmoles KCl, 30 μmoles potassium phosphate buffer, pH 7·4; 15 μmoles $MgCl_2$; 3 μmoles ATP; Sernyl as shown; and 0·5 ml 10% rat liver homogenate in 0·25 M sucrose. Substrates: 50 μmoles succinate; 56 μmoles DL-β-hydroxybutyrate; 28 μmoles α-ketoglutarate, citrate, or L-malate. The volume was 2·5 ml.

RESULTS AND DISCUSSION

The effects of Sernyl on the oxidation of various substrates by rat liver homogenates are shown in Table 1. With succinate as the substrate, oxygen uptake over a 20-min period in 0·2 mM Sernyl was 25 per cent greater than the control value; in a 0·5 mM concentration the increase was less marked. When the substrate was α-ketoglutarate,

the stimulation of oxygen uptake was 11 per cent and 25 per cent in concentrations of 0·2 and 0·5 mM Sernyl, respectively. Similar results were obtained for citrate. The oxidation of β-hydroxybutyrate was increased approximately 20 per cent by both concentrations of the drug. Malate was not significantly affected by 0·2 mM Sernyl but was somewhat inhibited at the 0·5 mM level.

The results of these experiments on homogenates using succinate and α-ketoglutarate as substrates are shown graphically in Fig. 1. Both concentrations of Sernyl increased the oxidation of α-ketoglutarate throughout the 30-min period; the higher

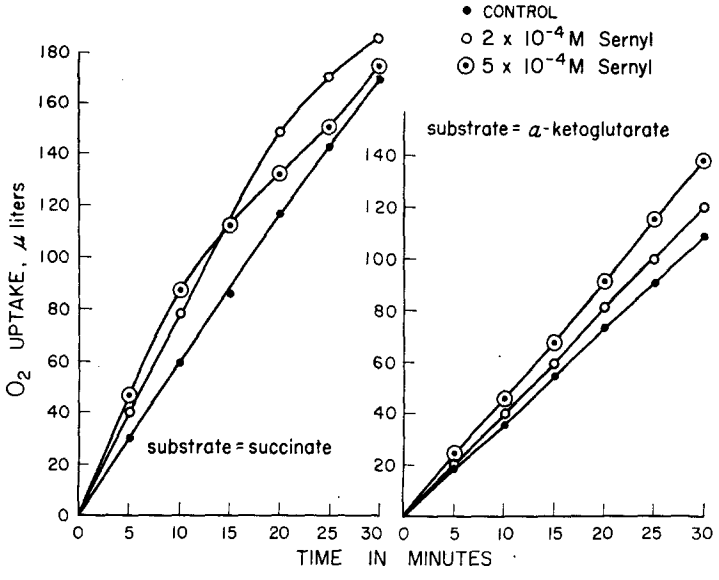


FIG. 1. The effects of Sernyl on the oxidation of succinate and α-ketoglutarate by liver homogenates

concentration was more effective. Initially the oxidation of succinate was stimulated more by 0·5 than by 0·2 mM Sernyl, but after 15 min the higher concentration had less effect than the lower one, although the values continued to be greater than those of the control flasks.

The effects of the drug on the oxidation of succinate and α-ketoglutarate by liver mitochondria are shown in Fig. 2. As the concentration of Sernyl was increased, oxygen consumption (20-min reading) was increased, reaching a maximum at about 0·8 mM for α-ketoglutarate and 0·5 mM for succinate, and dropping off at a concentration of 1 mM. Thus there is an optimum concentration for the stimulatory effect. It was also noted that, in higher concentrations of the drug, the longer the time of incubation of the mitochondria the less the stimulatory effect. For example, in the experiment using succinate as the substrate, oxygen uptake with 0·1 mM Sernyl during the 20 to 40-min period of incubation was 99 per cent of the value for the 0 to 20-min time interval. However, at concentrations of 0·2, 0·5, and 1·0 mM Sernyl, the 20 to 40-min values were 89, 73, and 68 per cent of the corresponding 0 to 20-min results, respectively. Similar results were obtained with homogenates.

Whether the drug acts by altering mitochondrial structure or through some other physical or ionic effect is not certain. If the increase in oxygen uptake is due to (or results in) an alteration of mitochondrial structure, a further change in structure, as might conceivably occur in higher concentrations of Sernyl, especially over a longer period of time, could cause a lessening of the stimulatory effect. The drug does seem to have an inhibitory effect on mitochondria which presumably already have an altered structure. When homogenates were prepared with a homogenizer with a tightly fitting Teflon pestle, concentrations of 0·2 and 0·5 mM Sernyl gave only 88 and 89 per cent of

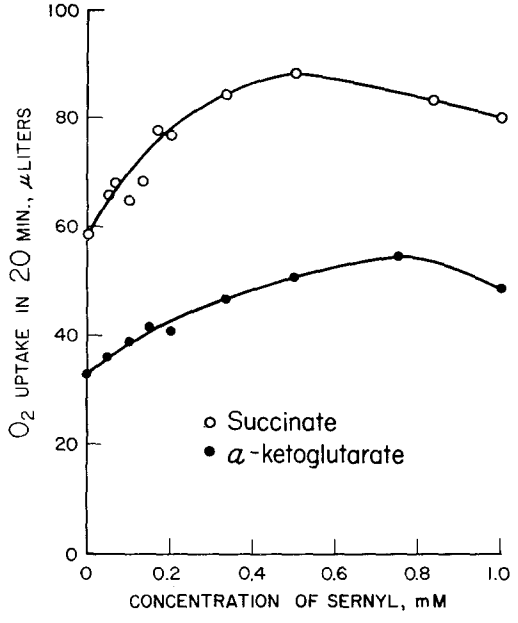


FIG. 2. The effects of Sernyl on the oxidation of succinate and α-ketoglutarate by mitochondria.

the control values (20-min reading), respectively—an inhibition. That the mitochondria in such preparations are damaged is suggested by the finding that P:O ratios for these were quite low (*e.g.* 0·8 for succinate). Aldridge[9] has stressed the importance of undamaged mitochondria in the measurement of a stimulatory effect. Although the stimulatory and inhibitory effects of the drug are not necessarily evoked through the same mechanism of action, it is probable that inhibition is due simply to a further exaggeration of whatever change produces stimulation.

The observation of these changes in oxidation prompted a study of the effects of the drug on P:O ratios which is reported in Table 2. Similar results were obtained with succinate, α-ketoglutarate, and β-hydroxybutyrate as substrates. A concentration of 0·1 mM Sernyl caused a slight decrease in P:O ratios and 0·5 mM produced a more marked decrease. The degree of uncoupling of oxidative phosphorylation was the same for the DPN-linked substrates as for succinate. So uncoupling does occur at or below the level of the flavoprotein in the respiratory chain. However, this is not sufficient absolutely to rule out uncoupling at the substrate level for α-ketoglutarate or β-hydroxybutyrate. Uncoupling also occurred when the substrate was ferrocytochrome

c (Table 3). These effects, though less marked, are very similar to those of chlorpromazine on liver mitochondria reported by several workers. Berger *et al.*[10] found that chlorpromazine inhibited phosphorylation coupled to the oxidation of cytochrome *c*. Dawkins *et al.*[11] also observed uncoupling when the substrate was succinate, β-hydroxybutyrate, or ferrocytochrome *c*.

TABLE 2, THE EFFECTS OF SERNYL ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF SUCCINATE, α-KETOGLUTARATE AND β-HYDROXYBUTYRATE

Experiment	Substrate	Concentration of Sernyl (mM)	$-\Delta P_i$ (μmole)	ΔO (μatom)	P : O
1	Succinate	none	7·3	4·6	1·6
		0·1	6·0	4·5	1·3
		0·5	4·0	3·6	1·1
2		none	9·9	5·9	1·7
		0·1	9·9	7·3	1·4
		0·5	6·0	5·7	1·1
3		none	9·5	5·6	1·7
		0·1	7·9	5·5	1·4
		0·5	4·5	4·6	1·0
4	α-Ketoglutarate	none	8·3	3·6	2·3
		0·1	7·4	3·5	2·1
		0·5	6·3	3·2	2·0
5		none	6·1	2·1	2·9
		0·1	5·7	2·3	2·5
		0·5	3·6	1·6	2·2
6		none	8·8	3·2	2·7
		0·1	8·0	3·1	2·6
		0·5	5·9	3·0	2·0
7	β-Hydroxybutyrate	none	6·8	3·1	2·2
		0·1	6·6	3·2	2·1
		0·5	6·2	4·1	1·5
8		none	6·9	3·3	2·1
		0·1	6·8	3·6	1·9
		0·5	5·3	3·8	1·4

Flask contents in 2·5 ml: 30 μmole phosphate buffer, pH 7·4; 40 μmole Tris-HCl buffer, pH 7·4; 100 μmole KCl; 50 μmole glucose; 15 μmole KF; 15 μmole $MgCl_2$; 0·020 μmole cytochrome *c*; 6 μmole ATP; 0·6 mg of hexokinase. Substrate: 30 μmole succinate, 21 μmole α-ketoglutarate, or 42 μmole DL-β-hydroxybutyrate. When the last two substrates were used, 2 μmole DPN were also added. Sernyl concentrations are shown. Mitochondria from 500 mg of liver suspended in 0·5 ml of 0·25 M sucrose were added. The side arm contained 0·5 ml of 20% TCA. The temperature was 30°.

Changes in oxidation and mitochondrial swelling appear to be related, therefore a few experiments on swelling were performed. An ionic medium was chosen as being closer in equivalence to the medium used for the oxidation studies. Spontaneous swelling in KCl-Tris buffer (Fig. 3) increased in the presence of 0·1 mM Sernyl. The stimulation of swelling obtained by the addition of succinate to the medium was enhanced by Sernyl. Although the effects in this medium might be related to that of KCl

(which causes a clumping of mitochondria), these results are compatible with the frequent finding that compounds which uncouple oxidative phosphorylation may stimulate oxygen uptake and increase mitochondrial swelling.

There is a question as to whether these changes in oxidation *in vitro* may be produced *in vivo*. Some experiments were performed to determine whether an injection of the drug could alter the oxidation of succinate by liver homogenates. Rats were given intraperitoneally 10 mg of Sernyl/kg of body weight and sacrificed 30 min later. The results were inconclusive. However, Domino did observe an increase in oxygen consumption by rats after the injection of Sernyl.[5]

TABLE 3. THE EFFECT OF SERNYL ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF FERROCYTOCHROME *c*

Experiment	Conc. of Sernyl (mM)	$-\Delta P_i$ (μmole)	ΔO (μatom)	P:O
1	none	1·50	3·6	0·42
	0·1	1·10	2·6	0·42
	0·5	0·88	3·2	0·28
2	none	1·70	5·9	0·29
	0·1	1·50	6·3	0·24
	0·5	0·60	5·7	0·11
3	none	1·41	4·7	0·30
	0·1	1·00	4·7	0·21
	0·5	0·89	5·1	0·17

Flask contents: same as for Table 2 except that 0·9 μmole of EDTA was added and the substrate was 0·120 μmole cytochrome *c* plus 30 μmoles L-ascorbic acid (neutralized with NaOH just before use). Mitochondria from 700 to 800 mg of liver were added in experiments 2 and 3. The equilibration time was 7 min and the temperature was 30°.

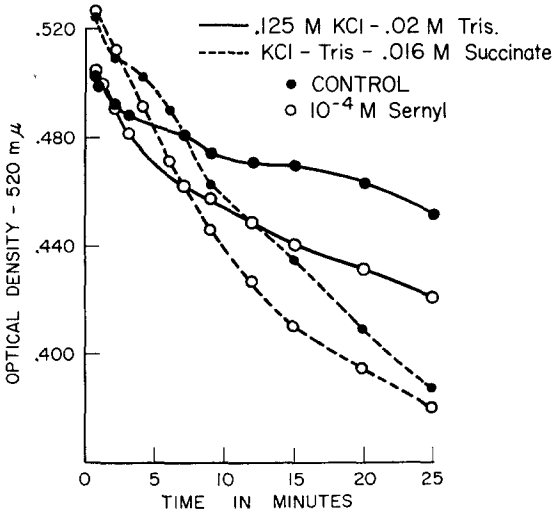
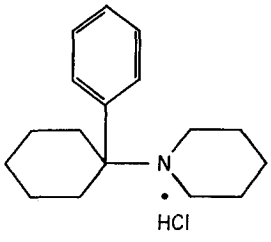
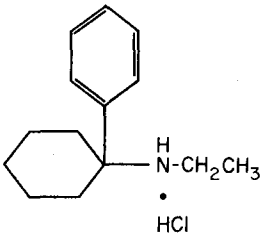


FIG. 3. The effects of Sernyl on mitochondrial swelling.

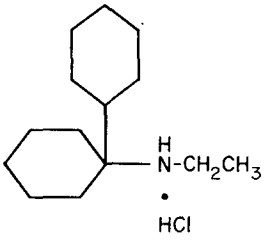
Many analogs of the drug were available for comparison. The structures of some of these are shown in Fig. 4. The effects of the compounds on the oxidation of succinate by liver homogenates are compared in Table 4. Both N-ethyl-1-cyclohexylcyclohexylamine·HCl and N-ethyl-1-phenylcyclohexylamine·HCl, at a concentration of 0·2 mM, were effective in stimulating oxygen uptake but less so than Sernyl. No significant effect was seen with N-cyclohexylpiperidine·HCl, N-ethylcyclohexylamine.



1-(1-phenylcyclohexyl) piperidine hydrochloride



N-ethyl-1-phenylcyclohexyl amine hydrochloride



N-ethyl-1-cyclohexylcyclohexyl amine hydrochloride

FIG. 4. The structural formulas of Sernyl and two analogs.

TABLE 4. A COMPARISON OF THE EFFECTS OF SERNYL AND ITS ANALOG ON THE OXIDATION OF SUCCINATE BY LIVER HOMOGENATES

Compound	Experiment 1 (μl O_2 uptake in 20 min)	Experiment 2 (μl O_2 uptake in 20 min)
None	91	123
1-(1-Phenylcyclohexyl)piperidine·HCl	120	154
N-Ethyl-1-cyclohexylcyclohexylamine·HCl	109	146
N-Ethyl-1-phenylcyclohexylamine·HCl	102	140
N-Ethylcyclohexylamine·HCl	92	
N-Ethylpiperidine·HCl	90	
N-Cyclohexylpiperidine·HCl		127

Flask contents are described in Table 1. Substrate: 50 μmole succinate. The concentration of Sernyl or analogs was 0·2 mM.

HCl, or N-ethylpiperidine·HCl. From this it is clear that a phenyl or cyclohexyl group attached to the 1-cyclohexyl position of N-ethylcyclohexylamine is essential for the stimulation of oxidation in this system and that the substitution of piperidine for the ethyl amine group (Sernyl) significantly enhances this effect. At physiologic pH these compounds are probably present mainly as the amine salts and have a positive charge. The presence of the nonpolar groups may change the physical properties sufficiently to allow a closer association of these ions with the mitochondrial lipids. Quastel[12] has suggested that narcotics may disturb electron transport because of specific association with lipid or lipoprotein. Sernyl may alter the mitochondrial structure or its permeability and thus affect electron transport or enzyme-substrate kinetics, or it may have a direct effect on these.

With respect to the question of how these effects on mitochondria may relate to those on the central nervous system, it is of interest that Levy *et al.*[13] reported that the psychological effects of N-ethyl-1-phenylcyclohexylamine·HCl administered to humans were milder than those elicited by Sernyl; in our experiments the former stimulated oxidation less than the latter. Certainly many compounds which do affect the nervous system also alter mitochondrial respiration. This may simply be related to the physical properties of these compounds which allow a close association with materials rich in lipids, a criterion satisfied by both mitochondria and the tissues of the nervous system.

REFERENCES

1. G. CHEN, C. R. ENSOR, D. RUSSELL and B. BOHNER, *J. Pharmacol. exp. Ther.* **127**, 241 (1959).
2. E. D. LUBY, B. D. COHEN, G. ROSENBAUM, J. S. GOTTLIEB and R. KELLEY, *Arch. Neurol. Psychiat., Chic.* **81**, 363 (1959).
3. G. ROSENBAUM, B. D. COHEN, E. D. LUBY, J. S. GOTTLIEB and D. YELEN, *Arch. gen. Psychiat.* **1**, 651 (1959).
4. B. D. COHEN, G. ROSENBAUM, E. D. LUBY and J. S. GOTTLIEB, *Arch. gen. Psychiat.* **6**, 395 (1962).
5. E. F. DOMINO. Personal communication.
6. W. C. SCHNEIDER, *J. biol. Chem.* **176**, 259 (1948).
7. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66**, 375 (1925).
8. A. L. LEHNINGER, *J. biol. Chem.* **234**, 2465 (1959).
9. W. N. ALDRIDGE, *Biochem. J.* **67**, 423 (1957).
10. M. BERGER, H. J. STRECKER and H. WAELSCH, *Nature, Lond.* **177**, 1234 (1956).
11. M. J. R. DAWKINS, J. D. JUDAH and K. R. REES, *Biochem. J.* **72**, 204 (1959).
12. J. H. QUASTEL, in *Enzymes: Units of Biological Structure and Function*, O. H. GAEBLER, Ed., p. 523. Academic Press, New York (1956).
13. L. LEVY, D. E. CAMERON and R. C. B. AITKEN, *Amer. J. Psychiat.* **116**, 843 (1960).