Biochemical Pharmacology, Vol. 16, pp. 1919–1931. Pergamon Press Ltd. 1967. Printed in Great Britain

STUDIES ON THE INTRACELLULAR LOCALIZATION AND EFFECTS OF 2-CHLORO-4′,4″-DI(2-IMIDAZOLIN-2-YL)TEREPHTHALANILIDE ON MITOCHONDRIA FROM P388 LYMPHOCYTIC LEUKEMIA AND RAT LIVER*

A. SIVAK,† A. J. MAHONEY, JR.‡ and W. I. ROGERS
with the technical assistance of S. A. SATTINGER

Life Sciences Division, Arthur D. Little, Inc., Cambridge, Mass., U.S.A.

(*Received* 29 *November* 1966; *accepted* 17 *March* 1967)

Abstract—2-Chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide (NSC 60339) is found primarily in the mitochondrial and nuclear fractions of P388 murine lymphocytic leukemia and rat liver cells which have been exposed to the drug. P388 cells which were exposed to the drug *in vivo* had higher endogenous oxygen uptake than untreated cells; this new rate of oxygen utilization was not affected by the subsequent addition of glucose or increased phthalanilide *in vitro*. The effects of the substituted phthalanilide on various other functions of isolated mitochondria were compared to those of spermine, tyramine, and 2,4-dinitrophenol. The phthalanilide was a more potent inhibitor than tyramine of soluble and mitochondria-bound rat liver choline dehydrogenase. Spermine inhibited the enzyme moderately but nitrogen mustard was ineffective. Choline dehydrogenase could not be demonstrated in mitochondria from P388 cells. At 10^{-4} M, the phthalanilide inhibited the succinic dehydrogenase complex in mitochondria from P388 cells resistant to NSC 60339. With succinate as a substrate, phosphorylation was inhibited completely in intact rat liver mitochondria and P388 mitochondria at 4×10^{-4} M and 5×10^{-5} M, respectively. However, succinate oxidation in the coupled systems was inhibited only 50 per cent at these and higher concentrations of drug.

The possible relationship between the effects of NSC 60339 on mitochondrial function and the toxic and chemotherapeutic properties of the drug are discussed.

2-CHLORO-4′,4″-DI(2-IMIDAZOLIN-2-YL)TEREPHTHALANILIDE (NSC 60339) has been localized in the mitochondrial and nuclear fractions of P388 lymphocytic leukemia cells and of rat kidney and liver after exposure to the drug *in vivo*.[1–3] This finding has prompted an investigation of the effects of the phthalanilide on various parameters of mitochondrial function to determine the possible relation of these effects to the toxic and chemotherapeutic properties of the drug. Choline dehydrogenase activity, succinate oxidation, and oxidative phosphorylation were assayed in mitochondria from rat liver and P388 lymphocytic leukemia cells. The effects of spermine were also determined because of the chemical's well known role as a swelling inhibitor[4] and because of its polyamine structure. Tyramine was used as an example of a potent inhibitor of choline oxidase[5] and 2,4-dinitrophenol was used as an example of a

* Supported by the Cancer Chemotherapy National Service Center Contract no. SA-43-ph-3789, National Cancer Institute, National Institutes of Health.

† Present address: New York University Medical Center, Institute of Environmental Medicine, 550 First Avenue, New York, N.Y., U.S.A.

‡ Present address: Department of Biochemistry, Brandeis University, Waltham, Mass., U.S.A.

classical uncoupler of oxidative phosphorylation. A preliminary report of these studies has appeared.[1]

MATERIALS AND METHODS

Preparation of mitochondria and other organelles

Cell fractions were prepared from P388 lymphocytic leukemia cells collected from BDF_1 mice 7 days after i.p. implanation of 10^6 cells per mouse. Drug was administered *in vivo* by a single i.p. injection of drug. The dihydrochloride, at 16 mg/kg, was injected into mice 24 hr prior to harvest of cells, unless otherwise noted. The ascites fluid was collected in oxalated tubes, diluted with an equal volume of 0·9 % saline, and centrifuged in the cold at 700 *g* for 10 min. The supernatant was discarded. The cell pellet was diluted to a known volume with 0·9 % saline and dispersed. The cells were counted in a hemocytometer in the presence of trypan blue. The number of cells which took up trypan blue due to altered permeability was usually 10–20 per cent of the total population of ascites cells. Erythrocyte contamination varied between 10 and 50 per cent of the total number of cells. However, the presence of red blood cells was not critical since these cells were lysed during the homogenization procedure. All further operations were performed at 1–4°.

The counted cells were centrifuged and resuspended at a concentration of 10^8 cells per ml in 0·005 M magnesium chloride. The cells were incubated for 10 min to allow swelling in the hypotonic medium. The cell suspension was then homogenized by hand with 25 strokes of the tight-fitting pestle in a Dounce homogenizer. A volume of 1·4 M sucrose was added to give a final sucrose concentration of 0·25 M. Nuclei and remaining whole cells were removed by centrifugation at 700 *g* for 10 min. Mitochondria were then collected from the supernatant at 10,000 *g* for 20 min. The supernatant was discarded and the mitochondrial fraction was dispersed in an appropriate medium.

Separation of the various cellular components for analysis was carried out by centrifugation of the P388 cell homogenate in three steps. The first two centrifugations were carried out as described above. The supernatant of the mitochondrial fraction was centrifuged at 100,000 *g* for 60 min to yield a microsomal pellet and a soluble supernatant. Each of the fractions was analyzed for phthalanilide with the column chromatographic isolation procedure described previously.[6] DNA and RNA were determined with the diphenylamine[7] and orcinol[8] reagents, respectively, after hydrolysis of the nucleic acids by treatment with hot perchloric acid. Protein determinations were made according to the method of Lowry.[9]

Rat liver mitochondria were prepared by the method of Schneider and Hogeboom,[10] except that the mitochondrial pellet was washed only once in 0·25 M sucrose. The supernatant was discarded, and the washed mitochondrial pellet was made to the desired concentration in 0·25 M sucrose or 0·1 M sodium phosphate buffer (pH 7·4).

Preparation of soluble enzymes from mitochondria

Extraction of soluble choline dehydrogenase from mitochondria was carried out essentially according to the method described by Williams and Sreenivasan.[11] The yield was 5 per cent based on the weight of dried mitochondria per weight of wet rat liver. This value agrees with the one reported by Williams and Sreenivasan. The extraction of choline dehydrogenase from rat liver mitochondria with sodium choleate

was essentially complete; there was no residue of enzymatic activity remaining in the particulate fraction. The extract also contained succinic dehydrogenase activity.

Assay methods

Choline dehydrogenase. The assay system for choline dehydrogenase contained 2,6-dichloro-indophenol,* choline chloride, and mitochondria, as described by Williams and Sreenivasan. The reduction of the dye was measured at 607 mμ with a Zeiss spectrophotometer. The soluble choline dehydrogenase was measured in the mitochondrial system except that the extracted enzyme was substituted for mitochondria.

Succinic dehydrogenase. Ferricyanide was used as an electron acceptor for some of the determinations of succinic dehydrogenase activity.[12] Reaction mixtures contained KCN (30 μmole), $K_3Fe(CN)_6$ (3 μmole), sodium succinate (40 μmole), and mitochondria in a final volume of 3 ml containing 0·1 M sodium phospahte buffer (pH 7·4).

Succinic dehydrogenase complex. The assay system was essentially that used for determining choline dehydrogenase,[11] with two exceptions: the reaction mixtures contained 18 μmole sodium succinate as substrate instead of choline chloride; the pH was 6·8 instead of 7·4.

Manometric experiments. Determination of oxygen uptake by rat liver and P388 cell mitochondria was made by standard manometric procedures.[13, 14] Determination of inorganic phosphate was made according to the method of Fiske and SubbaRow.[15] Phthalanilide analysis was made by the chromatographic method of Sivak *et al.*[6]

Reagents. NSC 60339,† 2-chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide, and NSC 762, methyl *bis*(β-chloroethyl)amine, were supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health. All other reagents were the best grades commercially available.

RESULTS

Intracellular distribution of NSC 60339 in P388 cells

The drug content of the subcellular fractions of sensitive and NSC 60339-resistant cells from mice treated for either 0·5 or 24 hr prior to harvest of cells is shown in Table 1. In all cases most of the drug was found in the nuclear and mitochondrial pellets. As the homogenization procedure was made more severe, more of the nuclear pellet was disrupted. After a 25-stroke homogenization, the mitochondrial and supernatant fractions contained considerable amounts of DNA, and concomitantly, a major fraction of NSC 60339.

With less severe homogenization techniques (10 strokes), there was an indication that at 0·5 hr the nuclei or an extranuclear component of the 700 *g* debris of sensitive cells may have contained most of the drug. After 24 hr the mitochondrial and nuclear fractions were essentially equivalent in drug concentration.

Preliminary results with the resistant line indicated that the nuclear and mitochondrial fractions also appeared to be the primary sites of drug accumulation. After a 0·5-hr treatment *in vivo*, resistant cells had a large amount of drug in the nuclear fraction as compared to the other experiments in Table 1. This was probably

* Synonym: 2,6-dichlorobenzenoneindophenol.

† NSC 60339 is the free base of NSC 38280.

TABLE 1. INTRACELLULAR DISTRIBUTION OF NSC 60339 IN P388 CELLS*

Cell fraction	Sensitive cells (½-hr treatment) 10-stroke homogenization				Sensitive cells (24-hr treatment) 6-stroke homogenization				10-stroke homogenization†				25-stroke homogenization			
	NSC 60339	DNA	RNA	Protein	NSC 60339	DNA	RNA	Protein	NSC 60339	DNA	RNA	Protein	NSC 60339	DNA	RNA	Protein
Nuclear, debris	66	69	30	45	36	82	27	35	43	57	25	39	7	6	2	8
Mitochondrial	27	9	8	12	60	4	7	17	52	12	8	14	77	57	11	37
Microsomal	7	6	44	11	4	4	37	14	5	8	40	15	5	8	14	20
Supernatant	0	16	18	32	0	10	29	34	0	23	27	32	11	29	73	35

Cell fraction	Resistant cells (½-hr treatment) 6-stroke homogenization)				Resistant cells (24-hr treatment 10-stroke homogenization)†			
	NSC 60339	DNA	RNA	Protein	NSC 60339	DNA	RNA	Protein
Nuclear, debris	83	86	40	62	43	58	17	20
Mitochondrial	11	2	10	6	39	29	4	16
Microsomal	6	2	40	8	16	4	43	17
Supernatant	0	10	10	24	8	9	46	47

* Values are given in per cent of total.

† The values in these experiments are averages of duplicate determinations. Ascites cells were harvested 7 days after implantation in BDF_1 mice. Animals were treated either 0·5 or 24 hr prior to harvesting cells (32 mg/kg, i.p.).

due to incomplete breakage of cells. Low levels of protein in the mitochondrial and microsomal fractions and the higher-than-normal levels in the nuclear fraction support this view. These results suggest that, after 0·5 or 24 hr of exposure to drug *in vivo*, the nuclear and the mitochondrial fractions contained most of the NSC 60339 bound to P388 cells.

Experiments were done to demonstrate that nuclei from cells which had not been exposed to drug did not affect the intracellular distribution of drug when added to homogenates of cells which had been exposed to drug *in vivo*. In the same experiment mitochondria from control cells were added to homogenates of cells which were exposed to drug *in vivo*. Nuclei or mitochondria from 3×10^8 saline-washed P388 cells in 0·25 M sucrose were added to equivalent volumes of 0·25 M sucrose containing 3×10^8 saline-washed, freshly homogenized P388 cells which had been exposed to drug in BDF_1 mice. Equivalent volumes of 0·25 M sucrose without organelles were added to other aliquots of cell homogenates as controls. No significant difference from control values of the intracellular distribution of NSC 60339 was found in experiments with P388 cells and NSC 60339-resistant P388 cells under the conditions of fractionation described in Materials and Methods.

Effect of NSC 60339 *on oxygen consumption by* P388 *leukemia cells*

Since a considerable fraction of the phthalanilide in the cells was associated with the mitochondrial pellet, it was of interest to determine the oxygen consumption of control and treated cells of sensitive and resistant leukemia lines. Exogenous glucose had no effect on the oxygen consumed during the course of the experiment. Addition of drug at zero time *in vitro* had no effect on the oxygen consumption of either treated or untreated cells of the sensitive line (Fig. 1). The data also show that a single 24-hr treatment of P388 leukemia cells *in vivo* (which did not result in prolonged survival of the host mice) did not inhibit the endogenous respiration of either the sensitive or resistant line. In fact, the endogenous respiration of treated cells was approximately double that of untreated cells. This unexpected finding led to an investigation of the effects of NSC 60339 on the function of isolated mitochondria from rat liver and P388 leukemia cells and a comparison with the effects of other antagonists of mitochondrial function.

Oxidative phosphorylation

P388 *leukemia mitochondria.* With succinate as substrate, the addition of NSC 60339 caused complete inhibition of phosphorylation and partial inhibition of oxidation. The P/O ratio was decreased about 50 per cent at 3×10^{-5} M NSC 60339. The data in Fig. 2 show that, although phosphorylation was completely inhibited at 5×10^{-5} M NSC 60339, oxidation was not inhibited more than 50 per cent with drug concentrations up to 10^{-4} M.

Rat liver mitochondria. Similar results were obtained with mitochondria from rat liver. However, the range of drug concentration from minimum to maximum inhibition of phosphorylation was about 10-fold greater. With succinate added, the P/O ratio was decreased about 50 per cent at about 4×10^{-5} M NSC 60339. The data in Fig. 2 show that, although phosphorylation was completely inhibited at 4×10^{-4} M drug, oxidation was not inhibited more than 50 per cent with drug concentrations up

to 7×10^{-4} M. Neither oxidation nor phosphorylation occurred in the presence of 4×10^{-4} M NSC 60339 without succinate.

Choline dehydrogenase

Rat liver mitochondria. The effects of NSC 60339, 2-4-dinitrophenol, spermine, and tyramine on choline dehydrogenase are summarized in Table 2. At concentrations of

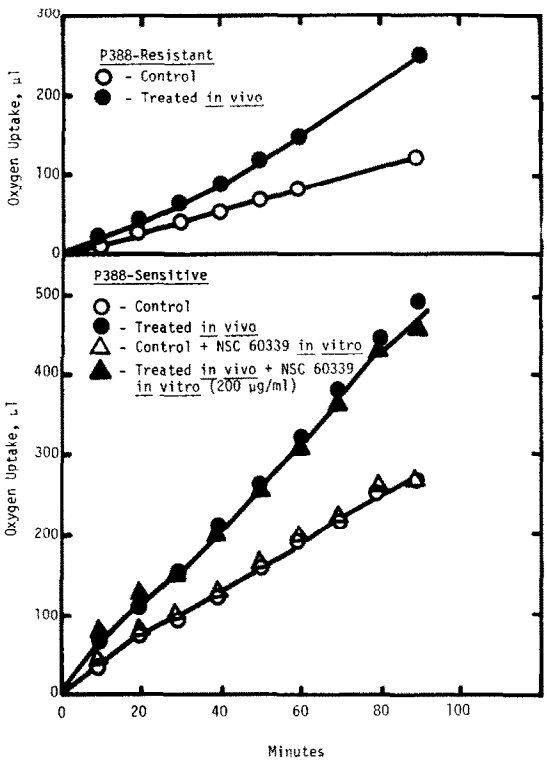

FIG. 1. Oxygen consumption by P388 cells treated with NSC 60339 *in vivo* or *in vitro*.

NSC 60339 which completely inhibited phosphorylation, 2,4-dinitrophenol had no inhibitory effect on choline dehydrogenase and spermine showed only a moderate inhibition (25–35 per cent). Tyramine, a previously reported inhibitor of choline oxidase,[5] was a potent inhibitor of choline dehydrogenase. Of the four compounds tested on a molar basis, NSC 60339 was the most potent inhibitor of choline oxidation.

Nitrogen mustard (NSC 762) has been reported to be a strong inhibitor of choline oxidation.[16] A manometric technique was used to determine the activity of the total oxidase system in liver homogenates with choline as substrate. Tyramine also was reported to be inhibitory in this system; however, tyramine protected against the action of nitrogen mustard.[5] With the spectrophotometric method for determining choline dehydrogenase, our experiments showed that nitrogen mustard had no effect on choline dehydrogenase in intact rat liver mitochondria and did not appear to affect the inhibition of dehydrogenase activity caused by tyramine or NSC 60339.

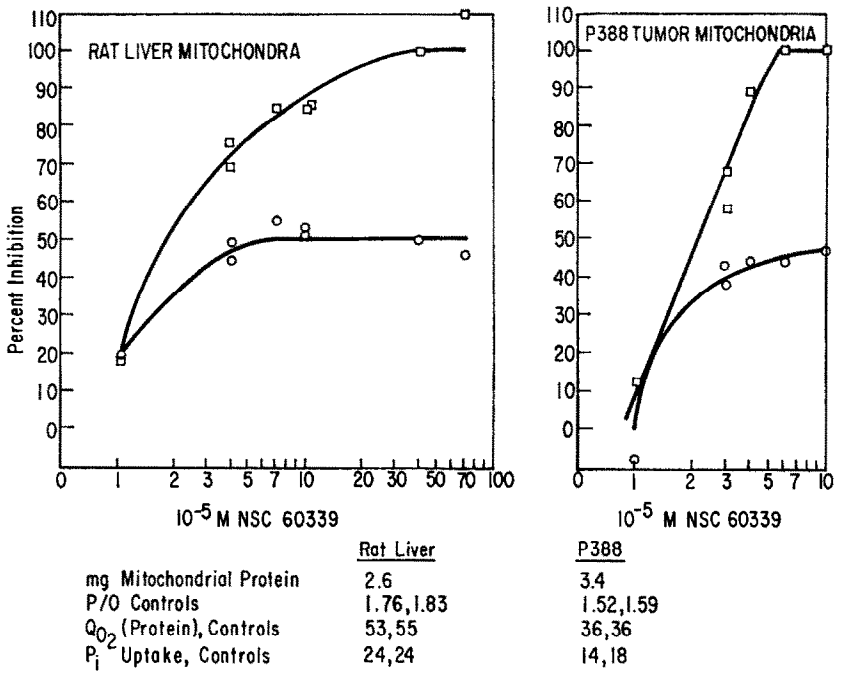

FIG. 2. Inhibition of oxidative phosphorylation by NSC 60339 *in vitro*. Square symbols indicate phosphorylation, circles indicate oxidation of succinate. Reaction vessels contained mitochondria in 0·25 M sucrose as indicated; potassium phosphate buffer (pH 7·4), 50 μmole; ADP, 10 μmole; NaF, 30 μmole; sodium succinate, 150 μmole; $MgCl_2$, 20 μmole, cytochrome c, 1 mg; the side-arm contained glucose, 60 μmole, and yeast hexokinase, 40 KM units; total volume 2·8 ml; the center well contained 10% KOH, 0·2 ml. Drug was added approximately 15 min prior to the addition of glucose and hexokinase.

TABLE 2. EFFECT OF 2,4-DINITROPHENOL, SPERMINE, TYRAMINE AND NSC 60399 ON CHOLINE DEHYDROGENASE ACTIVITY IN RAT LIVER MITOCHONDRIA*

	Inhibitor conc. (M)	$-\Delta A_{607m\mu} \times 10^3$/ 4·5 min	Minus endogenous	Per cent inhibition
Endogenous		66		
Choline control		228	162	0
+ 2,4-Dinitrophenol	10^{-4}	219	153	5
	2×10^{-4}	229	163	0
+ Spermine	3×10^{-3}	172	106	35
	9×10^{-3}	187	121	25
+ Tyramine	3×10^{-4}	102	36	78
	3×10^{-3}	36	−30	118†
Endogenous		70		
Choline control		239	169	0
+ NSC 60339	10^{-6}	191	121	29
	5×10^{-5}	105	35	79
	10^{-4}	49	−21	112†
	2×10^{-4}	15	−55	132†

* Each cuvette contained 1·7 mg protein in a total volume of 3·0 ml.
† Endogenous inhibited.

The soluble choline dehydrogenase was inhibited by NSC 60339 and by tyramine as effectively as the mitochondria-bound enzyme, but spermine was without effect at 6×10^{-3} M (Table 3).

TABLE 3. EFFECTS OF TYRAMINE, SPERMINE AND NSC 60339 ON SOLUBLE DEHYDROGENASES FROM RAT LIVER MITOCHONDRIA

	$-\Delta A_{607m\mu} \times 10^3$/ 4·5 min	$-\Delta A_{607m\mu} \times 10^3$/ 4·5 min minus endogenous	Per cent inhibition
Endogenous	219		
Choline control	349	130	0
+ Tyramine 6×10^{-3} M	224	5	97
+ Spermine 6×10^{-3} M	347	128	2
+ NSC 60339 3×10^{-4} M	263	44	56
5×10^{-4} M	75	−144	211*
Succinate control	355	136	
+ NSC 60339 3×10^{-4} M	300	81	40

* Endogenous inhibited.

P388 *leukemia mitochondria.* Neither mitochondria-bound nor soluble choline dehydrogenase was demonstrable in preparations from P388 leukemia cells of either sensitive or resistant lines.

Succinate oxidation

Rat liver mitochondria. Conventional manometric techniques were used to show that 10^{-3} M malonate exhibited its usual inhibitory effect on succinate oxidation in a system not coupled to phosphorylation (Fig. 3). There was no effect of NSC 60339, tyramine, spermine or 2,4-dinitrophenol at concentrations indicated in Fig. 3. (Each flask contained 9 mg mitochondrial protein.) At 2–4 mg mitochondrial protein per flask, the phthalanilide had a significant effect on succinate oxidation at 5×10^{-5} M in a system coupled to phosphorylation (Fig. 2).

P388 *leukemia mitochondria.* Succinic dehydrogenase activity of mitochondria isolated from P388 cells was determined with ferricyanide as an electron acceptor. This method measures the conversion of succinate to fumarate specifically.[17] After a typical homogenization and fractionation, 20 per cent of the enzymatic activity was found in the nuclear pellet (700 *g*, 10 min), 80 per cent was found in the mitochondrial pellet (10,000 *g*, 20 min), and none was found in the supernatant of the mitochondrial pellet. However, it was not possible to measure the effect of NSC 60339. Even at low concentrations of phthalanilide, a heavy precipitate was observed after the addition of NSC 60339 and ferricyanide to the incubation medium without added mitochondria.

To measure the effect of NSC 60339 on the oxidation of succinate, it was necessary to determine the enzymatic activity of mitochondria with 2,6-dichloroindophenol as the electron acceptor. This system measures succinate oxidation by determining the subsequent oxidation of components of the electron transfer chain which are coupled to the reduction of the indophenol.

The effect of NSC 60339 treatment *in vivo* on the succinic dehydrogenase complex in mitochondria from P388 cells is shown in Table 4. Oxidation of succinate was not inhibited as a result of the treatment *in vivo*. However, addition of NSC 60339 at

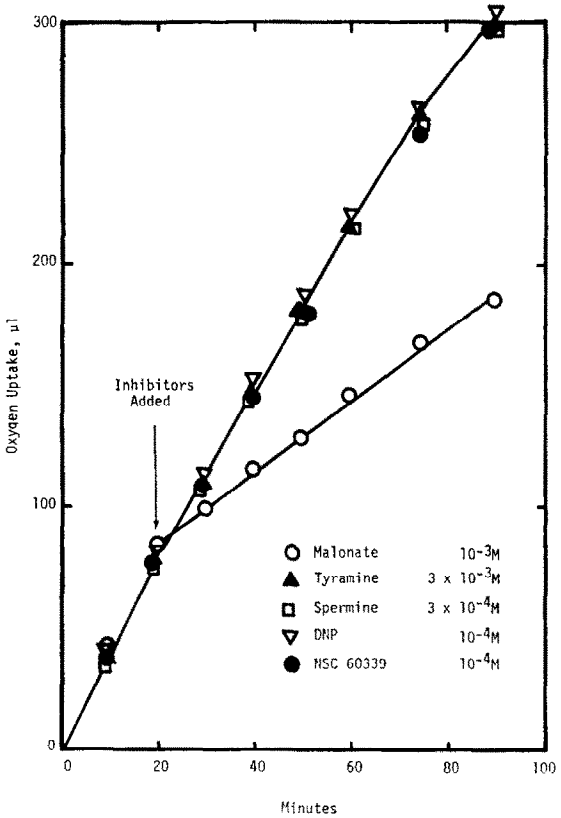

FIG. 3. Effect of malonate, tyramine, spermine, 2-4-dinitrophenol, and NSC 60339 on succinate oxidation by rat liver mitochondria. Reaction vessels contained mitchondria from 200 mg (wet wt) rat liver (9 mg mitochondrial protein); succinate, 150 µmole; phosphate buffer (pH 7·4), 100 µmole; $CaCl_2$, 1·2 µmole; $AlCl_3$, 1·2 µmole, cytochrome c, 0·04 µmole. The center well contained 0·2 ml of 10% KOH and the side-arm contained reagents (0·1 ml) added at 20 min. Total liquid was 3·0 ml.

TABLE 4. INFLUENCE OF NSC 60339 ON MITOCHONDRIAL SUCCINIC DEHYDROGENASE COMPLEX

Mitochondria origin	$-\Delta A_{607\ m\mu}$/4·5 min Endogenous	Succinate	Δ	NSC 60339* (µg drug/mg protein)
P388/NSC 60339 sensitive				
Control	83	241	129	
0·5-hr exposure *in vivo*	97	270	148	0·7
control	69	198	158	
24-hr exposure *in vivo*	72	220	173	0·5
P388/NSC 60339 resistant				
control	35	181	146	
0·5-hr exposure *in vivo*	54	177	123	0·7
control + NSC 60339 (10^{-4} M) *in vitro*	35	114	79	
0·5-hr exposure *in vivo* + NSC 60339 (10^{-4} M) *in vitro*	54	106	52	

* Cuvette contained 3 mg protein; total volume 3·0 ml.

10^{-4} M to control and treated mitochondria* resulted in approximately a 50 per cent inhibition of succinic dehydrogenase complex activity.

DISCUSSION

The substituted phthalanilide, 2-chloro-4′,4″-di(2-imidazolin-2-yl) terepthalanilide (NSC 60339), has been shown to be an effective inhibitor of several parameters of mitochondrial function. The results reported in this communication indicate that there are interesting, if general, effects of NSC 60339 on the biochemical activities of both rat liver and P388 leukemia mitochondria *in vitro*.

Oxidative phosphorylation

Inhibition of phosphorylation and coupled succinate oxidation was achieved with low drug concentrations. A 50 per cent decrease in P/O ratio with mitochondria from both rat liver and leukemia cells was obtained with 2–4 $\times$ 10^{-5} M NSC 60339 *in vitro*. However, there was a marked difference in the slopes of the dose-response curves. Phosphorylation by mitochondria from P388 leukemia was not affected by 10^{-5} M NSC 60339, but was completely inhibited by 5 $\times$ 10^{-5} M drug. Progressive inhibition in rat liver mitochondria extended over more than a 20-fold range of drug concentration and resulted in 100 per cent inhibition of phosphorylation at 3–4 $\times$ 10^{-4} M NSC 60339.

Our results with mitochondria of P388 cells and rat liver *in vitro* are in partial agreement with those of Pine and DiPaolo, who found that NSC 38280, the dihydrochloride salt of NSC 60339, decreased the P/O ratio substantially in rat liver mitochondria treated *in vitro*. However, they found that the drug had no effect on the respiration of L1210 lymphocytic leukemic cells after treatment *in vitro*, whereas exposure to NSC 38280 (2 $\times$ 33 mg/kg) *in vivo* markedly depressed respiration of L1210 cells as well as oxygen uptake and P/O ratio of L1210 mitochondria.[18, 19]

The apparent differences between the response of P388 and L1210 to NSC 60339 *in vivo* may be due to several factors. The dose used by Pine and DiPaolo (2 $\times$ 33 mg/kg) was approximately 4 times greater than the dose we used (1 $\times$ 16 mg/kg). Further, their results show that only a slight depression in respiration was demonstrable up to 24 hr after therapy, and that a second treatment resulted in a marked inhibition of respiration. Thus, the absence of an inhibition of mitochondrial function in P388 cells after drug exposure *in vivo* is probably related to the dose and time of exposure that we used. Preliminary experiments indicate that with dosages of drug that significantly prolong the life of host mice, oxidative phosphorylation by the leukemia cells is inhibited significantly.

An interesting aspect of the inhibition of oxidative phosphorylation by NSC 60339 is its effect on succinate oxidation. The first 50 per cent decrease in P/O ratio in both the rat liver and the P388 systems represents inhibition of both oxidation and phosphorylation. However, the inhibition from 50–100 per cent is due to inhibition of phosphorylation only and there is essentially no further inhibition of oxidation at higher drug levels. The remaining 50 per cent oxidation was apparently not due to endogenous oxygen uptake or stimulation of oxygen uptake by drug in liver mitochondria, since

* Although the cells were from the NSC 60339-resistant line, it has been established that the intracellular concentration[2] and distribution (Table 1) of NSC 60339 are equivalent in the sensitive and resistant lines at 0·5 hr after administration of the drug to host mice.

without succinate no oxygen uptake was found in the presence of sufficient drug to inhibit phosphorylation completely (4×10^{-4} M NSC 60339). Further, the oxidation of succinate in an uncoupled system was not inhibited by 10^{-4} M NSC 60339. These results suggest that one effect of NSC 60339 may be an inhibition of the energy coupling or phosphorylation reactions of the enzymatic chain.

The reduction of cellular levels of ATP as a result of exposure *in vivo* of L1210 cells to a high level of the dihydrochloride salt of NSC 60339 has been reported, although this reduction was partially reversed by glucose. Moreover, lower levels of phthalanilide that were therapeutic did not reduce ATP levels and still exerted an inhibitory effect on mitochondrial function, according to Pine and DiPaolo.[19] Based on the available evidence, the precise nature of the inhibitory effect of NSC 60339 on oxidative phosphorylation in mitochondria is not clear. However, further information on this point is presented in the following paper.[20]

Oxidations not coupled to phosphorylation

Choline oxidation by intact rat liver mitochondria or by soluble enzymes derived from these mitochondria was inhibited markedly by NSC 60339. On a molar basis, NSC 60339 was approximately 10 times as effective as tyramine in the inhibition of solubilized or mitochondrial choline dehydrogenase.

Succinate oxidation by intact mitochondria of P388 cells also was inhibited *in vitro* by NSC 60339. However, mitochondria isolated from cells exposed *in vivo* to NSC 60339 did not show any inhibition of succinate oxidation. Treatment of tumor-bearing mice with a single dose of NSC 60339 (16 mg/kg), which did not cause increased survival compared with untreated controls, did not affect the oxidative phosphorylation with succinate as a substrate or the indophenol-coupled oxidation of succinate by the mitochondria from P388 cells. Because the concentration of NSC 60339 in mitochondria after such exposure to drug *in vivo* was only about 5 per cent of that required to elicit a minimal biochemical effect *in vitro*, these results are not surprising.

Possible sites of action

A possible common denominator of both the antitumor and toxic effects of NSC 60339 may be associated with its inhibition of mitochondrial function.[1, 19, 21] The processes of RNA, DNA, protein, and lipid biosynthesis all require energy of mitochondrial origin, and all these processes are inhibited in murine lymphocytic leukemia cells by NSC 60339.[1, 22, 25, 28] As noted above, although cellular ATP levels are depressed at high drug dosages, normal ATP levels obtain at lower phthalanilide doses that are thereapeutic.[19] Thus, reduction of this ATP pool and the source of energy associated with it probably does not represent the proximal biochemical inhibition due to NSC 60339. Meloni and Rogers[20] have reported that oligomycin and NSC 60339 are equivalent in their effects on mitochondrial swelling. Therefore, the action of these two compounds may be to inhibit the formation of a high-energy intermediate not associated with primary oxidative phosphorylation. An alteration of the energy balance in such a manner could account for the inhibitions of biosynthetic reactions that have been observed.

The finding of Yesair *et al.*[2, 23–25] that a substantial portion of the NSC 60339 associated with P388 cells can be extracted as a drug–lipid complex suggests that

NSC 60339 has a strong affinity for biological membranes.[29] Thus, the mitochondrial inhibitions caused by NSC 60339 may be related to the binding of the phthalanilide to some essential component of the mitochondrial membranes.

NSC 60339 has been shown to bind to DNA *in vitro* and *in vivo*.[26-28] This drug–DNA affinity is also evident from our results reported here (Table 1). The role of this interaction in the biological effects of NSC 60339 is not clear from the information now available, although the inhibition by NSC 60339 of synthetic functions in a cell could be attributed to a DNA–phthalanilide complex.

The affinity of NSC 60339 for several components in the cell (nucleic acids, lipids) may explain the difficulty in focusing on a particular primary site of action. Moreover, the binding of the phthalanilide to various lipoid membranes (plasma membrane, endoplasmic reticulum, lysosomes, mitochondria) would be a sufficient condition to account for the observed toxic and therapeutic effects of NSC 60339.

Acknowledgements—The authors wish to thank Dr. C. J. Kensler for his continued encouragement and support throughout this study. We are grateful to Mr. Isidore Wodinsky for his generosity in supplying animals.

REFERENCES

1. W. I. ROGERS, D. W. YESAIR, I. WODINSKY, A. J. MAHONEY, JR., A. SIVAK and C. J. KENSLER, *Proc. Am. Ass. Cancer Res.* **5**, 54 (1964).
2. D. W. YESAIR, F. A. KOHNER, W. I. ROGERS, P. E. BARONOWSKY and C. J. KENSLER, *Cancer Res.* **26**, 202 (1966).
3. C. J. KENSLER, P. E. PALM, H. M. DAY, S. P. BATTISTA, W. I. ROGERS, D. W. YESAIR and I. WODINSKY, *Cancer Res.* **25**, 1622 (1965).
4. H. TABOR, C. W. TABOR and S. M. ROSENTHAL, *A. Rev. Biochem.* **30**, 579 (1961).
5. J. S. COLTER and J. H. QUASTEL, *Archs Biochem. Biophys.* **41**, 305 (1952).
6. A. SIVAK, W. I. ROGERS, I. WODINSKY and C. J. KENSLER, *J. natn. Cancer Inst.* **33**, 457 (1964).
7. Z. DISCHE, *Mikrochemie* **8**, 4 (1930).
8. W. MEJBAUM, *Z. phys. Chem.* **258**, 117 (1939).
9. E. LAYNE, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 3, p. 447. Academic Press, New York (1957).
10. W. C. SCHNEIDER and G. H. HOGEBOOM, *J. biol. Chem.* **183**, 123 (1950).
11. J. N. WILLIAMS, JR. and A. SREENIVASAN, *J. biol. Chem.* **203**, 899 (1953).
12. W. D. BONNER, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 1, p. 722. Academic Press, New York (1955).
13. F. E. HUNTER, JR., in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 2, p. 610. Academic Press, New York (1955).
14. W. E. UMBREIT, R. H. BURRIS and J. F. STAUFFER, in *Manometric Techniques*. Burgess, Minneapolis, Minn. (1957).
15. L. F. LELOIR and C. E. CARDINI, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 3, p. 840. Academic Press, New York (1957).
16. E. S. G. BARRON, G. R. BARTLETT and Z. B. MILLER, *J. exp. Med.* **87**, 489 (1948).
17. T. P. SINGER and E. B. KEARNEY, *Meth. biochem. Analysis* **4**, 307 (1957).
18. M. J. PINE, *Proc. Am. Ass. Cancer Res.* **5**, 51 (1964).
19. M. J. PINE and J. A. DIPAOLO, *Cancer Res.* **26**, 18 (1966).
20. M. L. MELONI and W. I. ROGERS, *Biochem. Pharmac.* **16**, 1835 (1967).
21. A. J. MAHONEY, JR., W. I. ROGERS, A. SIVAK and C. J. KENSLER, 6th *Int. Cong. Biochem.* abstract VIII-66 (1964).
22. M. OCHOA, JR., A. GELLHORN and W. B. BENJAMIN, *Cancer Res.* **24**, 480 (1964).
23. D. W. YESAIR, A. SIVAK, W. I. ROGERS and C. J. KENSLER, *Proc. Am. chem. Soc.* abstract 96-C (1963).
24. D. W. YESAIR, W. I. ROGERS, J. T. FUNKHOUSER and C. J. KENSLER, *J. Lipid Res.* **7**, 492 (1966).

25. D. W. YESAIR, W. I. ROGERS, P. E. BARONOWSKY, I. WODINSKY, P. S. THAYER and C. J. KENSLER, *Cancer Res.* **27**, 314 (1967).
26. A. SIVAK, W. I. ROGERS and C. J. KENSLER, *Biochem. Pharmac.* **12**, 1056 (1963).
27. A. SIVAK, W. I. ROGERS, I. WODINSKY and C. J. KENSLER, *Cancer Res.* **25**, 902 (1965).
28. J. BOOTH, E. BOYLAND and A. GELLHORN, *Cancer Chemother. Rep.* **43**, 11 (1964).
29. R. HIRT and R. BERCHTOLD, *Cancer Chemother. Rep.* **18**, 5 (1962).