Biochemical Pharmacology, Vol. 28, pp. 2471–2478.

0006-2952/79/0815–2471 $02.00/0

SELECTIVE INHIBITORS OF LECITHIN BIOSYNTHESIS IN MOUSE PERITONEAL MACROPHAGES

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(*Received* 19 *October* 1978; *accepted* 26 *January* 1979)

Abstract—Mouse peritoneal macrophages turn over their membrane components rapidly. The biosynthetic pathway for phosphatidylcholine synthesis from choline can be inhibited in a non-toxic manner by a series of diphenylsulfones. The parent compound of this series, dapsone, and its derivative, 1-[4-(4-sulfanilyl)phenyl]urea (AUS), inhibit the synthesis of lecithin from choline by mouse peritoneal macrophages in a dose- and time-dependent manner. This inhibition of choline incorporation does not appear to be due to an effect on transport of choline into the cells nor is it an effect on general membrane synthesis. In addition, the sulfones inhibit the incorporation of [^{32}P]phosphate into phospatidylcholine but do not significantly inhibit its incorporation into sphingomyelin, phosphatidylinositol or phosphatidylethanolamine. This inhibition of lecithin synthesis is reversible and appears to require functionally intact membranes.

Macrophages continuously internalize large amounts of their plasma membranes [1], indicating a high rate of turnover of membrane components. Indeed, plasma membrane proteins of rabbit alveolar macrophages were found to have an average half-life of 8 hr [2]. In this study, Nachman *et al.* [2] also showed that radioactive choline was incorporated into phosphatidylcholine (PC)* and lysophosphatidylcholine of alveolar macrophage membranes. The biosynthetic pathway for the formation of PC and phosphatidylethanolamine (PE) has been well studied in liver [3–5]. Phosphate esters of choline and ethanolamine are transferred to diacylglycerol acceptors with CDP-choline and CDP-ethanolamine as obligatory intermediates (Fig. 1). These transfer reactions are catalyzed by separate cytidylyltransferases [6]. In addition, PC can be formed in liver from PE and *S*-adenosylmethionine via successive phospholipid *N*-methylations [7]. The relative importance of these pathways for PC formation by macrophages is unknown. PC represents 33 per cent and PE 23 per cent of the phospholipid content of rabbit alveolar macrophages [8]. It is expected, therefore, that changes in the polar head group composition could alter charge and fluidity of the membrane [9]. These changes could, in turn, affect various cellular functions in which plasma membranes participate.

The macrophage is a phagocytic and secretory cell which plays important roles in defense against tumour cells and infections agents and in chronic inflammation. The secretory products of these cells include neutral proteinases [10–12], lysosmal acid hydrolases [13], prostaglandins [14], factors which influence the proliferation of lymphocytes [15, 16], pyrogen [17], and complement components [18]. Little is known about the regulation of secretion or release of these products but two where controls could be exerted are at the levels of the lysosomal and plasma membranes, although other sites cannot be excluded.

One approach to the study of mechanisms controlling macrophage secretion of products is to correlate changes in membrane composition with secretory activity. This can be accomplished by using specific, non-toxic inhibitors of the biosynthesis of membrane components of cultured cells. In this report, we show that the diphenylsulfones specifically inhibit the formation of PC from choline in mouse peritoneal macrophages.

MATERIALS AND METHODS

All animals used in this study were 6-week-old male Swiss Webster mice obtained from Hilltop Lab Animals, Scottdale, PA.

Hela cells were obtained from the laboratory of Dr. C. C. Wang of this Institute. Tissue culture medium 199 (M199), Dulbecco's phosphate-buffered saline (PBS), penicillin–streptomycin solution, sodium heparin and porcine serum were obtained from GIBCO, Grand Island, NY. The porcine serum was inactivated by heating at 56° for 30 min. Reduced nicotinamide adenine dinucleotide (NADH), human serum albumin and lysozyme (salt free) were from the Worthington Biochemical Co., Freehold, NJ. *Micrococcus lysodeikticus* was purchased as freeze-dried cells from Cal-Biochem, San Diego, CA. Nunclon tissue culture dishes were purchased from Vanguard International, Inc., Neptune, NJ. Redi-Coat silica gel thin-layer chromatography plates were purchased from Supelco, Inc., Belletente, PA, and SG81 paper for paper chromatography was from Whatman, Ltd. 1,2-Diolein was obtained from the Sigma Chemical Co., St. Louis MO. The labeled compounds, [methyl-^{14}C]-CDP-choline (49 mCi/m-mole), [1,2-^{14}C]choline chloride (64 mCi/m-mole), [1,2-^{14}C]-ethanolamine hydrochloride

*Abbreviations used in this paper are: PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin; PE, phosphatidylethanolamine; CDP, cytidine diphosphate; HIPS, heat-inactivated porcine serum; AUS, (1-[4-(4-sulfanilyl)phenyl]urea; M199, medium 199; PBS, Dulbecco's phosphate-buffered saline; TCA, trichloroacetic acid; DMSO, dimethylsulfoxide; and EGTA, ethyleneglycol-bis(aminoethylether)tetra-acetate.

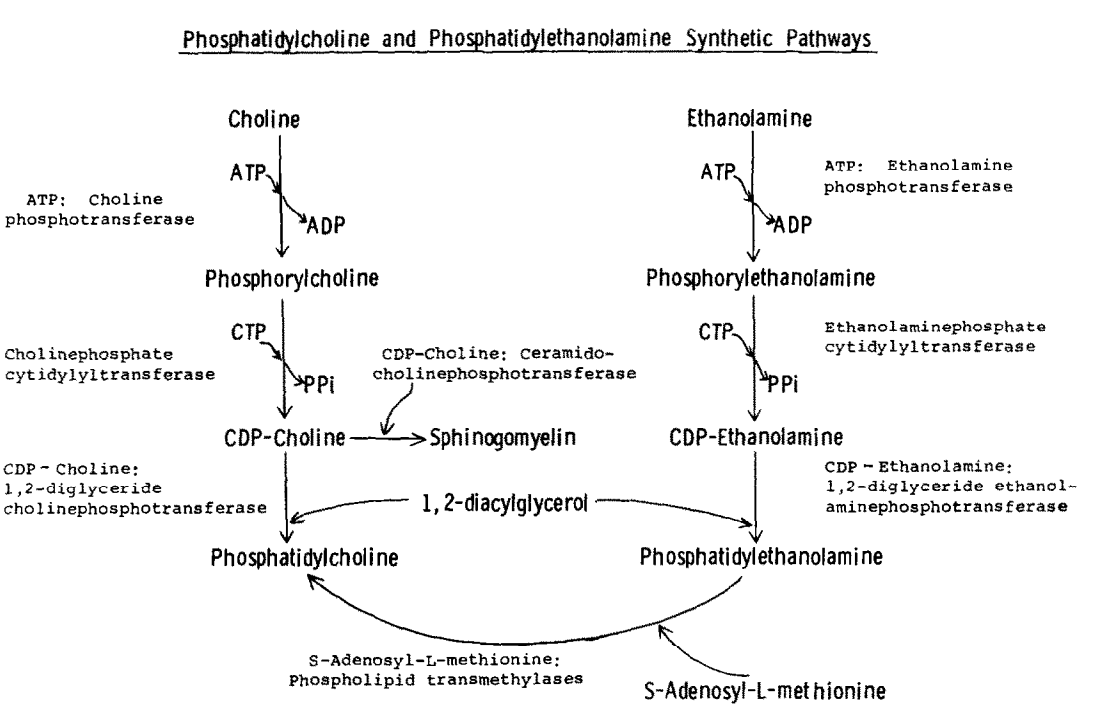

Fig. 1. Pathway of phosphatidylcholine and phosphatidylethanolamine biosynthesis.

(3.9 mCi/m-mole), [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (10 Ci/m-mole), [9,10^{3}H(N)]oleic acid, (6.4 Ci/m-mole), D-[6-^{3}H(N)]glucosamine hydrochloride (10 Ci/m-mole) and [^{32}P]orthophosphoric acid (carrier-free) were obtained from New England Nuclear, Boston, MA.

The diphenylsulfones [1-[4-(4-sulfanilyl)phenyl]-urea (AUS) and *P,P'*-diaminodiphenylsulfone (dapsone)] were obtained from N. P. Jensen of this Institute. Radioactive derivatives bore the ^{14}C-moiety in the benzene ring.

All spectrophotometric determinations were performed on a Gilford Stasar III spectrophotometer equipped with a model 4009 recorder. Liquid scintillation counting was performed using a Beckman model LS3150T liquid scintillation counter.

Macrophage culture

Peritoneal macrophages were isolated by peritoneal lavage from untreated mice or from mice stimulated 4 days previously by intraperitoneal injection of 2 ml of thioglycollate broth. The lavage medium was M199 containing 1% heat-inactivated procine serum (HIPS), 200 units penicillin and 200 μg streptomycin/ml, and 20 units sodium heparin/ml. The peritoneal exudates were pooled, and cells were counted in a hemocytometer chamber, plated in 50 mm dishes at a density of 5×10^6 cells per dish, and incubated in a humidified atmosphere of 5% CO_2 in air at 37°. The cells were allowed to adhere for 2 hr. Each dish was then washed three times with PBS to remove the non-adhering cells and the medium was replaced with fresh M199 containing 10% HIPS. Cell cultures were then incubated for 24 hr under the conditions described above. After this period the medium was removed and replaced with M199 containing 1% HIPS and antibiotics and incubated for an additional 24 hr. Studies of label incorporation were initiated with replacement of the medium with serum-free M199 including antibiotics plus various additions as prescribed by experimental protocol.

Assay of [^{14}C]Choline incorporation

[^{14}C]Choline incorporation into cultured peritoneal macrophages was determined in the following way. Serum-free M199 containing antibiotics plus [^{14}C]choline (0.125 μCi/ml) was added to the cultures, which were then incubated for 4 hr. The medium was removed and assayed for lactate dehydrogenase. Leakage of this enzyme into the media was used as a measure of toxicity [13]. The cells were washed three times with PBS and removed from the dish in 4 ml of ice-cold TCA after scraping with a rubber policeman. This material was then centrifuged for 10 min at 2000 rev/min, and the precipitate was resuspended in 2 ml of ice-cold TCA and again centrifuged. This step was repeated three times. The TCA-soluble material was pooled after each centrifugation and counted for radioactivity. The final precipitate (TCA-insoluble material) was dissolved in 1 ml of 1 N NaOH. Protein was measured by the method of Lowry *et al.* [19] and 0.5 ml was added to a scintillation vial containing 0.5 ml of 1 N HCl, 1 ml H_2O and 15 ml Aquasol-2 and counted.

Experiments measuring the incorporation of other radiolabeled membrane precursors were as described above with the substitution of [^{14}C]choline by one of the other precursors.

Extraction and identification of labeled phospholipids

Cells labeled with [^{14}C]choline or [^{14}C]ethanolamine were harvested in cold PBS with a rubber police-

man. The phospholipid material was extracted by the method of Folch *et al.* [20] and chromatographed on SG81 paper in chloroform–methanol–acetic acid–H_2O (50:25:8:4) with authentic phospholipid standards. The standards were located by iodine vapor and the entire chromatogram was scanned with a Packard Strip Scanner. Quantitation was achieved by cutting out the labeled areas and counting them in the liquid scintillation counter.

Characterization of ^{32}P labeled material was accomplished by extraction by the Folch method followed by separation of the products using two-dimensional thin-layer chromatography. The products were located by autoradiography and identified by their migration with appropriate standards. Quantitation was achieved by subsequent scraping and counting of the areas of the thin-layer plates corresponding to the chromatographic standards. System I was chloroform, methanol and concentrated ammonia (65:35:8) and system II was chloroform, acetone, methanol, acetic acid and H_2O (30:40:10:1:5).

Enzyme assays

Lysozyme. Lysozyme was assayed according to the method of Gordon *et al.* [21]. In brief, intracellular lysozyme activity and lysozyme activity released into the culture medium were determined by monitoring the rate of lysis of *M. lysodeikticus* in a Gilford spectrophotometer at 550 nm.

Lactate dehydrogenase. Lactate dehydrogenase was assayed by measuring the rate of decrease of optical density at 340 nm due to oxidation of 0.18 mM NADH in the presence of 2.6 mM pyruvate and 50 mM phosphate buffer (pH 7.5).

Cholinephosphotransferase. Peritoneal exudate cells were isolated by peritoneal lavage with 5 ml of heparinized PBS from male Swiss Webster mice stimulated 4 days previously with 2 ml of thioglycollate broth. Exudates were pooled and centrifuged for 10 min at 1500 rev/min. Contaminating erythrocytes were removed by hypotonic lysis and the remaining cells were washed three times in cold PBS. The final pellet was resuspended in 6 ml of cold PBS and homogenized by sonication for 10 min on ice. The homogenate was then centrifuged for 15 min at 1000 *g* to sediment nuclei and cell debris. Greater than 95 per cent of the cells were disrupted by this procedure, as determined by the presence of the plasma membrane marker enzyme 5′-nucleotidase [22] and the cytoplasmic enzyme lactate dehydrogenase [23] in the supernatant fracture. Protein content of the supernatant fracture was determined by the method of Lowry *et al.* [19].

The cholinephosphotransferase activity was assayed using a modification of the method described by Wang *et al.* [24]. The reaction mixture contained 1 mg of homogenate protein, 0.175 M Tris/HCl (pH 8.5), 0.5 mM EGTA, 0.01 M $MgCl_2$, 3.2 mM 1,2-diolein in ethanol (final concentration, 2.5%), 0.2 mM (0.08 μCi) [^{14}C]-CDP-choline, and H_2O to give a final volume of 1 ml. The reaction was terminated after 20 min by the addition of 1 ml of cold saturated *n*-butanol. Each reaction tube was agitated vigorously on a Vortex mixer for 30 sec and centrifuged for 10 min at 2000 rev/min. Three hundred μl of the upper *n*-butanol phase was removed and added to a scintillation vial containing 15 ml of Aquasol-2, and counted.

RESULTS

[^{14}C]Choline incorporation by mouse macrophages

Since the major phospholipid component of macrophage membranes is PC [8], we examined the biosynthesis of this class of lipid as a measure of total phospholipid synthesis under a variety of conditions. The incorporation of exogenously added [^{14}C]choline into PC by macrophages was linear for at least 4 hr (Fig. 2,

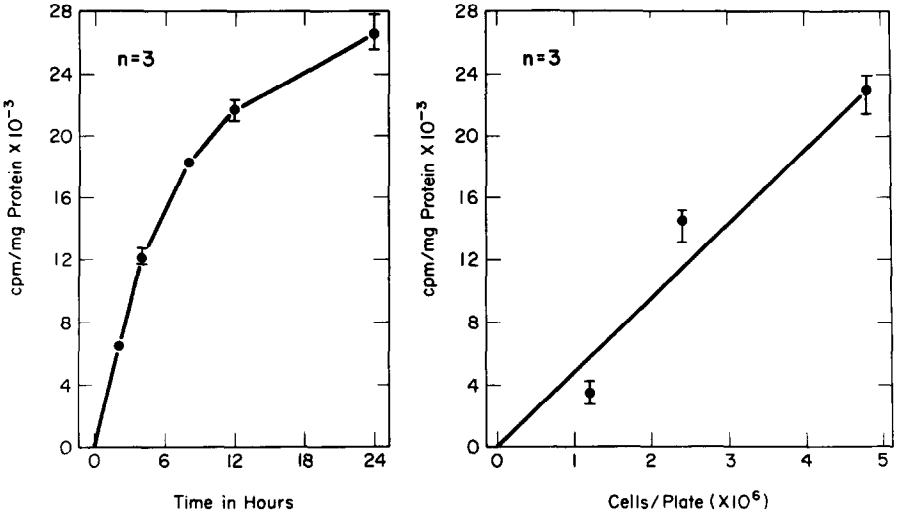

Fig. 2. Incorporation of [^{14}C]choline into cultured macrophages. Thioglycollate-stimulated macrophages were cultured for 48 hr, as described in Materials and Methods. At this time, fresh M199 devoid of serum and containing 0.125 μCi/ml of [^{14}C]choline was added and the cultures were incubated at 37° in 5% CO_2 in air. The results are the averages ±S.D.; N = 3. The left panel shows incorporation as a function of time, 5 × 10^6 cells/plate. The right panel shows incorporation over a 4-hr period as function of cell number.

Table 1. Inhibition by AUS and dapsone of the cellular incorporation of acid-insoluble but not acid-soluble [^{14}C]choline*

Addition	Concn (μg/ml)	Acid-insoluble (cpm × 10^2)	Protein (mg)	Cellular uptake Acid-insoluble (cpm × 10^3/mg protein)	% Inhibition	Acid-soluble (cpm × 10^2)	% Inhibition
Control		457	0.325	141 ± 1.6		15.7 ± 1.0	
AUS	10	384	0.300	128 ± 5.4	9.2	15.9 ± 1.0	−1.2
	20	342	0.303	113 ± 2.2	19.9	13.3 ± 0.4	15.2
	40	316	0.320	98 ± 2.1	30.5	14.8 ± 1.1	5.7
	80	262	0.300	88 ± 7.1	37.6	12.9 ± 0.1	17.7
Dapsone	10	399	0.288	139 ± 12.4	1.5	16.7 ± 0.6	−0.5
	20	387	0.307	126 ± 8.1	10.1	17.9 ± 0.4	−14.0
	40	340	0.300	113 ± 8.1	19.3	16.4 ± 1.5	−4.4
	80	284	0.317	89 ± 4.1	26.9	15.9 ± 1.3	−1.2
	160	323	0.280	84 ± 7.6	40.5	16.8 ± 0.7	−7.0

* Macrophages (5×10^6) from thioglycollate-stimulated mice were cultured as described in Materials and Methods. M199 media (4 ml) containing 1% HIPS and 0.5 μCi of [^{14}C]choline was added to the plates. AUS or dapsone dissolved in dimethylsulfoxide (DMSO) was added as described. All cultures contained a final concentration of 0.1% DMSO. Cells were harvested after 4 hr and radioactive uptake was determined. The results are the averages ±S.D.; N = 3.

left panel). Furthermore, the incorporation was linear (correlation coefficient of 0.96) with cell number from 1×10^6 to 5×10^6 per 50 mm dishes (Fig. 2, right panel). Neither the rate nor the total amount of [^{14}C]choline incorporation was influenced by the presence of serum in the media. In addition, the incorporation was not quantitatively different in macrophages from untreated mice or from mice which had received an inflammatory stimulus in the form of an intraperitoneal injection of thioglycollate broth 4 days previously. Therefore, all assays of [^{14}C]choline incorporation were performed over a 4-hr period with a cell density between 2 and 5×10^6/plate.

Specific inhibition of macrophage PC synthesis by diphenylsulphones

It has been reported that the diphenylsulfones, AUS and dapsone, inhibit [^{14}C]choline incorporation into PC of avian macrophages [25]. We now show that these compounds also inhibit the incorporation of [^{14}C]choline by mammalian macrophages in a dose-dependent manner. There appears to be little or no inhibition of the uptake of choline by the cells as measured by the amount of acid-soluble cell associated radioactivity (Table 1). Therefore, it seems likely that these compounds inhibit the incorporation of [^{14}C]choline into PC and not its transport into the cells. These compounds are equally effective in inhibiting choline incorporation into macrophages from untreated mice and from thioglycollate-stimulated mice.

In order to show that the incorporation of the phospholipid precursor, [^{14}C]choline, into TCA-insoluble material is a measure of PC synthesis, we examined [^{14}C]choline incorporation into TCA-insoluble material and into extracted lipid, and determined the effects of the diphenylsulfones. The extracted lipid containing [^{14}C]choline was identified as PC by chromatography. The results, shown in Table 2, demonstrate that AUS inhibits the incorporation of choline into TCA-insoluble material as well as the incorporation into extracted lipid material. Since all of the lipid extractable material co-chromatographed with PC, it is likely that the incorporation of [^{14}C]choline into TCA-insoluble materials reflects PC synthesis from choline. The sulfones, AUS and dapsone, did not affect the incorporation of ethanolamine in TCA-insoluble material or into lipid ex-

Table 2. Effect of AUS on choline and ethanolamine incorporation into phospholipids of macrophages*

Additions	TCA-insoluble (cpm × 10^2/plate)	Protein (mg)	TCA-insoluble (cpm × 10^3/mg protein)	Lipid extract (cpm × 10^2)	PL component PC (%)	PE (%)
Choline alone	197	0.14	141.3 ± 14	74.9 ± 7.7	100	0
+AUS, 40 μg/ml	107	0.124	86.5 ± 2.0 (38.8)	46.6 ± 2.7 (37.0)	100	0
Ethanolamine alone	61.8	0.299	21.5 ± 1.7	5.2 ± 1.8	9.1	90.9
+AUS, 50 μg/ml	58.3	0.274	21.3 ± 1.6 (0.1)	5.5 ± 0.9 (0)	10.5	89.5
+Dapsone, 50 μg/ml	58.2	0.279	20.9 ± 4.1 (0.3)	4.8 ± 1.0 (7.7)	9.7	90.3

* Radioactive choline and ethanolamine were added to cultures of macrophages in M199 + 1% HIPS. After 4 hr the amount of precursor incorporated into TAC-insoluble material was determined. A duplicate set of cultures containing labeled choline and ethanolamine was also harvested at 4 hr and the labeled lipids were extracted and separated by chromatography. The results are the averages ±S.D.; N = 3. Per cent inhibition with respect to control is given in parentheses.

Table 3. Effect of diphenylsulfones on phospholipid synthesis by macrophages as measured by ^{32}P-incorporation *

	Total counts (cpm)	PC (cpm)	PE (cpm)	PI (cpm)	SM (cpm)
(A) 4 Hr with compound, ^{32}P added at 2 hr					
Control (DMSO)	5090 ± 999	1021 ± 235	34 ± 8	92 ± 47	30 ± 13
AUS (50 μg/ml)	2585 ± 452 (50)	475 ± 99 (54)	40 ± 5	87 ± 26	34 ± 12
Dapsone (50 μg/ml)	4408 ± 2229 (14)	484 ± 228 (52)	50 ± 24	85 ± 31	30 ± 9
(B) 24 Hr with compound, ^{32}P added at 22 hr					
Control (DMSO)	4524 ± 348	1079 ± 91	69 ± 10	100 ± 31	41 ± 13
AUS (50 μg/ml)	2868 ± 514 (37)	476 ± 203 (56)	51 ± 8	143 ± 30	44 ± 16
Dapsone (50 μg/ml)	3645 ± 816 (20)	534 ± 100 (51)	57 ± 22	140 ± 40	34 ± 6

* Macrophages were cultured as described in Materials and Methods. In A, ^{32}P was added 2 hr after the compounds and in B, ^{32}P was added 22 hr after the compounds. Following 2 hr in the presence of the label the cells were harvested and the lipids extracted and chromotographed as described. Blank counts obtained from areas of the plates that did not contain standards (15–25 cpm) were substracted. The results are the averages ±S.D.; N = 3. The inhibition of total incorporated acid-insoluble counts and counts found in PC are in parentheses. No radioactivity was found with phosphatidylserine, cardiolipin, lysolecthin or phosphatidic acid.

Table 4. Effects of AUS and dapsone on the incorporation of various membrane precursors by macrophages *

Additions	Precursor			
	Choline	Oleate (cpm/mg protein × 10^3)	Arachidonate (cpm/mg protein × 10^3)	Glucosamine
Control	79 ± 6	392 ± 20	418 ± 27	7.9 ± 0.2
AUS (60 μg/ml)	56 ± 1 (29)	500 ± 50	398 ± 21	8.2 ± 0.3
Dapsone (60 μg/ml)	64 ± 1 (19)	462 ± 11	371 ± 17	7.3 ± 0.8

* To cultures of macrophages was added 0.5 μCi of [^{14}C]choline, [^{3}H]oleate, [^{3}H]arachidonate or [^{3}H]glucosamine. After 4 hr the amount of precursor incorporated into TCA-insoluble material was determined. The per cent inhibition is given in parentheses. The results are the averages ±S.D.; N = 3.

Table 5. Effect of AUS on macrophage viability *

Drug	Enzyme activity					
	Cells (4 hr)	Media (4 hr)	Cells (24 hr)	Media (24 hr)	Cells (48 hr)	Media (48 hr)
	Activity of LDH (mU/ml)					
Control	131 ± 7	0	96 ± 6	2.6 ± 3.2	122 ± 10	5.1 ± 6.4
AUS, 20 μg/ml	119 ± 5	10 ± 11	104 ± 9	4.9 ± 6.2	111 ± 8	5.1 ± 10.0
AUS, 40 μg/ml	127 ± 19	10 ± 11	95 ± 12	5.1 ± 3.3	120 ± 8	5.9 ± 2.9
	Amount of lysozyme (μg)					
Control		0.12	1.03	2.7 ± 0.4	1.23	6.3 ± 0.3
AUS, 20 μg/ml			1.04	2.6 ± 0.4	1.03	6.0 ± 0.7
AUS, 40 μg/ml		0.10		2.3 ± 0.5	1.20	6.2 ± 0.1

* Stimulated macrophages (3×10^6) were cultured in M199 + 1% serum for 4, 24 and 48 hr. The activity of LDH in cells and media, as well as the amount of lysozyme in the media, was determined as described in Materials and Methods. The results are the averages ±S.D.; N = 3.

Table 6. Effects of AUS and dapsone on choline and ethanolamine incorporation by Hela cells and macrophages*

Cells	Concn (μg/ml)	Choline (cpm/mg protein × 10^{-3})	Ethanolamine	C/E†
Macrophage				
Control		129 ± 0.83	21.5 ± 1.7	6.09
AUS	40	84 ± 1.7	21.3 ± 1.6	3.94
Dapsone	40	94 ± 0.50	20.9 ± 4.1	4.49
Hela				
Control		33.6 ± 2.4	50.9 ± 8.8	0.66
AUS	40	21.9 ± 3.3	49.8 ± 4.6	0.44
Dapsone	40	27.1 ± 2.5	51.2 ± 1.0	0.43

* Macrophage and Hela cells were maintained in M199 1% HIPS. [^{14}C]choline or [^{14}C]ethanolamine, 0.125 μCi/ml, was added to cell cultures and DMSO or drug added at the indicated concentration. After 4 hr, the amount of radioactive-insoluble material was determined. The results are the average ±S.D.; N = 3.

† The C/E ratio is calculated by dividing the choline incorporation value by the ethanolamine incorporation value.

tractable material (Table 2). In addition, these compounds do not effect the methylation of PE to form PC.

The biosynthesis of phospholipids can also be estimated by measuring the incorporation of [^{32}P]PO_4 followed by extraction and separation of the radioactive products [26]. This technique allows the simultaneous determination of the amount of this precursor incorporation into various phospholipids. Utilizing this technique we again found that the diphenlysulfones specifically inhibit PC synthesis (Table 3). Treatment of the cells for 4 or 24 hr before measuring the biosynthesis of phospholipids gave identical results. There was significant incorporation of ^{32}P into PC, PE, PI and sphingomyelin, but only the incorporation of label into PC was reduced due to the diphenylsulfones (Table 3). Therefore, by utilizing two procedures to measure phospholipid synthesis, it is clear that AUS and dapsone specifically inhibit PC synthesis from choline by macrophages.

This inhibition of lecithin synthesis from choline by the diphenylsulfones appears to be specific. In addition to ethanolamine (Table 2), incorporation of arachidonic acid or glucosamine into macrophages was also not affected by the diphenylsulfones, whereas oleic acid incorporation was slightly elevated (Table 4).

It was necessary to show that these compounds did not exert their effects through toxicity. Therefore, we examined the ability of the cells exposed to the drugs to retain the cytoplasmic enzyme, lactate dehydrogenase, and their ability to secrete lysozyme. Treatment of macrophages with AUS (40 μg/ml) for 48 hr did not cause significant leakage of LDH nor affect its cellular level. In addition, lysozyme secretion was unaffected in the presence of the compound (Table 5).

Effect of diphenylsulfones on PC synthesis in human cells

AUS and dapsone inhibited the incorporation of [^{14}C]choline by Hela cells but no effect on the incorporation of ethanolamine (Table 6). It is of interest to note that macrophages incorporated choline at a rate greater than ethanolamine, whereas the reverse was true for Hela cells. Thus, the sulfones not only inhibit choline incorporation into human cell membranes but do so in cells with widely different rates of phosphatidylcholine synthesis.

Table 7. Reversible inhibition of [^{14}C]choline incorporation into macrophage PC by AUS and dapsone*

Additions	[^{14}C]Choline incorporation (cpm × 10^3/mg protein) Control†	20-hr Wash‡
Control	75.3 ± 3.6	77.6 ± 4.8
Dapsone, 50 μg/ml	64.7 ± 4.4 (14.1)	80.7 ± 4.9 (−4.0)
Dapsone, 100 μg/ml	58.5 ± 0.8 (22.3)	79.1 ± 7.7 (−1.9)
AUS, 50 μg/ml	61.9 ± 4.5 (17.8)	79.6 ± 5.1 (−2.6)

* [^{14}C]Choline, 0.5 μCi, was added to cultured macrophages. After 4 hr the amount of TCA-insoluble material was determined. The results are the averages ±S.D.; N = 3. The per cent inhibition is given in parentheses.

† Compound and choline added together and cells harvested at 4 hr.

‡ Compound added at zero time and removed by washing after 4 hr. Cells were reincubated for an additional 20 hr in fresh medium. Choline was then added and cells were harvested after 4 hr.

Reversible inhibition of choline incorporation by diphenylsulfones

The diphenylsulfones used in this study must be present in the culture media along with the precursor to exert their effects. This conclusion is based on the finding that the inhibition of choline incorporation by AUS and dapsone is fully reversible (Table 7). The data shown in Tables 4 and 7 suggest that the cells do not accumulate the compounds. Therefore, we prepared radioactive dapsone and AUS and examined their interactions with cultured macrophages. [^{14}C]dapsone was not accumulated by the cells and only 356 out of 270×10^3 cpm of [^{14}C]AUS was associated with the cells after 6 hr.

Studies on the mechanism of inhibition of PC synthesis by AUS and dapsone

The inhibition of PC formation is not due to a general inhibition of membrane component synthesis (Tables 1 and 4), nor is it due to an effect on the transport of precursor into the cells (Table 1). Therefore, a limited number of explanations exist for the above findings. The level of the terminal enzyme of the pathway could be decreased either by increased turnover or decreased synthesis due to the drugs. Alternatively, the compounds could exert a direct inhibition of choline:1,2-diglyceride cholinephosphotransferase or of choline–phosphate cytidylyltransferase. Finally, compartmentalization involving membrane bound cholinephosphotransferase [24], enzyme substrates and compounds with interference of substrate access to enzyme may be related to the inhibition seen in the cell culture experiments. We tested two of these possibilities.

First, cholinephosphotransferase was assayed in crude extracts prepared from macrophages cultured for 24 hr in the presence of 200 μg/ml of AUS and without compound to determine if total enzyme activity was affected. We found identical specific activities in the control and in treated cells.

Second, in order to test the effect of the compounds directly on the enzyme activity, we assayed choline phosphotransferase in crude homogenates prepared from peritoneal exudates. The K_m for CDP-choline was found to be 43 μM and the V_{max} 0.835 nmole/min/mg of protein. The apparent K_m for 1,2-diolein was 0.64 mM. AUS was tested for its ability to inhibit the enzyme when either CDP-choline or α,β-diglyceride was at the K_m and the other at its saturating concentration. The results shown in Table 8 show that this compound does not inhibit cholinephosphotransferase in broken cell preparations.

Table 8. Effects of AUS on phosphotransferase activity*

AUS (μg/ml)	DCP-Choline† Dis./min ±S.D.	E/C	Dioleate‡ Dis./min ±S.D.	E/C
0	3101 ± 123		1469 ± 260	
25	3442 ± 220	1.11	1398 ± 176	0.95
50	3234 ± 184	1.04	1572 ± 82	1.07
100	2855 ± 83	0.92	1343 ± 226	0.91
200	2914 ± 48	0.94	1267 ± 266	0.86
400	2412 ± 113§	0.78	1080 ± 64	0.74

* CDP-choline phosphotransferase was assayed as described in Materials and Methods. AUS was dissolved in DMSO and added to the reaction tubes giving a final DMSO concentration of 0.25%. Results expressed are corrected for background (104 dis./min) and are the averages ±S.D.; N = 3. E/C is the activity of the drug-treated sample over that of the control value. The values for dis./min compared to the control are not statistically significant except for the one with the § symbol.

† CDP-choline, 43 μM, and α,β-dioleate, 3.2 mM.

‡ α,β-Dioleate, 0.64 mM, and CDP-choline, 0.2 mM.

§ Statistically significant at the P = 0.005 level.

DISCUSSION

Dapsone and AUS have been reported to inhibit choline incorporation into PC of avian macrophages [25]; however, it was not fully established whether a similar effect would be found in mammalian cells.

Choline was found to be incorporated exclusively into PC by cultured mouse peritoneal macrophages in a linear fashion for at least 4 hr. Formation of this phospholipid, as measured by the incorporation of [^{14}C]choline or ^{32}P, was inhibited by the diphenylsulfones, dapsone and AUS. Although this inhibition is between 20 and 50 per cent at moderate drug levels, this could dramatically alter membrane phospholipid composition and subsequent function. This inhibition of phospholipid biosynthesis by AUS and dapsone is specific for PC from choline, since PC synthesis from ethanolamine was not affected (Table 2) nor was the incorporation of ^{32}P into sphingomyelin. In addition, these compounds did not inhibit the incorporation of several other precursors into membrane components. The effect of this inhibition on macrophage functions is under study.

The data presented in this report suggest that the inhibition of PC formation from choline might be due to a direct inhibition of the terminal enzyme of the pathway. Specifically, the sulfones did not affect the accumulation of acid soluble counts, suggesting that the transport of choline is not the target. The reversibility of the inhibition and the lack of significant cellular accumulation of the compounds suggest that the target is a membrane-bound component. The enzyme converting phosphorylcholine to CDP-choline is found in the cytosol fraction of liver [27], whereas cholinephosphotransferase is located in the plasma membrane of alveolar macrophages [24]. However, we have established experimentally that the diphenylsulfones have no effect on the synthesis or function in a broken cell environment of CDP-choline:1,2-diglyceride choline phosphotransferase (Table 8). It should be noted that Shigeura *et al.* [25] reported that the levels of phosphorylcholine were found to accumulate in AUS-treated cultures of chicken macrophages with no traces of CDP-choline found. Thus, it is likely that the specific inhibition by the diphenylsulfones is not at the level of the terminal enzyme unless the inhibition is manifested only when the enzyme is present in the membranes of functionally intact cells.

Acknowledgements—We wish to thank Dr. Roger Sundler for his assistance with the ^{32}P-labeling experiments, Ms. M. E. Dahlgren for her assistance with the cell cultures, and Miss Olympia Huneycutt for typing the manuscript.

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