Biochemical Pharmacology, Vol. 32, No. 11, pp. 1737–1744, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

INHIBITION OF PHOSPHOLIPID DEGRADATION AND CHANGES OF THE PHOSPHOLIPID-PATTERN BY DESIPRAMINE IN CULTURED HUMAN FIBROBLASTS

RUPERT FAUSTER*, ULRICH HONEGGER† and ULRICH WIESMANN‡

*Fellow of the European Molecular Biology Organization (EMBO), †Department of Pharmacology and ‡Department of Pediatrics, University of Berne, CH 3010 Berne, Switzerland

(*Received* 10 *September* 1982; *accepted* 13 *December* 1982)

Cationic amphiphilic drugs produce profound changes in phospholipid metabolism when administered in high doses over an extended period of time [1]. In a variety of tissues of animals and man and also in cultured cells they form microscopically visible lamellar bodies within the cell. These bodies have been identified as lysosomes containing excess membrane phospholipids. Analyses of the accumulated individual phospholipids have shown a disproportionally high content of acidic phospholipids. It has been speculated that cationic drugs including tricyclic antidepressants change the physico-chemical properties of phospholipids in biological membranes by complex formation which alters their turnover rates. Thus, these drugs could interfere with membrane-linked phenomena such as fusion of vesicles, permeability and receptor functions [2]; e.g. tricyclic antidepressants have been shown to reduce the number of beta-adrenergic receptor sites in brain and peripheral organs of rats as part of their regular drug effects [3].

Two different mechanisms have been discussed to explain lysosomal phospholipid accumulation in drug-treated cells. One involves storage of drug–phospholipid complexes in the lysosomes as the result of a decreased breakdown of these phospholipids [1, 4, 5].

The other hypothesis suggests a drug-induced increase of the selective synthesis of acidic phospholipids together with an inhibition of lysosomal phospholipid degradation [6, 7].

In the present paper the effect of desipramine (DMI) on the phospholipid metabolism in cultured human skin fibroblasts was studied using [^{14}C]-glycerol and [^{3}H]-inositol as markers for phospholipids. The aim of the study was to differentiate between possible hypothetic mechanisms of drug effects leading to phospholipid accumulation in living cells.

MATERIALS AND METHODS

Tissue culture. Skin biopsies for fibroblast cultures were obtained after informed consent from healthy persons at occasion of minor surgery. Fibroblasts were grown from the biopsies and cultured as previously described [8]. Fibroblast stock cultures were maintained as monolayers in 75-cm^2 Falcon tissue culture flasks. The cultures were fed twice a week with Eagle MEM supplemented with 10% fetal calf serum, nonessential amino acids, penicillin 100 U/ml and aureomycin 15 μg/ml. The medium was buffered at pH 7.4 with 15 mM $NaHCO_3$ and 5% CO_2 in air. The stock cultures were grown to confluency at 37° in a CO_2-incubator. Cultures were subcultured every 8 days at a subculture ratio of 1:2.

For experiments cells were used between the 5th and 10th subculture. 3.2×10^5 cells suspended in 4 ml of culture medium were seeded into 60 mm ∅ Falcon Petri dishes and grown to confluency for 6 days and fed 3 and 1 days before beginning of the experiment.

Experimental cultures. [2-^{14}C]-glycerol (16.5 mCi/mmol) and myo-[2-^{3}H]-inositol (12.5 Ci/mmol) were obtained in sterile aqueous or ethanol/water solutions. These were diluted directly with the culture medium. 1 μCi of radioactive phospholipid precursor in 4 ml medium was added to each Petri dish. A stock solution of 1 mM DMI was prepared in culture medium and sterilized by Millipore filtration. Aliquots were then diluted with the radiolabelled incubation medium to a final concentration of 10, 30 and 50 μM DMI, respectively. After the incubation the culture medium was removed, the monolayer washed twice with ice-cold Hank's solution and scraped with a rubber "policeman" into a 50 ml Falcon tube. The suspension was centrifuged at 1000 *g* for 5 min, the supernatant discarded and the pellet homogenized by ultrasonication in a Branson ultrasonicator in 1 ml of distilled water. Aliquots of the homogenate were used for protein determination according to the method of Lowry *et al.* [9] and for DNA measurement according to the method of Hinegardner *et al.* [10].

Extraction and separation of lipids. Lipids were extracted from the homogenized cells with chloroform/methanol, 2:1 (v/v) as described by Folch *et al.* [11]. The chloroform layer was collected, evaporated to dryness under a stream of N_2, redissolved in chloroform/methanol, 1:1 (v/v) and stored at −20° under N_2. Aliquots were taken for counting total lipid radioactivity and for lipid phosphorus

† Author to whom correspondence should be addressed: Dr. U. Honegger, Dept. of Pharmacology, University of Bern, Friedbühlstr. 49, 3010 Bern, Switzerland.

determination using a test kit (Boehringer GmbH, Mannheim, Germany).

Individual lipids were separated by TLC on Silica gel G plates. Prior to the use, the plates were activated for 1 hr at 120°. The samples were applied to the plates together with authentic phospholipid standards. TLC was performed in paperlined chromatography tanks which were allowed to pre-equilibrate for 1 hr. Neutral lipids, free fatty acids and phospholipids, were separated by one-dimensional TLC using chloroform/methanol, 95:5 (v/v) as solvent. Individual phospholipids were separated by two-dimensional TLC. After developing in solvent system I with chloroform/methanol/water/concentrated ammonia, 130:70:8:0.5 (v/v) the plates were dried with a stream of N_2 at room temperature for 30 min. The plates were then developed in the second dimension with chloroform/acetone/methanol/acetic acid/water, 100:40:40:20:10 (v/v). Individual lipids were detected with iodine vapour and identified according to the co-chromatographed standards. The areas representing the respective lipids were scraped into counting vials and 10 ml of Aquassure was added. Measurement of radioactivity was performed in an Intertechnique liquid-scintillation counter equipped with an absolute activity analyser.

Materials. Radioactive compounds and Aquassure for scintillation counting were purchased from NEN Chemicals GmbH, Dreieichenhain, Germany, lipid standards were obtained from Supelco S.A. Crans, Switzerland, fetal calf serum and the test kit for phosphorus determination were from Boehringer GmbH, Mannheim, Germany. Eagle MEM was obtained from Gibco, Basel, Switzerland and trypsine was from North America Biologicals, Miami, Florida, U.S.A.

All disposable cell culture material was obtained from Becton Dickinson AG, Basel, Switzerland. Reagents of purest grade available and Silica gel G TLC plates were purchased from Merck AG, Darmstadt, Germany.

RESULTS

Effect of DMI on the labelling of total lipids

Incubation of confluent fibroblast cultures with medium containing 10, 30 and 50 μM DMI, respectively for 48 hr increased the total cellular protein and DNA content per culture. In control cultures protein and DNA remained constant throughout the experimental period. The protein/DNA ratio remained unchanged in treated and in control cultures. This increased cellular growth exerted by 10 or 30 μM DMI became evident after 24 hr. The effect was less pronounced or even suppressed in presence of 50 μM (results not illustrated).

Total lipid phosphorus per mg of protein increased during the DMI exposure. This phospholipid-accumulation appeared after 12 hr and was dose-dependent (Fig. 1). Simultaneously a change of the cellular morphology occurred. The cells became increasingly granulated as shown in Fig. 2. After removal of the DMI-containing media and incubation with normal culture medium the granulation within the cells persisted for several days and concomitantly the

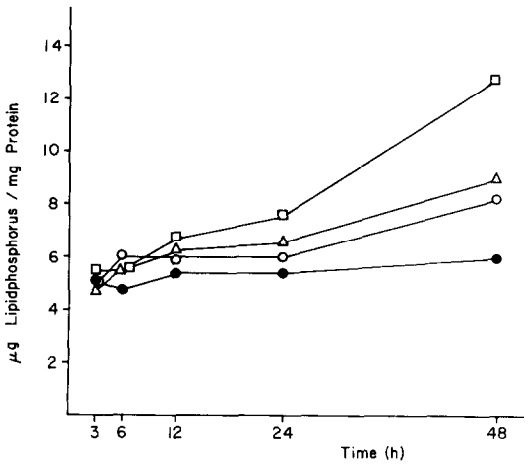

Fig. 1. Phospholipid accumulation in DMI-treated cultured human fibroblasts. Replicate confluent cultures were incubated with medium containing 10, 30 or 50 μM DMI up to 48 hr. Lipid phosphorus was determined in aliquots of the FOLCH extracts and referred to cellular protein. ●, Control: ○, 10 μM DMI; △, 30 μM DMI and □, 50 μM DMI. Each symbol represents the mean of three separate cultures. All determinations were made in duplicate. Standard deviations of the mean values were calculated for each experiment and were found to be generally within the size of the symbols (Figs. 1–8).

phospholipid content remained elevated as long as the granulation was visible.

The effect of DMI on the incorporation of [^{14}C]-glycerol into phospholipids and triglycerides of confluent fibroblasts is shown in Fig. 3(a). DMI was added together with the [^{14}C]-labelled glycerol. In control cultures the incorporation rate into phospholipids was linear only for about 3–6 hr. In DMI-treated cultures the [^{14}C]-glycerol incorporation was same as in the control up to 6 hr. However, a significant increase of [^{14}C]-labelled phospholipids after 24 hr of incubation ($P < 0.005$) resulted from prolonged linearity of the incorporation. The incorporation of [^{14}C]-glycerol into neutral lipids (triglycerides and cholesterolesters) was similar in DMI-treated and control cells. The specific labelling of phospholipids, [^{14}C]-glycerol/total phospholipid content, in DMI-treated and in control cultures was the same during the incubation (Fig. 3b) indicating a true increase of phospholipid content after drug treatment.

In cultures that were preincubated for 3 and 7 days with 10 μM DMI before they were labelled with [^{14}C]-glycerol in the presence of 10 μM DMI, the specific radioactivity of the phospholipids in the DMI-pretreated cultures were lower than in control cultures and in cultures that received DMI during the labelling only (Fig. 4).

The effect of DMI on the rate of disappearance of [^{14}C]-glycerol labelled phospholipids from confluent fibroblast cultures is shown in Fig. 5. After pretreatment of the cultures with medium containing [^{14}C]-glycerol, the medium was removed and the cells incubated up to 48 hr with fresh medium containing 0, 10, 30 or 50 μM DMI. In control cells [^{14}C]-glycerol labelled phospholipids had a half-life of about 24 hr.

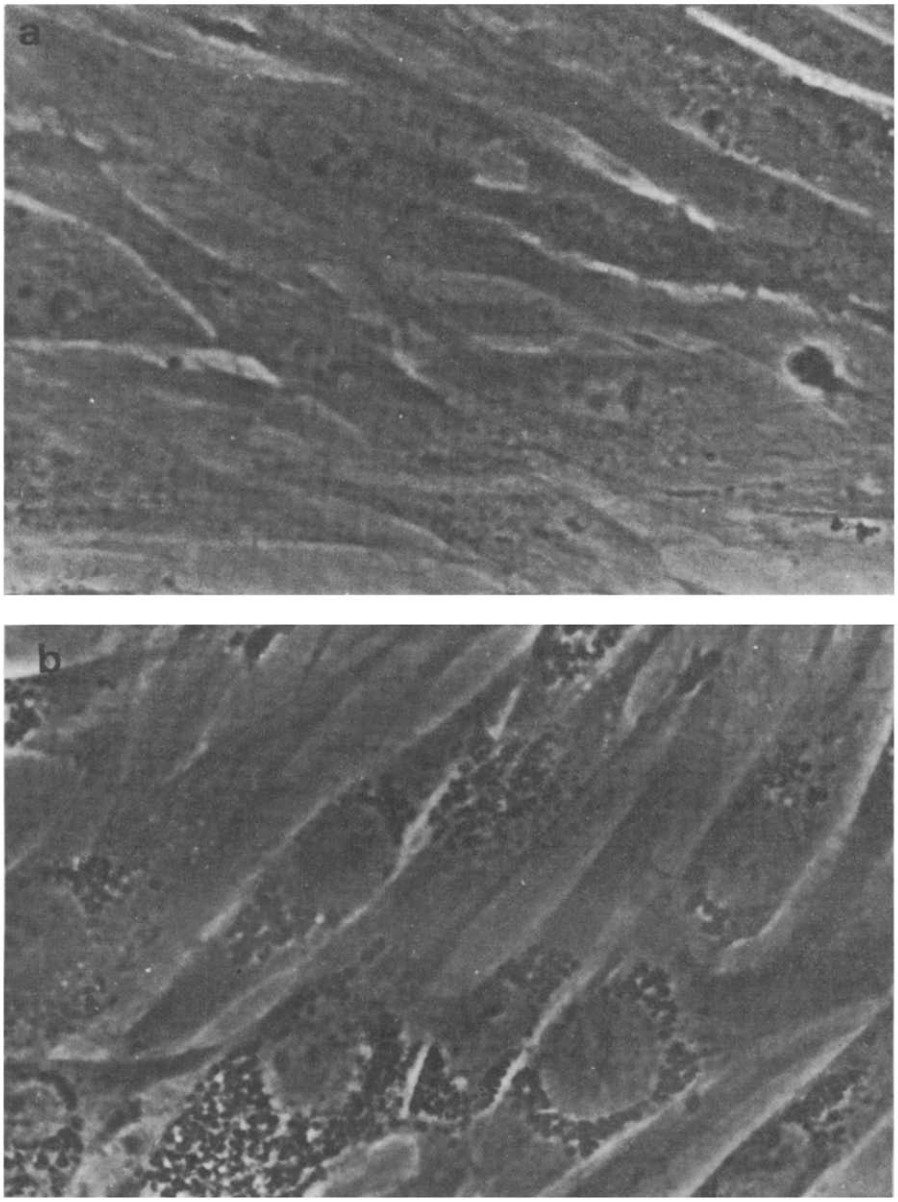

Fig. 2. Morphology of DMI-treated cultured human fibroblasts. Original magnification ×400. Photographs were taken after a 48-hr exposure to drug. (a) Control; (b) 10 μM DMI.

DMI, regardless of the concentration doubled the half-life of the phospholipids ($P < 0.001$) (Fig. 5a). The specific radioactivity of the phospholipids, however, decreased in the same way in control and in DMI-treated cultures (Fig. 5b).

Other cultures were preincubated for 3 and for 7 days, respectively with 10 μM DMI and then labelled with [^{14}C]-glycerol for 24 hr still in the presence of 10 μM DMI. In drug-pretreated cultures the specific radioactivity of the phospholipids was again lower than in control cultures. The decrease of specific phospholipid radioactivity during a subsequent 48 hr chase period was slower the longer the cultures had been pretreated with the drug (Fig. 5c).

Effect of DMI on the labelling of individual phospholipids

[^{14}C]-glycerol labelled phospholipids were separated into the major individual phospholipids by thin layer chromatography and their percent distribution and their individual radiolabelling were calculated (Fig. 6). Up to 6 hr the rate of [^{14}C]-glycerol incorporation into individual phospholipids was approximately the same in presence and absence of 30 μM DMI. The incorporation of [^{14}C]-glycerol increased linearly in control and drug treated cultures.

The highest incorporation was observed into phosphatidylcholine (PC) and phosphatidylethanolamine

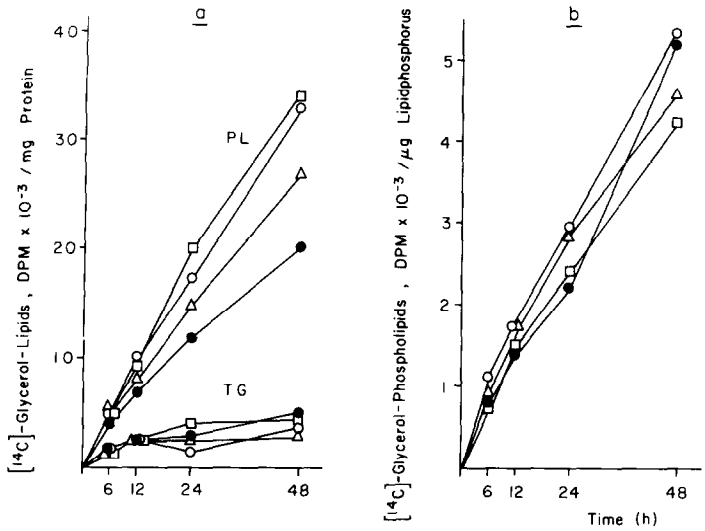

Fig. 3. (a) Effect of DMI on [^{14}C]-glycerol incorporation into phospholipids (PL) and triglycerides (TG) of cultured human fibroblasts. Radioactivity was measured in the chloroform phase of the FOLCH extract and calculated for PL and TG according to the percent distribution obtained by one-dimensional TLC. These data are referred to cellular protein. ●, Control; ○, 10 µM DMI; △, 30 µM DMI and □, 50 µM DMI. Each symbol represents the mean of three separate cultures. All determinations were made in duplicate. (b) Effect of DMI on the specific radioactivity of [^{14}C]-glycerol labelled PL in cultured human fibroblasts. Conditions and calculations as in Fig. 3(a), lipid phosphorus determination is given in Methods.

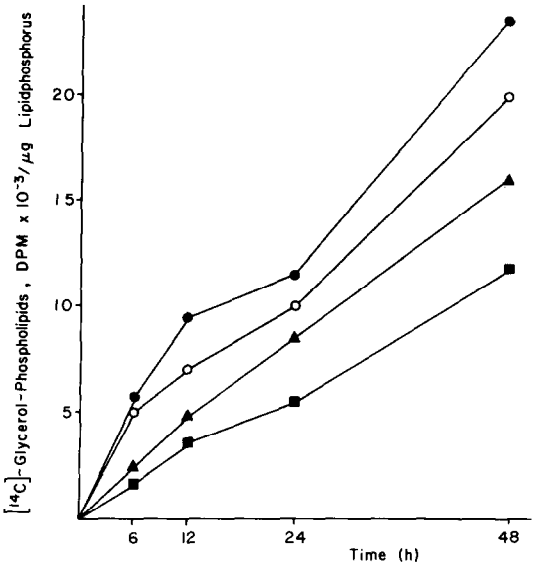

Fig. 4. Effect of DMI-pretreatment on the specific radioactivity of [^{14}C]-glycerol labelled PL in cultured human fibroblasts. Replicate confluent cultures were pretreated for 3 and 7 days with one or two changes of medium containing 10 µM DMI. Then the medium was removed and 4 ml of incubation medium containing 10 µM DMI together with 1 µCi [2-^{14}C]glycerol was added for up to 48 hr. Analytical procedures and calculation as in Figs. 3(a) and (b). Lipid phosphorus determination is given in Methods. ●, Control; ○, no DMI-pretreatment; △, 3 days DMI-pretreatment and ■, 7 days DMI-pretreatment.

(PE) whereas the labelling of phosphatidylinositol (PI) and phosphatidylserine (PS) was 5–6 times lower. After 12 hr of incubation, the incorporation rate of the label into PI and PE decreased in control cultures while it remained almost constant in DMI treated cultures. Therefore, at 48 hr the incorporation of label into PI reached 200% of the controls. For PC and PS the increase was only 120–130% and for PE 150% over control values.

The effect of DMI on the disappearance rate of [^{14}C]-glycerol label from individual phospholipids is shown in Fig. 7. In control cells PC and PS as well as PI had an estimated half-life of 24 hr as calculated for total phospholipids whereas the half-life for PE ranged between 24 and 48 hr. DMI had no significant effect on the half-life of PC and PS, relatively little effect on PE, but a marked immediate effect on PI which appeared to be dose related. There was an immediate temporary increase in labelling of PI and a markedly decreased disappearance rate.

Similar labelling kinetics as for the incorporation and the disappearance of [^{14}C]-glycerol in PI were obtained by labelling the fibroblast cultures with myo-[^{3}H]-inositol as shown in Figs. 8(a) and (b).

DISCUSSION

DMI is a metabolite of imipramine. Both drugs are widely used as antidepressants. They are tricyclic compounds and are cationic and amphiphilic in nature. Imipramine was shown to produce phospholipidosis in animals as a side-effect upon prolonged administration [12]. Similar side-effects have been described for many other amphiphilic drugs [12] in animals and in man as well as in cultured cells [4].

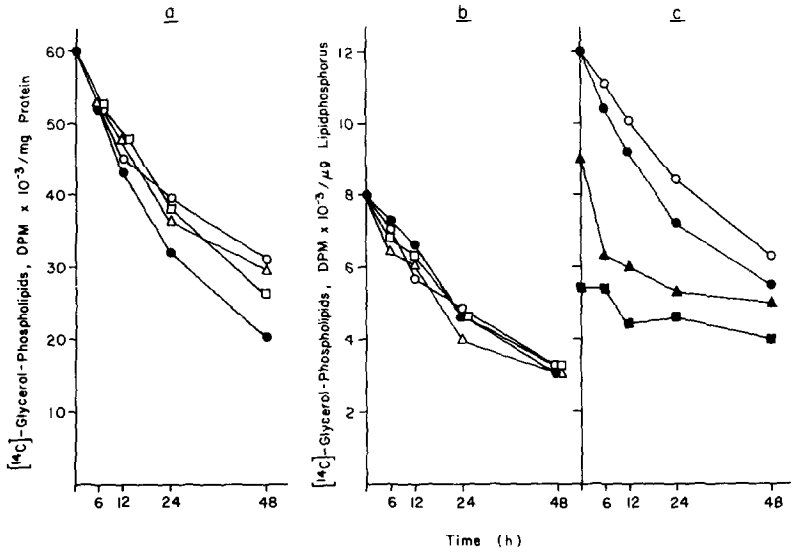

Fig. 5. (a) Effect of DMI on the rate of disappearance of [^{14}C]-glycerol labelled PL from confluent human fibroblast cultures. Replicate confluent fibroblast cultures were each incubated with 4 ml of medium containing 1 μCi [2-^{14}C]-glycerol for 24 hr. Then the medium was removed, the monolayer washed twice with 5 ml HANK'S balanced salt solution and incubated with fresh medium with or without DMI up to 48 hr. At times indicated, cells were harvested, lipids were extracted, and PL were separated from TG by one-dimensional TLC. Radioactivity was measured in the chloroform phase of the FOLCH extract calculated according to the percent distribution obtained by TLC and referred to protein values obtained from aliquots of the homogenized cells. ●, Control; ○, 10 μM DMI; △, 30 μM DMI and □, 50 μM DMI. Each symbol represents the mean of three separate cultures. All determinations were made in duplicate. (b) Effect of DMI on the specific radioactivity of [^{14}C]-glycerol labelled PL of cultured human fibroblasts during a 48 hr "chase" period. Experimental and analytical procedure, calculation, and symbols as in Fig. 5(a), radioactivity was referred to lipid phosphorus values obtained from aliquots of the chloroform phase of the Folch extract. (c) Effect of DMI-pretreatment on the specific radioactivity of [2-^{14}C]-glycerol labelled PL during a 48 hr "chase" period. Replicate confluent cultures were pretreated for 3 and 7 days with one and two changes of medium containing 10 μM DMI. Then the medium was removed and 4 ml of medium containing 1 μCi [2-^{14}C]-glycerol together with or without 10 μM DMI was added for 24 hr. Then again the medium was discharged, the monolayer washed twice with HANK'S solution, and cells were incubated with fresh medium only or with medium supplemented with 10 μM DMI. Analytical procedure and calculation as in Fig. 5(b). ●, Control; ○, 10 μM DMI added only during 48 hr chase period, ▲, 10 μM DMI pretreatment for 3 days (■ 7 days) drug remaining in the culture medium to the end of experiment.

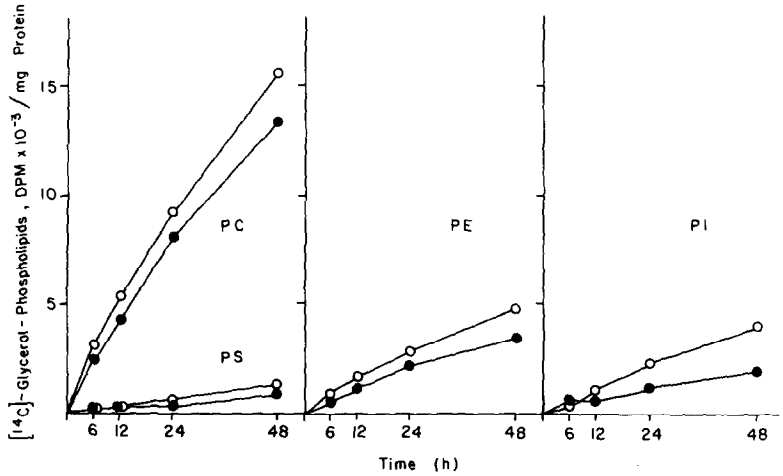

Fig. 6. Effect of DMI on the incorporation of [^{14}C]-glycerol into the four major phospholipids of cultured human fibroblasts. Replicate confluent cultures were incubated in the presence of 1 μCi [2-^{14}C]-glycerol with and without 30 μM DMI up to 48 hr. Cells were harvested, lipids were extracted, and individual phospholipids separated as in Methods. Radioactivity in the individual phospholipids was calculated from total phospholipid radioactivity according to the percent distribution obtained by two-dimensional TLC. Phosphatidylcholine (PC), Phosphatidylserin (PS), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI). ●, Control; ○, 30 μM DMI; each symbol represents the mean of three separate cultures. All determinations were made in duplicate.

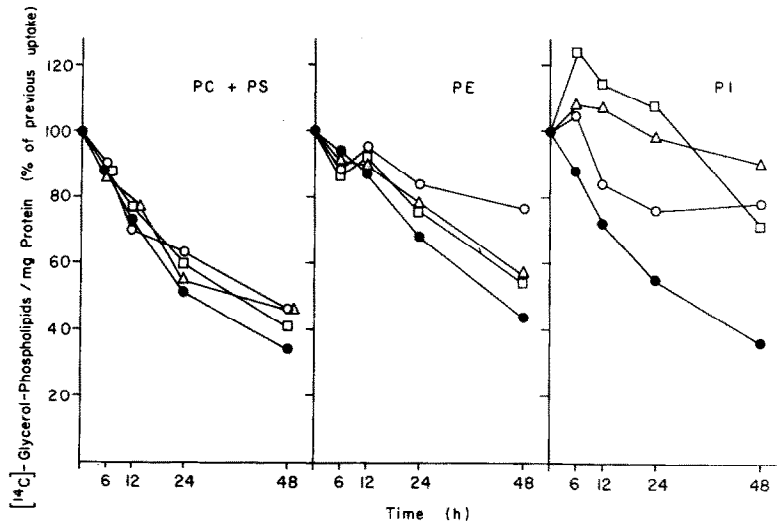

Fig. 7. Effect of DMI on the disappearance of [^{14}C]-glycerol label from individual major PL. Replicate confluent fibroblast cultures were incubated with 4 ml of medium containing 1 μCi [2-^{14}C]-glycerol for 24 hr. Then medium was removed, cells were washed twice with HANK'S solution and incubated with fresh medium with and without DMI up to 48 hr. Cells were harvested, lipids extracted, and PL separated as in Methods. Total PL radioactivity was taken as 100%. The amount of radioactivity in the individual PL was calculated from total phospholipid radioactivity according to the percent distribution obtained by TLC. ●, Control; ○, 10 μM DMI; △, 30 μM DMI and □, 50 μM DMI. Each symbol represents the mean of three separate cultures. All determinations were made in duplicate. Phosphatidylcholine and -serine, phosphatidylethanolamine, phosphatidylinositol.

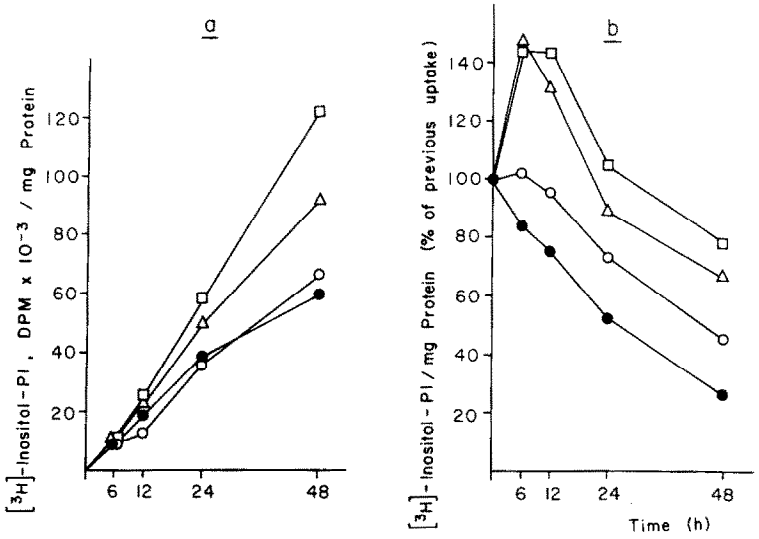

Fig. 8. (a) Effect of DMI on the incorporation of myo-[^{3}H]-inositol into PI. Replicate confluent cultures were incubated with medium containing about 1 μCi [2-^{3}H]-inositol with and without DMI. Cells were harvested and lipids extracted as given in Methods. Two-dimensional TLC showed that label occurred only in PI. Radioactivity measured in the FOLCH extract was referred to total cellular protein. ●, Control; ○, 10 μM DMI; △, 30 μM DMI and □ 50 μM DMI. Each symbol represents the mean of three separate cultures. All determinations were made in duplicate. (b) Effect of DMI on the disappearance rate of myo-[2-^{3}H]-inositol-label from PI of cultured human fibroblasts. Replicate confluent cultures were incubated with 4 ml of medium containing about 1 μCi of myo-[2-^{3}H]-inositol for 24 hr. Then the labelled medium was removed, the cells washed twice with HANK'S solution, and incubated with fresh medium with and without DMI up to 48 hr. At times indicated cells were harvested, lipids were extracted and the radioactivity measured in the FOLCH extract and referred to cellular protein. The value obtained after the 24 hr labelling period was taken as 100%. Symbols as in Fig. 8(a).

The excessive phospholipid accumulation in the cells appears to be exclusively located in lysosomes [13].

DMI is avidly taken up by the fibroblasts from the incubation medium and already after 30 min a steady state is reached.*

Initially the drug may be bound to membrane phospholipids [14] but after 1–2 hr the drug became concentrated within lysosomes.*

Effects of lysosomotropic drugs can be conveniently studied in cultured human fibroblasts [15, 16, 17]. In these cells little or no drug metabolism is to be expected. In cultured fibroblasts [^{14}C]-glycerol is specifically incorporated into the glycerol moiety of phospholipids and neutral lipids [18]. [^{14}C]-glycerol is incorporated into phospholipids by de novo synthesis in microsomes and transferred to other intracellular membranes which lack the capacity for de novo synthesis of phospholipids. During the initial labelling period with [^{14}C]-glycerol DMI had no apparent effect on total and/or individual phospholipid synthesis and on the specific phospholipid radioactivity. Neither did pretreatment of the cultures with DMI increase the synthesis of subsequently labelled phospholipids, although it increased the total content of phospholipids. After incubation longer than 6–12 hr there was an apparent accumulation of [^{14}C]-glycerol labelled total phospholipids, especially of PI and PE. The phospholipid accumulation was dependent on the dose and on the number of repetitive exposures to the drug. At no time and at none of the concentrations of DMI used, could a change of the rate of synthesis of triglycerides be observed in the fibroblasts. The "chase" experiments demonstrated that the accumulation of [^{14}C]-glycerol labelled phospholipids was due to a decrease in the breakdown of phospholipids in the presence of DMI. DMI prolonged the overall half-life of phospholipids from 24–48 hr. For non-labelled phospholipid accumulation it took about 24–48 hr to become measurably increased according to the DMI concentration.

In most cases of drug-induced phospholipidosis, as well as in our experiments, a disproportionate accumulation of acidic phospholipids, e.g. PI has been observed.

Different hypotheses have been formulated to explain this phenomenon.

(1) Acidic phospholipids may preferentially form non-degradable complexes with cationic drugs [4] and specific phospholipases for acidic phospholipids may be preferentially inhibited by cationic drugs [19].

(2) Acidic phospholipids may be accumulated by drug-induced redirection of phospholipid synthesis by a partial inhibition of the phosphatidyl phosphohydrolase [2, 7, 20]. Thereby, the synthesis of the more acidic phospholipids would be favoured at the expense of the synthesis of the neutral phospholipids and of the triglycerides. In addition, drug-phospholipid complexes formed may be resistant to degradation in lysosomes [20].

The observed increase of PI among the accumulating phospholipids in DMI-treated fibroblasts does not favour the latter hypothesis. There is no evidence for increased synthesis although a DMI dose-dependent instant increase of PI in both [^{14}C]-glycerol and in [^{3}H]-inositol labelled cultures has been observed. In the chase experiments with [^{14}C]-glycerol prelabelled cultures, a relative increase and in [^{3}H]-inositol prelabelled cultures even an absolute increase of PI was almost instantly observed in the presence of DMI. This immediate change in the disappearance rate of the label is unique for PI and is not seen for the other phospholipids.

This observation is compatible with the inhibition by DMI of the rapid turnover of PI. A rapid turnover of PI has been described to be stimulated by numerous agents, among them by growth-promoting substances like fetal calf serum [21]. The products of this rapid turnover are diacylglycerol and inositolphosphate. Both appear to be reutilized for the synthesis of PI via phosphatidic acid [21]. DMI could interfere with the breakdown of PI most likely by inhibition of phospholipase C. This enzyme isolated from rat liver lysosomes has been shown to act preferentially on PI and to a lesser extent on other phospholipids [13]. A rapid lysosomal turnover of labelled PI with partial reutilization of the labelled degradation products [^{14}C]-glycerol and [^{3}H]-inositol [22] would result in a much slower apparent overall turnover similar to the one of the other phospholipids. The inhibition of phospholipase C by DMI and possibly by other cationic drugs would explain both the non-proportional increase of PI as well as the overall increase of phospholipids in cultured human fibroblasts treated by such drugs.

In conclusion, our results suggest that DMI inhibits phospholipid degradation in the lysosomes. Since phosphatidylinositol appears to have much faster turnover rates than other phospholipids a rapid increase of phosphatidylinositol is resulting from inhibition of lysosomal phospholipases, confirming the work of Matsuzawa and Hostettler (19).

Acknowledgements—This work was supported by a grant from the Austrian Ministry of the Interior and by the Swiss National Science Foundation, grant No. 3.054.81. The authors would like to thank Prof. M. H. Bickel and Prof. H. Reuter for their helpful comments on this manuscript. We are also indebted to Miss S. Beeri for her invaluable technical assistance.

* U. Honegger *et al.*, submitted for publication.

REFERENCES

1. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Germ. Med.* **3**, 128 (1973).
2. D. N. Brindley, D. Allan and R. H. Michell, *J. Pharm. Pharmac.* **27**, 462 (1975).
3. U. E. Honegger and M. H. Bickel, in *Phenothiazines and Structurally Related Drugs* (Eds. E. Usdin, H. Eckert and I. S. Forrest) Vol. 7 p. 245 Elsevier/North-Holland (1981).
4. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).
5. Y. Matsuzawa and K. Y. Hostettler, *J. biol. Chem.* **255**, 5190 (1980).
6. D. N. Brindley and M. Bowley, *Biochem. J.* **148**, 461 (1975).
7. D. N. Brindley, M. Bowley, R. G. Sturton, P. H. Pritchard, S. L. Burditt and J. Cooling, *Biochem. Soc. Trans.* **5**, 40 (1977).

8. U.N. Wiesmann and N. N. Herschkowitz, *Pediatr. Res.* **8**, 865 (1974).
9. O. H. Lowry, J. H. Rosenbrough, L. A. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. R. T. Hinegardner, *Analyt. Biochem.* **39**, 197 (1971).
11. J. Folch, M. Lees and G. H. S. Standley, *J. biol. Chem.* **226**, 497 (1957).
12. R. Lüllmann-Rauch and D. Scheid, *Virchows Arch. B. Cell Pathol.* **19**, 225 (1975).
13. Y. Matsuzawa and K. Y. Hostettler, *Biochim. biophys. Acta* **620**, 592 (1980).
14. C. Di Francesco and M. H. Bickel, *Chem. biol. Interactions* **16**, 335 (1977).
15. C. De Duve, Th. De Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof, *Biochem. Pharmac.* **23**, 2495 (1974).
16. U. N. Wiesmann, St. Di Donato and N. N. Herschkowitz, *Biochem. biophys. Res. Commun.* **66**, 1338 (1975).
17. St. Di Donato, U. N. Wiesmann and N. N. Herschkowitz, *Biochem. Pharmac.* **26**, 7 (1977).
18. R. D. Lynch, E. E. Schneeberger and R. P. Geyer, *Biochemistry* **15**, 193 (1976).
19. Y. Matsuzawa and K. Y. Hostettler, *J. biol. Chem.* **255**, 5190 (1980).
20. R. H. Michell, D. Allan, M. Bowley and D. N. Brindley, *J. Pharm. Pharmac.* **28**, 331 (1976).
21. R. H. Michell, *TIBS* **4**, 128 (1979).
22. A. J. R. Habenicht, J. A. Glomset, W. C. King, C. Nist, C. D. Mitchell and R. Ross, *J. biol. Chem.* **256**, 12329 (1981).