Br. J. Pharmac. (1984), **83**, 293–298

A comparison of dimaprit, nordimaprit, methylamine and chloroquine as inhibitors of mitogen-induced lymphocyte activation

M. Maureen Dale & R. Ladd*

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT and Department of Physiology*, University of Queensland, St. Lucia, Brisbane, Queensland, Australia

1 Methylamine and chloroquine both 'lysosomotropic' agents (i.e. agents which sequester in lysosomes) caused a dose-related inhibition of mitogen-induced lymphocyte activation in the concentrations which have previously been shown to increase the pH of lysosomes.

2 The dose-response curves of inhibition of mitogen-induced lymphocyte activation for chloroquine and methylamine are very steep and are similar to the dose-response curves obtained with dimaprit and nordimaprit, but very different from the flat dose-response curves previously described for histamine. Approximate IC_{50} values were methylamine 6.4 mM, dimaprit 0.13 mM, nordimaprit 0.03 mM and chloroquine 18 μM.

3 It is suggested that the mechanism of action of methylamine and chloroquine may be related to their lysosomotropic action and consequent interference with ligand-receptor processing, and that dimaprit and nordimaprit but not histamine may act by a similar mechanism.

Introduction

Several drugs are known to inhibit mitogen-induced lymphocyte activation. These include the autacoid, histamine, (Wang & Zweiman, 1978; Beets & Dale, 1979) histamine analogues (Vickers *et al.*, 1980; Gordon *et al.*, 1981) and chloroquine and its congeners (Hurvitz & Hirschhorn, 1965; Trist & Weatherall, 1981). It has been suggested that histamine produces its effects by an action on an H_2-receptor (Wang & Zweiman, 1978), though adequate pharmacological evidence for this is difficult to obtain (Beets & Dale, 1979). The picture is complicated by the fact that though dimaprit, an H_2-receptor agonist, causes marked inhibition, this action is not affected by H_2-receptor antagonists. Furthermore, nordimaprit, a chemical congener of dimaprit with virtually no H_2-receptor agonist activity on other tissues (see Ganellin, 1982), is even more effective as an agonist than dimaprit in inhibiting lymphocyte activation (Vickers *et al.*, 1980; Gordon *et al.*, 1981). The mechanism of action of these agents is at present a mystery.

It has been pointed out that the action of dimaprit resembles, in some respects, the action of chloroquine (Gordon *et al.*, 1981). Chloroquine and mepacrine, like dimaprit, have very steep dose-response curves and are able to produce total inhibition of activation (Trist & Weatherall, 1981). Possible mechanisms of action suggested for chloroquine and/or mepacrine include inhibition of glycolysis (Frazer & Kermack, 1957), of phospholipase A_2 (Vigo *et al.*, 1980), of RNA and DNA polymerase (O'Brien *et al.*, 1966), and of ATP-requiring membrane pumps (Trist, 1979). One of the cellular effects of chloroquine which may well be involved in its inhibitory action on lymphocyte activation and which has not been emphasized is its 'lysosomotropic' property, i.e. its propensity to sequester in lysosomes (de Duve *et al.*, 1974), and to increase lysosomal pH (Ohkuma & Poole, 1978) which would interfere with the action of the acid hydrolases. Indeed, chloroquine and other lysosomotropic amines have been shown to inhibit the mitogenesis of fibroblasts induced by epidermal growth factor (King *et al.*, 1980). It is possible that degradation of the internalized ligand-receptor complex by the acid hydrolases in the lysosomes may be an essential step in the transduction mechanism of the mitogenic response to a ligand (Das, 1980). If this is so, then other simpler amines should be equally effective in inhibiting lymphocyte activation. Methylamine, for example, is extremely effective in increasing lysosomal pH – a concentration of 10 mM producing in macrophage lysosomes,

an increase from pH 4.7 to approximately 6.2 (Ohkuma & Poole, 1978). It has also been shown to inhibit the intracellular proteolytic processing of complexes of receptor and epidermal growth factor in cultured Swiss mouse 3T3 cells (Michael *et al.*, 1980).

We describe the effect of methylamine on mitogenic lymphocyte transformation along with comparisons with the effect of dimaprit, nordimaprit, chloroquine and histamine, and data on the relative toxicity of these agents. We suggest that the results obtained with methylamine may provide a clue to a possible mechanism of action of dimaprit and nordimaprit, which differentiates their action from that of histamine.

Methods

Preparation of lymphocytes

Blood, 40–100 ml, was withdrawn from the antecubital vein of human volunteers and mixed immediately with 1/10 of its volume of sterile 0.1 M EDTA (ethylenediaminetetraacetic acid) and diluted with three volumes of sterile 0.9% w/v NaCl solution (normal saline). The cell suspension was then partially separated by centrifugation at 2000 *g* and 24°C for 30 min over a mixture of 24 parts of 9% Ficoll to 10 parts of 34% Isopaque 440 adjusted to a specific gravity of 1.078. The blood mononuclear cells were then collected from the buffy layer, washed three times in Hank's balanced salt solution and resuspended in 5 ml tissue culture medium, RPMI 1640. A 100 μl aliquot was diluted 1/10 in 0.4% Trypan blue and the cells counted in a Neubauer chamber. The remainder of the suspension was diluted in RPMI 1640 to a final concentration of 2.5×10^6 cells ml^{-1}.

The viability of the final lymphocyte suspension was always better than 95% as assessed by Trypan blue exclusion.

Lymphocyte cultures

The culture plates (Nunc, Denmark) were set up as described by Beets & Dale (1979) each well receiving 200 μl of lymphocyte suspension (500,000 cells) and 10 μl aliquots of mitogen (either Concanavalin A or phytohaemagglutinin) and of drug in varying concentrations, made up in RPMI 1640; control wells received 10 μl aliquots of the suspension medium so that all wells contained equal final volumes of mixture. The distribution of the treatments in the plate was a partial Latin Square design.

After gassing as described by Beets & Dale (1979) the cultures were incubated for 48 h at 37°C in a humidified 92% air, 8% CO_2 atmosphere and then withdrawn for the addition to each well of 0.5 μCi of [^{3}H]-thymidine in 10 μl of sterile saline. The incubation chamber was re-gassed and incubated for a further 18 h.

Harvesting of tritiated lymphocytes

Harvesting was as described by Beets & Dale (1979) except that for the first wash, Hank's balanced salt solution was used at 4°C and the plates were centrifuged at 300 *g*. The plates were centrifuged at 350 *g* and 4°C after subsequent washings with 5% trichloroacetic acid and then methanol. The results were expressed as percentage inhibition using the following formula:

$$\frac{\begin{array}{l}(\text{mitogen-stimulated} \\ \text{control samples})\end{array} - \begin{array}{l}(\text{drug-treated,} \\ \text{mitogen-stimulated,} \\ \text{test samples})\end{array}}{(\text{mitogen-stimulated control samples})} \times 100$$

Mortality assessment by ^{51}Cr assay

Three eighths of the undiluted lymphocyte suspension from each experiment was used for this assay. It was diluted to contain 10^7 cells per ml of medium to which had been added ^{51}Cr-labelled sodium chromate at a concentration giving 5 μCi per 10^6 cells. This mixture was then incubated at 37°C for one hour with gentle shaking every 15 min to resuspend the cells. After incubation the suspension was washed four times with Hank's balanced salt solution, resuspended in RPMI 1640 and the cells counted again in Trypan blue, to determine viability. Low viability suspensions were discarded. The suspension was diluted in RPMI 1640 to give 2.5×10^6 cells ml^{-1} and 200 μl aliquots were distributed among the wells of a micro-test plate and the treatments, as described above, were applied in similar Latin Square design to that used for the accompanying lymphocyte culture plate except that only three replicates were used, as opposed to the four to six used for assessment of thymidine uptake. The ^{51}Cr-labelled lymphocytes were incubated in the same way as for the thymidine-uptake assay – the incubation was interrupted at 48 h as if for the addition of thymidine but the incubation chamber was merely re-gassed.

For harvesting, the plates were centrifuged at 300 *g* for 10 min at 24°C and 110 μl of each supernatant (one half of the total volume added) was removed from each well and placed in counting tubes. The cells in the control wells were lysed with equal volumes of 10% Triton X100 for estimation of total ^{51}Cr added, and 110 μl (one quarter of the total volume) transferred to counting tubes. Each counting tube was then topped with wax for safety and the radioactivity measured in a gamma-counter (Packard 5210).

The results were expressed as percentage cell mortality by multiplying by 100 the ratio of the difference between the mean counts for drug-treated and untreated mitogen-stimulated cells to the difference between the mean counts for total ^{51}Cr added and mitogen-stimulated cells; i.e.,

$$\frac{(\text{drug-treated, mitogen-stimulated}) - (\text{untreated, stimulated})}{(\text{total added}) - (\text{untreated, stimulated})} \times 100$$

Drugs

Concanavalin A (Con A), methylamine and chloroquine were obtained from Sigma U.K. and phytohaemagglutinin (PHA) from Wellcome Reagents Ltd. Dimaprit and nordimaprit were generously donated by Smith, Kline & French Ltd. All drug solutions were filtered using 0.22 μm Millipore filters.

Reagents

RPMI 1640 culture medium containing 40 mM L-glutamine and buffered with 25 mM HEPES (N-2 hydroxyethylpiperazine-N'-2 ethane sulphonic acid) was obtained from Gibco. This was supplemented with ampicillin, 200 μg ml^{-1} (Beechams), streptomycin sulphate, 400 μg ml^{-1}, benzylpenicillin, 240 μg ml^{-1} (Glaxo), and 10% heat decomplemented foetal calf serum (Gibco). ^{51}Cr was obtained as [^{51}Cr]-Na_2CrO_4 (100–350 Ci μg^{-1}) from Amersham International Ltd.

Results

In experiments on lymphocyte activation methylamine was tested against three different doses of Con A, 5, 10 and 20 μg ml^{-1}. In all cases methylamine produced a dose-related inhibition of lymphocyte activation though there was some degree of variation in sensitivity between subjects. The full results are given in Table 1. The pooled results of the 7 experiments in which Con A 10 μg ml^{-1} and all 4 doses of methylamine were used, are plotted in Figure 1 for comparison with results with other inhibitors. The IC_{50} was 6.4 mM. The inhibition of activation was only partly due to a toxic effect of methylamine as is shown by ^{51}Cr release experiments (Table 2) which were carried out concomitantly with some of the experiments indicated in Table 1. The figures for percentage inhibition produced by 20 mM methylamine were 95.2, 99.3, 98.7 and 98.9. The concomitant figures for mortality as shown by ^{51}Cr release were 41.8%, 37.5%, 39.7% and 21.9%, respectively, with a mean of 35 ± 9%.

The results obtained with dimaprit and nordimaprit were in line with those previously found and were not very different for 3 different concentrations of

Table 1 The effect of methylamine on Concanavalin A (Con A) induced lymphocyte activation (% inhibition)

	Methylamine (mM)			
Con A	*2*	*5*	*10*	*20*
5 μg ml^{-1}	—	49.0 ± 4.7	—	88.3 ± 1.7
	0	0	50.2 ± 4.6	95.2 ± 0.5†
	32.9 ± 3.7	79.2 ± 1.7	92.3 ± 0.5	97.7 ± 0.6
	0	0	65.1 ± 4.6	95.5 ± 0.3
	28.4 ± 2.5	63.8 ± 6.6	93.1 ± 1.1	99.3 ± 0.1‡
	—	63.4 ± 7.3	87.6 ± 1.0	98.9 ± 0.2
10 μg ml^{-1}	—	35.1 ± 7.7	—	91.3 ± 2.0
	0	32.2 ± 4.4	75.3 ± 4.7	99.0 ± 0.2
	0	30.9 ± 6.7	86.7 ± 0.3	98.8 ± 0.2
	5.8 ± 4.7	70.5 ± 4.2	90.1 ± 0.7	98.4 ± 0.4
	0	0	53.2 ± 4.3	92.4 ± 2.6
	12.0 ± 5.3	45.2 ± 7.7	90.3 ± 0.9	98.7 ± 0.2*
	0	40.7 ± 6.7	82.9 ± 2.1	98.3 ± 0.2
20 μg ml^{-1}	23.3 ± 10.0	31.1 ± 9.8	77.7 ± 2.9	97.9 ± 0.3
	12.0 ± 4.5	71.1 ± 1.5	92.4 ± 0.8	98.9 ± 0.1
	0	0	25.6 ± 10.2	89.1 ± 2.6
	4.3 ± 4.0	47.7 ± 4.2	90.9 ± 2.4	98.9 ± 0.3**
	—	27.2 ± 9.3	82.4 ± 2.1	98.3 ± 0.5

The figures given are the mean ± s.e.mean of four replicates. For meaning of symbols, see Table 2.

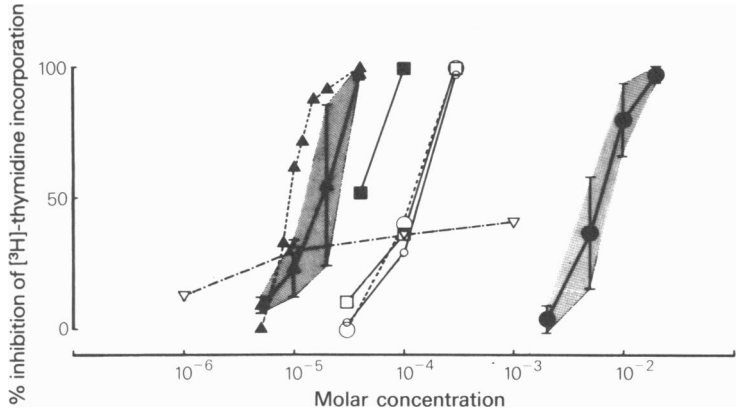

Figure 1 The effect of drugs as inhibitors of mitogen-stimulated [^{3}H]-thymidine incorporation into human lymphocytes. Methylamine with Concanavalin A (Con A) (●——●); chloroquine with phytohaemagglutinin (PHA) (▲——▲); nordimaprit with Con A (■——■); dimaprit with Con A (□——□); dimaprit with PHA (○——○). Results from other work are given for comparison: Chloroquine with PHA (▲-----▲ from Trist & Weatherall, 1981); dimaprit with PHA (○-----○ from Gordon *et al.*, 1981); histamine with PHA (▽-.-.-▽ from Vickers *et al.*, 1980). The shaded areas show the variation between experiments as indicated by standard errors (given by vertical bars) at each concentration of methylamine ($n = 7$) and chloroquine ($n = 8$).

Con A – 5, 10 and 20 μg ml^{-1}. When 20 μg Con A was used to stimulate the lymphocytes the figures for percentage inhibition with dimaprit, used in concentrations of 0.03, 0.1, 0.3 and 1 mM were 0, 36, 99 and 100, respectively (Figure 1), with an IC_{50} of 0.13 mM, and the concomitant percentage mortality as measured by ^{51}Cr release was 0, 0, 44.6 ± 1.2 and 55.7 ± 7.2. The average figures for percentage inhibition with nordimaprit used in the same concentrations with 5 μg ml^{-1} Con A were 52, 100, 100 and 100 (Figure 1) with an IC_{50} of 0.03 mM and the mean figures for percentage mortality with the same 4 concentrations of nordimaprit in 2 experiments were 10.2, 35.4, 56.1 and 71.2, respectively. Thus, as with methylamine, the lowest concentrations of dimaprit and nordimaprit which resulted in 99–100% inhibition also caused about 35% mortality, as measured by ^{51}Cr release.

When PHA was used as the mitogen in a 1 in 6 dilution, the figures for mean percentage inhibition from 2 experiments with dimaprit in concentrations of 0.03, 0.1, 0.3 and 1 mM were 1.5, 29, 100 and 100, respectively (Figure 1) with an IC_{50} of 0.14 mM.

The figure for mean percentage inhibition in 8 experiments with chloroquine used in concentrations of 2, 5, 10, 20 and 40 μM with PHA diluted 1 in 6 were 0, 9.3, 23, 55 and 98, respectively (Figure 1) with an IC_{50} of 18 μM.

Discussion

The present results show that methylamine is extremely effective in inhibiting lymphocyte activation in the concentrations previously shown to increase lysosomal pH (Ohkuma & Poole, 1978), to inhibit

Table 2 Percentage mortality of mitogenically-stimulated lymphocytes as measured by ^{51}Cr release

	Methylamine (mM)			
Con A	*2*	*5*	*10*	*20*
5 μg ml^{-1}	—	7.0 ± 0.5	13.9 ± 1.0	41.8 ± 5†
	2.2 ± 1.3	8.9 ± 0.8	21.6 ± 0.5	37.5 ± 2.3‡
10 μg ml^{-1}	4.2 ± 1.5	10.1 ± 3.0	31.1 ± 1.0	39.7 ± 2.1*
20 μg ml^{-1}	0	0	18.4 ± 3.4	21.9 ± 4.5**

The figures were obtained on the cells used in 4 of the experiments recorded in Table 1 and indicated by the following symbols: †, ‡, *, **.

fibroblast proliferation (King *et al.*, 1981), and to inhibit the intracellular processing of ligand-receptor complexes (Michael *et al.*, 1980). Because of its simple structure it is exceedingly unlikely that methylamine could affect DNA or RNA polymerase by intercalation in the DNA, as has been suggested for chloroquine, and it is not known to affect phospholipase A_2 or glycolysis. The most likely mechanism of action is related to its lysosomotropic effect. The unionized moiety crosses into the lysosomes, becomes protonated and is thus sequestered. As shown by Ohkuma & Poole (1978) this process results in an increase of pH to values outside the optimal range of the acid hydrolases and may thus interfere with lysosomal function. It has been proposed that continuous degradation or limited processing of ligand-receptor complexes by lysosomal enzymes is required to stimulate cell growth, one hypothesis being that this results in the production of a necessary intracellular messenger (Das, 1980; King *et al.*, 1980). It is also possible that general lysosomal activity is essential for the lymphocyte to reprocess its constituents during activation and that inhibition of the acid hydrolases prevents this. Certainly there is an increase in the number of lysosomes during transformation (Allison & Mallucci, 1964; Hirschhorn *et al.*, 1967) and it has been shown that lysosomotropic amines inhibit protein degradation (Seglen & Gordon, 1980) which could be necessary for transformation.

Chloroquine could inhibit lymphocyte activation by a similar mechanism or by any of the other mechanisms described in the Introduction, while the mechanism of action of dimaprit and nordimaprit is essentially unknown. A comparison of the dose-response curves for chloroquine, methylamine, dimaprit and nordimaprit with that previously found for histamine (Figure 1) strongly suggests that histamine is likely to act by a different mechanism from the other agents. There is a striking similarity in the dose-response curves of the other four drugs and in contrast to histamine, all four can produce total inhibition of lymphocyte activation regardless of mitogenic stimulus. The dose-response curve for histamine is very flat. The histamine curve shown in Figure 1 is taken from a paper by Vickers *et al.*, 1980, but all the published dose-response curves for histamine inhibition of mitogen-induced lymphocyte activation show the same pattern (Wang & Zweiman, 1978; Beets & Dale, 1979; Gordon *et al.*, 1981). In the case of methylamine and chloroquine the concentrations in which they inhibit lymphocyte activation correlate exactly with the concentrations at which they increase lysosomal pH (Okhuma & Poole, 1978) and inhibit the mitogenesis of fibroblasts induced by growth factors (King *et al.*, 1981). It is within the bounds of possibility that dimaprit and nordimaprit may also be lysosomotropic and have an inhibitory effect on lysosomal function and that this may explain part if not all of their immunosuppressive effect. Dimaprit has two basic centres of pKa 8.23 and 9.23, and it is largely ionized at body pH (see Ganellin, 1982). This also applies to chloroquine which has two basic centres of pKa 10.8 and 8.4. It is noteworthy that dimaprit and nordimaprit are both non-specific in their inhibition – suppressing lymphocyte responses to phorbol myristate acetate and pokeweed mitogen as well as Con A and PHA (Gordon *et al.*, 1981) – which would be expected if they had a non-specific effect on cellular proliferation. A further point is that there is a close similarity in toxicity between dimaprit, nordimaprit and methylamine; all three causing about 35% mortality of the cell suspension at the concentrations which result in 95–100% inhibition of lymphocyte activation. Thus for these compounds, part of the apparent total inhibition of lymphocyte activation is actually due to cell lysis. In this respect, too, these compounds are different from histamine.

It would be of interest to measure the effect of dimaprit, nordimaprit and homodimaprit on lysosomal pH in macrophages and polymorphs.

The authors wish to acknowledge some technical assistance provided by Dr L. Al-Imara and Mr R.E. Muid. Running expenses for this work were derived in part from a grant from the British Technology Group.

References

ALLISON, A.C. & MALLUCCI, L. (1964). Lysosomes in dividing cells with special reference to lymphocytes. *Lancet*, **ii**, 1371–1373.

BEETS, J.L. & DALE, M.M. (1979). Inhibition of lymphocyte activation by histamine and histamine analogues. *Br. J. Pharmac.*, **66**, 365–372.

DE DUVE, C., DE BARSY, T., POOLE, B., TROUET, A., TULKENS, P. & VAN HOOF, F. (1974). Lysosomotropic agents. *Biochem. Pharmac.*, **18**, 2495–2531.

DAS, M. (1980). Mitogenic hormone-induced intra cellular message: Assay and partial characterization of an activator of DNA replication induced by epidermal growth factor. *Proc. natn. Acad. Sci.* U.S.A., **77**, 112–116.

FRAZER, D.M. & KERMACK, W.O. (1957). The inhibitory actions of some antimalarial drugs and related compounds on the hexokinase of yeast and of *plasmodium berghei*. *Br. J. Pharmac.*, **12**, 16–23.

GORDON, D., LEWIS, G.P. & NOURI, A.M.E. (1981). Inhibi-

tion of lymphocyte proliferation by histamine and related compounds not mediated via H_1- or H_2-receptors. *Br. J. Pharmac.*, **74,** 137–141.

GANELLIN, C.R. (1982). Chemistry and structure-activity relationships of drugs acting at histamine receptors. In *Pharmacology of Histamine Receptors*, ed. Ganellin, C.R. & Parsons, M.E., pp. 10–102. Bristol, London: Wright, P.S.G.

HIRSCHHORN, R., HIRSCHHORN, K. & WEISSMANN, G. (1967). Appearance of hydrolase-rich granules in human lymphocytes induced by PHA and antigens. *Blood*, **30,** 84–102.

HURVITZ, D. & HIRSCHHORN, K. (1965). Suppression of *in vitro* lymphocyte responses by chloroquine. *New Engl. J. Med.*, **273,** 23–26.

KING, A.C., HERNAEZ, L.J. & CUATRECASAS, P. (1980). Lysosomotropic amines cause intracellular accumulation of receptors for epidermal growth factor. *Proc. natn. Acad. Sci.* U.S.A., **77,** 3283–3287.

MICHAEL, H.K., BISHAYEE, S. & DAS, M. (1980). Effect of methylamine on internalization, processing and biological activation of epidermal growth factor. *FEBS Letts.*, **117,** 125–130.

O'BRIEN, R.L., OLENICK, J.G. & HAHN, F.E. (1966). Reactions of quinine, chloroquine and quinacrine with DNA and their effects on the DNA and RNA polymerase reactions. *Proc. natn. Acad. Sci.* U.S.A., **55,** 1511–1517.

OHKUMA, S. & POOLE, B. (1978). Fluoresence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. natn. Acad. Sci.* U.S.A., **75,** 3327–3331.

SEGLEN, P.O. & GORDON, P.B. (1980). Effects of lysosomotropic monoamines, diamines, amino alcohols, and other amino compounds on protein degradation and protein synthesis in isolated rat hepatocytes. *Molec. Pharmac.*, **18,** 468–475.

TRIST, D.G. & WEATHERALL, M. (1981). Inhibition of lymphocyte transformation by mepacrine and chloroquine. *J. Pharm. Pharmac.*, **33,** 434–438.

TRIST, D.G. (1979). Effects of ouabain and mepacrine on ion movements in horse granulocytes. *Ph.D. Thesis University of London*, 120–121.

VICKERS, M.R., ARLINGTON, S.A., JONES, C., MARTIN, D. & MELVIN, M.A. (1980). The role of histamine in immune responses. In *H_2-Receptor Antagonists*, ed. Torsoli, A., Lucchelli, P.E. & Brimblecombe, R.W. pp. 310–326. Amsterdam, Oxford and Princeton: Excerpta Medica.

VIGO, C., LEWIS, G.P. & PIPER, P.J. (1980). Mechanisms of inhibition of phospholipase A_2. *Biochem. Pharmac.*, **29,** 623–627.

WANG, S.R. & ZWEIMAN, B. (1978). Histamine suppression of human lymphocyte response to mitogens. *Cell. Immunol.*, **36,** 28–36.

(*Received March 21, 1984.*
Revised April 9, 1984.)