INFLUENCE OF PROTEIN-CALORIE MALNUTRITION ON THE ANTI-INFLAMMATORY ACTIVITY OF SALICYLATE IN RATS

by

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ABSTRACT

The influence of protein-calorie malnutrition (PCM) on the anti-inflammatory activity (suppression of carrageenan-induced paw edema and pleurisy) of salicylate was studied in Sprague-Dawley male rats. For this purpose, animals were fed a 21% (control) or a 5% (PCM) protein diet ad libitum or a control diet in restricted amounts (pair-fed). A significant increase in the anti-inflammatory activity of salicylate was observed in PCM but not in pair-fed rats. PCM caused a decrease in plasma salicylate (assayed by HPLC) concentration (due to increased synthesis of glycine conjugate and elimination), no change in tissue distribution, and a moderate decrease in serum protein binding of salicylate. PCM exerted inconsistent effects on the activities of liver lysosomal enzymes (activities of β-glucuronidase decreased, of acid phosphatase did not change, and of aryl sulfatase increased) and there was no relationship between the lysosome stabilizing and anti-inflammatory activities of salicylate and other aspirin-like drugs (indomethacin and oxyphenbutazone). It was therefore concluded that the PCM-induced increase in the anti-inflammatory activity of salicylate was primarily not due to any changes in its pharmacokinetics or in its effects on lysosomal membranes. The biosynthesis of prostaglandins and leukotrienes was studied because of their important role in inflammatory processes. PCM decreased the biosynthesis by pleural neutrophils of both cyclooxygenase-dependent (major products: thromboxane B_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid) and lipoxygenase-dependent (leukotriene B_4) metabolites of arachidonic acid. The biosynthesis of prostaglandins E_2 and $F_{2\alpha}$ by renal medulla homogenates and of prostaglandin D_2, and 12-hydroxy-5,8,10,14-eicosatetraenoic acid by spleen homogenates were also reduced by PCM. At all concentrations of aspirin studied, the amounts of these metabolites were less in preparations from PCM

than from control animals. However, the concentration of aspirin required to inhibit arachidonic acid metabolism by 50% of the maximum (IC_{50}) was similar in both groups of animals. It is concluded that the increase in the anti-inflammatory activity of salicylate in PCM animals is mainly the result of a net decrease in the formation of arachidonic acid metabolites and might also be contributed by a decrease in serum protein-salicylate binding. The study suggests that a rationale salicylate therapy requires appropriate consideration of the nutritional status of patients.

RESUME

L'influence de la malnutrition protéique et calorique (MPC) sur l'activité anti-inflammatoire du salicylate, (suppression de l'oedème de la patte et de la pleuresie induits par la carragénine) a été étudiée chez le rat mâle Sprague-Dawley. Les animaux recevaient une diète contenant soit 21% de protéines (contrôle), soit 5% de protéines (MPC) ad libitum, soit la diète contrôle mais limitée aux quantités consommées par les malnourris (contrôle limité). La MPC augmente significativement l'activité anti-inflammatoire du salicylate, ce qui n'est pas le cas lors d'une carence calorique seule (contrôle limité). Chez le rat MPC, la concentration plasmatique du salicylate (mesurée par HPLC) est diminuée (augmentation de la synthèse de conjugués-glycine et élimination); la distribution tissulaire reste inchangée et la fraction de salicylate liée aux protéines sériques est légèrement diminuée. La MPC n'exerce pas d'effets consistants sur les activités des enzymes lysosomales hépatiques (diminution de l'activité de la β-glucuronidase, aucun changement de celle de la phosphatase acide et augmentation dans le cas de l'aryl-sulfatase). On n'observe aucune relation entre les activités de stabilisation du lysosome et de l'effet anti-inflammatoire du salicylate et des autres médicaments semblables à l'aspirine (indométhacine et oxyphenbutazone). Pour ces différentes raisons, il est conclu que l'augmentation de l'effet du salicylate induite par la MPC n'est pas dûe à des changements pharmacocinétiques ni à son effet sur la membrane lysosomale. L'influence de la MPC sur la biosynthèse des prostaglandines et des leukotriènes a été étudiée en raison du rôle important joué par ces substances dans le processus inflammatoire. La MPC diminue la biosynthèse par les neutrophiles pleuraux des métabolites dépendants de la cyclooxygénase (produits principaux: thromoboxane B_2 et acide 12-hydroxy-5,8,10-heptadecatriènoique) et de la lipoxygénase (leukotriène B_4) de

l'acide arachidonique. La biosynthèse des prostaglandines E_2 et $F_{2\alpha}$ dans des homogénats de médulla rénale et celle de la prostaglandine D_2 et de l'acide 12-hydroxy-5,8,10,14-eicosatéraènoique dans des homogénats de rate sont aussi réduites par la MPC. A toutes les concentrations d'aspirine étudiées, les quantités de ces métabolites étaient moindres chez les rats MPC que chez les contrôles. Par contre la dose d'aspirine requise pour inhiber le métabolisme de l'acide arachidonique à 50% de son maximum (CI50) est identique dans les deux groupes d'animaux. Il est conclu que l'augmentation de l'activité anti-inflammatoire du salicylate provoquée par la MPC est dûe principalement à une nette diminution de la formation de métabolites de l'acide arachidonique et pourrait aussi être expliquée par une diminution de la liaison du salicylate aux protéines sériques. Cette étude suggère que l'état nutritionnel des patients doit être pris en considération pour le choix d'une thérapie rationnelle au salicylate.

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LIST OF ABBREVIATIONS

PCM	Protein-calorie malnutrition (syn. Protein deficiency, Protein-deficient)
Cl_p	plasma clearance
GC-MS	gas chromatography-mass spectrometry
5-HETE	5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid
12-HETE	12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid
HHT	12-hydroxy-5, 8, 10-heptadecatrienoic acid
5-HPETE	5-hydroperoxy-6, 8, 11, 14-eicosatetraenoic acid
HPLC	high pressure liquid chromatography
K_{el}	elimination rate constant
K_{ex}	excretion rate constant
K_{mf}	metabolite formation constant
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PMNLs	polymorphonuclear leukocytes (neutrophils)
LT	leukotriene
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
LTF_4	leukotriene F_4
m/z	mass-to-charge ratio
TX	thromboxane
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2

$t_{½}$	half-life
V_d	apparent volume of distribution

1. INTRODUCTION

1. INTRODUCTION

According to a United Nations estimate of 1974, approximately 500 million people, mostly in the developing countries, suffered from varying degrees of protein-calorie malnutrition (PCM) (Joint FAO/WHO, 1976). The incidence of protein-calorie malnutrition in industrialized societies is not rare, although it is usually secondary to renal, hepatic, or neoplastic diseases or surgery. For instance, 40-50% of the hospitalized patients in the U.S. and U.K. were found to suffer from varying degrees of protein-calorie malnutrition (Bistrian et al., 1976; Hill et al., 1977).

Although it has been long recognized that nutritional factors may modify drug effects (Drill, 1952; Opie and Alford, 1915a, 1915b), this realization has not stimulated sufficient investigation into the influence of clinical and experimental malnutrition on the therapeutic efficacy of drugs. Studies in the area of protein deficiency and drug interactions have been largely confined to an evaluation of toxicity and metabolism of chemicals and environmental pollutants (Boyd, 1972; Campbell and Hayes, 1974; Williams, 1978). A few studies have also dealt with the influence of dietary protein deficiency on the plasma protein-drug binding, pharmacokinetics and biological activity of drugs (Alvares et al., 1979; Campbell et al., 1979; Conney, 1967; Mehta et al., 1975; Samuel et al., 1976; Shastri and Krishnaswamy, 1976, 1979; Varma, 1979, 1980; Varma and Mulay, 1980; Varma et al., 1982).

Protein-calorie malnutrition is associated with several physiological and biochemical changes, which can modify the biological activity of drugs in different ways. Some of these consequences of protein-calorie malnutrition, which have been shown to alter the effects of drugs, are described below.

Quantitative or qualitative dietary protein deficiency is associated with a decrease in the activity of several hepatic drug-metabolizing enzymes (Anthony, 1973; Campbell, 1977, Campbell and Hayes, 1976; Campbell et al., 1979; Ericksson et al., 1975; Hospador and Manthei, 1968; Kato et al., 1962, 1968; Marshall and Mclean, 1969; Mclean and Mclean, 1966; Mgbodile and Campbell, 1972; Miranda and Webb, 1973; Newberne et al., 1978; Varma, 1980a; Thabrew et al., 1982; Hoyumpa and Schenker, 1982; Basu, 1982). Enzymes of intestinal endoplasmic reticulum (Chhabra and Tredger, 1978), serum and liver cholinesterase (Casterline and Williams, 1971) and acetyl-CoA synthetase (Gerson and Wong, 1978) are also affected by protein deficiency. The metabolism of carbon tetrachloride (Seawright and Mclean, 1967), Heptachlor (Weatherholtz et al., 1969), octamethylpyrophosphoramide (Kato et al., 1968), ethanol (Horn and Manthei, 1965), antipyrine and theophylline (Alvares et al., 1979), sulphadiazine (Shastri and Krishnaswamy, 1979) and phenylbutazone (Varma, 1979) are reduced in protein-malnourished patients or animals. However, all drug-metabolizing enzymes are not equally affected by protein deprivation. Although the conjugation of chloramphenicol with glucuronide was reduced in malnourished children (Mehta et al., 1975), an association of dietary protein deficiency in rats with an increase in the activity of UDP-glucuronyl transferase and no change in sulphotransferase has been reported (Woodcock and Wood, 1971), suggesting that the enzymes involved in the synthetic conjugation of drugs might differ from the group of NADPH-dependent drug metabolizing enzymes. Besides, drug-metabolizing enzymes can still be induced during PCM (Anthony, 1973; Campbell and Hayes, 1974; Hospador and Manthei, 1968; Marshall and Mclean, 1969; Mclean and Mclean, 1966; Miranda and Webb, 1973; Varma, 1980a). The significance of the change in the activity of many drug-metabolizing enzymes on drug effects is obvious. However, the metabolite(s) of a drug can possess greater or lesser biological activity than the parent compounds (Conney, 1967; Williams, 1978);

consequently, protein-calorie malnutrition may lead to a decrease or an increase in the pharmacological effects of drugs.

Protein-calorie malnutrition is associated with a decrease in the hepatic synthesis and the serum concentration of albumin in humans as well as in experimental animals (James and Hay, 1968; Kelman et al., 1972; Kirsch et al., 1968; Morgan and Peters, 1971; Pain et al., 1978; Varma, 1979, 1980b). A decrease in plasma protein binding of drugs in malnourished humans and experimental animals has also been observed (Shastri and Krishnaswamy, 1976; Varma, 1979). For agents with high affinity for plasma albumin, the degree of fractional binding is considered an important factor in their biological action and metabolic degradation (Goldstein, 1949; Bridges and Wilson, 1976). Since most drugs bind to plasma albumin, a decrease in its concentration during protein deficiency is likely to result in an increased availability of the biologically active fractions of drugs. If the drug is metabolized by an enzyme(s) which has a lower activity during protein malnutrition, a decrease in the fractional binding may lead to a considerable increase in the biological effect of drugs, as is probably the case with Warfarin (Colvin and Wang, 1974). If, on the other hand, the drug is not metabolized in the body, the decrease in binding may lead to an increase in its clearance as has been reported for tetracyclines (Shastri and Krishnaswamy, 1976). A decrease in the binding of salicylates in malnourished children below the age of one year (Monckeberg et al., 1978), and phenylbutazone and oxyphenbutazone in protein-deficient rats has also been reported (Varma, 1979, 1980c).

Protein deficiency has been shown to alter the pharmacokinetics of certain drugs (Alleyne and Young, 1967; Conney et al., 1977; Mehta et al., 1975; Monckeberg et al., 1978; Shastri and Krishnaswamy, 1976; Varma, 1979, 1980c). Several changes induced by protein-calorie malnutrition, such as the

activities of drug metabolizing enzymes, protein-binding, tissue-binding, renal function and changes in other body functions can influence the pharmacokinetics of drugs. Protein-calorie malnutrition in adults is associated with a decrease in the plasma half-life, volume of distribution and absorption, and an increase in the clearance of tetracycline (Shastri and Krishnaswamy, 1976). The clearance and metabolism of chloramphenicol, however, is reduced in malnourished children (Mehta et al., 1975). A low protein-high carbohydrate diet led to an approximately two-fold increase in the plasma half-life of antipyrine and theophylline in human volunteers (Conney et al., 1977; Alvares et al., 1979). On the other hand, the plasma half-life of digoxin and ouabain and the relationship of the tissue concentration to the myocardial effects of digoxin and ouabain in guinea pigs (Varma, 1980b) and of catecholamines in rats (Benfey et al., 1983), was not influenced by protein deficiency. Enough data are not available to permit any generalization about the relationship between chemical structure of drugs and protein deficiency-induced changes in pharmacokinetics and pharmacodynamics.

Clinical and experimental malnutrition is also associated with marked changes in many physiological and biochemical processes which alone or in combination can influence drug behaviour. These include changes in the function of various endocrine organs (Aldard and Smart, 1972; Alleyne and Young, 1967; Atinmo et al., 1976; Coward et al., 1977; Whitehead and Lunn, 1979; Youlton et al., 1972; Mulay et al., 1982), immune competence (Chandra, 1977), fluid and electrolyte balance (Kagan et al., 1972; Mann et al., 1975) and nucleic acid synthesis (Haider and Tarver, 1969; Umana, 1965). It has also been shown that protein-calorie malnutrition is associated with changes in glucocorticoid receptors and actions (Varma and Mulay, 1981; Varma et al., 1982) and placental transfer of drugs such as salicylate (Varma and Yue, 1983).

Although salicylates are among the world's most commonly used drugs, there are very few studies on the influence of protein-calorie malnutrition on their pharmacology. Plasma protein binding of salicylate was found to be reduced in malnourished children (Monckeberg et al., 1978). Maternal protein deficiency (West, 1964) or food restriction (Bell and Klein, 1967) has also been reported to increase the fetal toxicity of salicylates in animals. No systematic study on the influence of protein-calorie malnutrition on the anti-inflammatory effects of salicylates has been reported.

Presentation of the Problem

Nonsteroidal and steroidal anti-inflammatory agents are commonly used in countries with widespread protein malnutrition. However, only a few studies have dealt with the influence of dietary protein deficiency on the pharmacological activities of these agents (Varma, 1981).

In a study on the influence of dietary protein deficiency on the anti-inflammatory and ulcerogenic effects of dexamethasone in rats, it was found that a low protein diet decreased both the anti-inflammatory and ulcerogenic effects of the steroid, which could not be attributed to any pharmacokinetic changes (Varma and Mulay, 1980). Conversely, phenylbutazone exerted a greater ulcerogenic (Varma, 1979) and oxyphenbutazone a greater ulcerogenic and paw edema suppressant activity in rats fed a low protein diet than in control rats (Varma, 1980c). A low protein diet in rats was associated with increases in the plasma half-lives and decreases in the plasma protein binding of both drugs, and a decrease in the metabolism of phenylbutazone (Varma, 1980a). The observed changes in the pharmacological effects of these drugs in animals with protein-calorie malnutrition might have been caused by changes in the pharmacokinetics of these agents, but the exact mechanism was not clear from these studies.

These findings indicate that protein calorie malnutrition might alter the phamacology of other nonsteroidal anti-inflammatory agents. We selected salicylates as the test agents since they are still the most extensively employed anti-inflammatory and analgesic-antipyretic agents (Flower et al., 1980). For instance, 12-25 million kg of aspirin are consumed annually in North America (Mustard, 1982). Also, salicylates are the standard of reference for comparison and evaluation of other drugs with similar effects. Moreover, considerable advances have been made in the understanding of the mechanism of actions of salicylates subsequent to the demonstration that these agents inhibit prostaglandin synthesis (Vane, 1971). However, the influence of dietary protein deprivation on the pharmacological activity of salicylates has not been fully studied. In the present study we have investigated the influence of dietary protein deficiency on the anti-inflammatory activity of salicylates and have attempted to define the mechanism of the observed increase in this activity.

2. SALICYLATES: A REVIEW OF PHARMACOLOGY

2. SALICYLATES: A REVIEW OF PHARMACOLOGY

The main pharmacological properties of aspirin and sodium salicylate are their anti-inflammatory and analgesic-antipyretic activities. These agents relieve mild pain of diverse causes and lower an elevated body temperature. The anti-inflammatory effect of salicylates is fundamental both to their analgesic and to their antirheumatic effects. The most common toxic effect of salicylates is gastrointestinal damage.

2.1. Pharmacokinetics and Metabolism of Salicylates

Orally ingested salicylates are absorbed rapidly and completely (Davidson, 1971; Levy and Leonards, 1966) partly from the stomach but mostly from the upper small intestine. Appreciable concentrations are found in plasma in less than 30 minutes. After absorption, salicylates are distributed into most body tissues and transcellular fluids, primarily by pH-dependent passive processes. Aspirin is rapidly converted to sodium salicylate, with as much as 32-50% of orally administered aspirin hydrolyzed before it enters the circulation. The absorbed ester is rapidly hydrolyzed to salicylic acid (Riegelman, 1971; Rowland et al., 1972). As a result of the rapid hydrolysis, plasma levels of aspirin are always low at ordinary therapeutic doses and can be detected only for a short time. The plasma half-life of aspirin in man is only about 15-20 minutes (Levy, 1965: Rowland and Reigelman, 1968). At concentrations encountered clinically, from 70 to 90% of salicylates are bound to plasma proteins, mainly albumin (Levy and Leonards, 1966; Hucker et al., 1980).

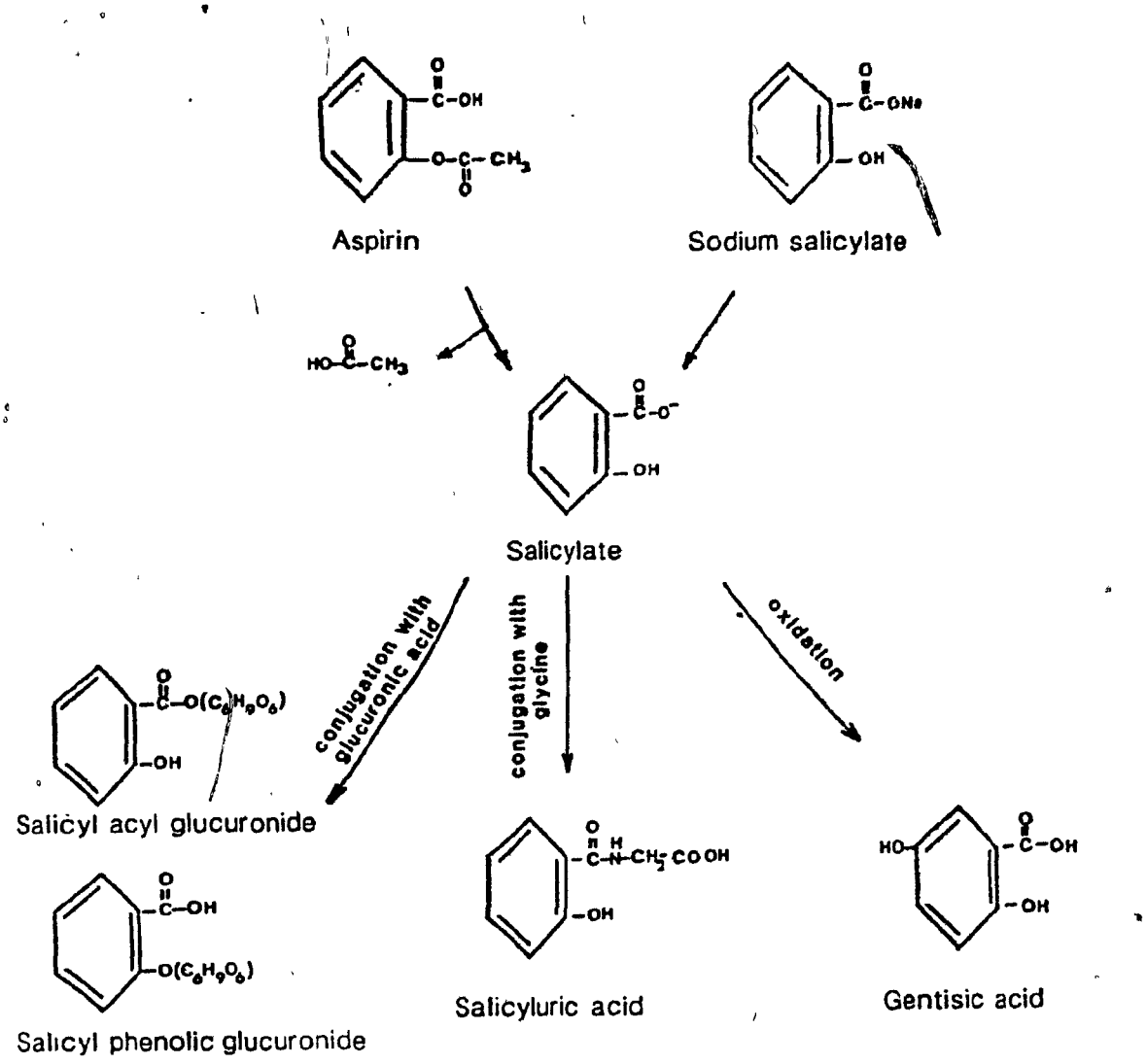
The biotransformations of salicylates take place in many tissues, but particularly in hepatic endoplasmic reticulum and hepatic as well as renal mitochondria (Forman et al., 1971). The three chief metabolites are the conjugate with glycine yielding salicyluric acid (SU), the salicylphenolic

glucuronide (SPG) and the salicyl acyl glucuronide (SAG); a small fraction is oxidized to gentisic acid (Flower et al., 1980) (Figure 1). The formation of salicyluric acid is capacity limited and proceeds by easily saturable Michaelis-Menten kinetics. Similarly, phenolic glucuronide formation has also been shown to be saturable although at somewhat higher salicylate levels. Therefore, the metabolism of salicylates is dose-dependent (Davison, 1971; Levy et al., 1972; Levy, 1979). The plasma half-life for salicylate in humans is 2 to 3 hours at low doses and 15 to 20 hours at high doses (Levy, 1979). The rat, like humans, has only limited capacity for the synthesis of salicylurate from salicylate and glycine. However, salicylate elimination by rat follows apparent first-order kinetics after administration of a dose as high as 100 mg/kg although salicylurate formation proceeds at a constant rate. This could be due to the fact that glycine conjugation is only one of the major routes of salicylate metabolism in rats (Nelson et al., 1966). It has also been found that plasma concentration profiles in dog and swine resemble those in man with indications of saturable elimination while those in the pony, the goat and the cat follow first-order kinetics (Hucker et al., 1980).

Salicylates are excreted mainly by the kidney. Studies in man indicate that salicylate is excreted in the urine as free salicylic acid, salicyluric acid, salicyl phenolic and acyl glucuronides, and gentisic acid. However, excretion of free salicylic acid is extremely variable (Levy and Leonards, 1966; Nelson, 1966; Furst et al., 1979).

2.2. Mechanisms of Action of Salicylates

Several hypotheses have been advanced to explain the actions of aspirin-like drugs. These include an interference with oxidative phosphorylation (Whitehouse and Haslam, 1962), the displacement of an endogenous anti-inflammatory peptide from plasma protein (McArthur et al., 1971a.b; Smith et al., 1971), interference with the migration of leukocytes (Di Rosa et al.,



Metabolism of salicylates

Figure 1. Metabolic pathways of salicylates. The major metabolites of salicylate are salicyluric acid and salicyl glucuronides (salicyl phenolic glucuronide and salicyl acyl glucuronide). A variable fraction of salicylate is excreted unchanged and a small fraction is oxidized to gentisic acid.

1971a.b), inhibition of leukocyte phagocytosis (Chang, 1972), inhibition of generation of lipoperoxides (Sharma et al., 1972), and hyperpolarization of neuronal membranes (Barker and Levitan, 1971; Levitan and Barker, 1972).

These hypotheses have obvious discrepancies and ignore the possibility that many observations could be due to an inhibition of the metabolism of arachidonic acid (Ferreira and Vane, 1974). For instance, higher concentrations of salicylate are needed to uncouple oxidative phosphorylation than that achieved by therapeutic doses and no convincing evidence relating uncoupling potency to anti-inflammatory activity has been obtained (Smith and Dawkins, 1971). Inhibition of leucocyte phagocytosis also occurs only with high concentrations of aspirin-like drugs (Chang, 1972). Interference with migration of leucocytes is no longer a tenable hypothesis, because salicylate and indomethacin affect only migration of monocytes and not of polymorphonuclear cells (Van Arman et al., 1970). Confirmation of displacing anti-inflammatory peptides from serum proteins will depend upon isolating the peptides from serum (Ferreira and Vane, 1974). The interference with generation of lipoperoxides could be explained by inhibition of prostaglandin synthetase.

In addition, a number of reports have proposed that anti-inflammatory agents produce their effects by stabilizing lysosomal membrane and thereby inhibiting the release of lysosomal enzymes (Ignarro, 1974; Weissmann, 1967, 1972; Gryglewski, 1979).

Since Vane and associates demonstrated that low concentrations of aspirin and indomethacin inhibited the enzymatic production of prostaglandins (PGs) in different biological systems (Ferreira et al., 1971; Smith and Willis, 1971; Vane, 1971), the concept that the inhibition of biosynthesis of PGs is the main mechanism of aspirin-like drugs has been widely accepted (Moncada and

Vane, 1979; Kuehl and Egan, 1980; Oates, 1982; Flower, 1983). It is now possible to explain why such heterogenous agents have the same basic therapeutic activities and often the same basic side effects.

2.2.1. Inhibition of Biosynthesis of Prostaglandins (PGs)

2.2.1.1. Metabolism of Arachidonic Acid

Arachidonic acid (AA) is released from membrane phospholipids by the action of the enzyme phospholipase A_2. Once released, AA is rapidly metabolized to oxygenated products by a cyclooxygenase and several lipoxygenases (Figures 2, 3, 4). All available evidence indicates that cells do not store PGs and their release depends on de novo biosynthesis (Oates, 1982).

Cyclooxygenase Pathway. As the first step, AA is oxygenated and cyclized to form the cyclic endoperoxide derivatives, PGG_2 and PGH_2. These endoperoxides, which are chemically unstable, are then isomerized enzymatically or non-enzymatically into different products PGE_2, $PGF_{2\alpha}$ or PGD_2.

The endoperoxide PGH_2 is also metabolized into two unstable and biologically highly active compounds. One of these is thromboxane A_2 (TXA_2), formed by an enzyme, thromboxane synthetase. TXA_2 is unstable and breaks down nonenzymatically into the stable thromboxane B_2 (TXB_2). PGH_2 is also converted to prostacyclin (PGI_2), which is hydrolyzed nonenzymatically to a stable compound, 6-oxo$PGF_{1\alpha}$ (6-Keto-$PGF_{1\alpha}$).

The endoperoxides are also transformed into a 17-carbon hydroxy acid: 12-hydroxy-5, 8, 10-heptadecatrienoic acid (HHT) with the concomitant formation of malondialdehyde. It has been found that all mammalian cell types studied, with the possible exception of the erythrocyte, possess enzymes for the synthesis of PGs.

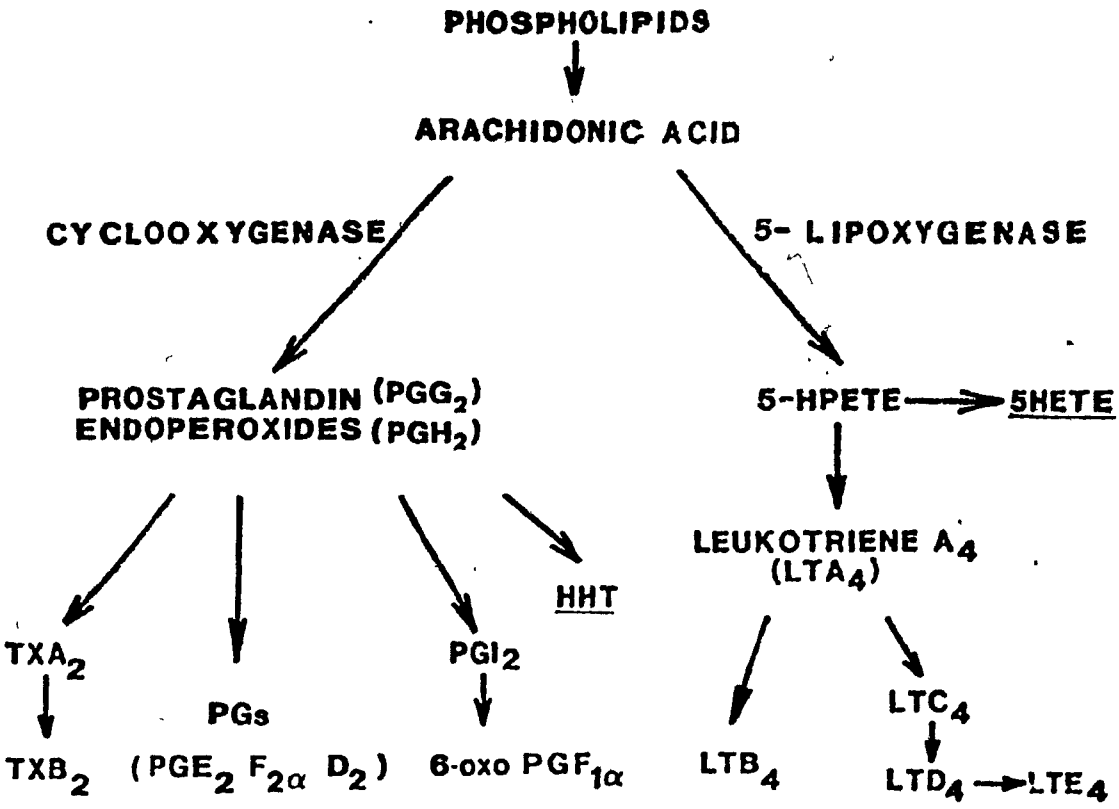


Figure 2. Transformation of arachidonic acid into prostaglandins, thromboxanes and leukotrienes.

PG: prostaglandin

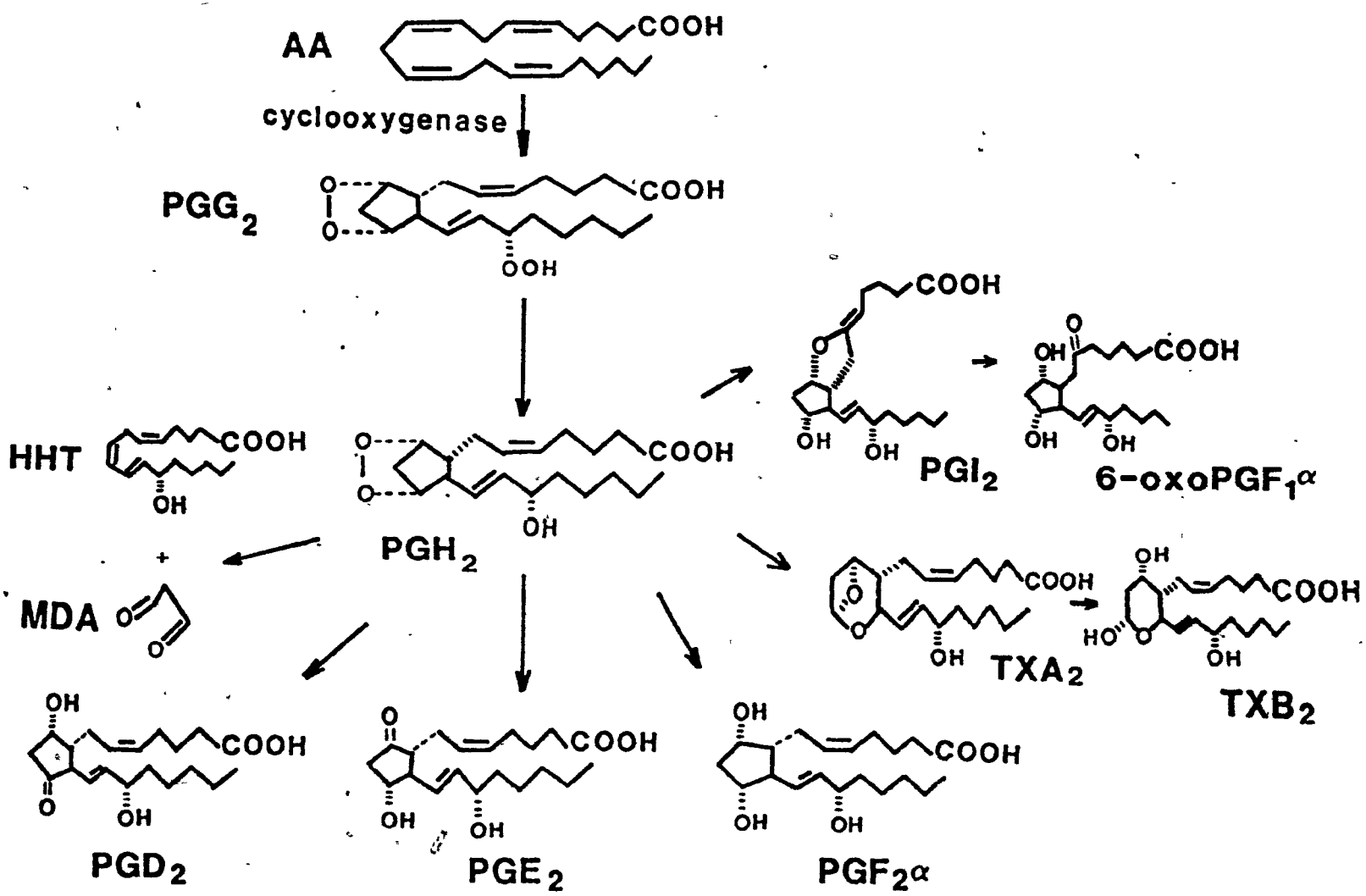
TX: thromboxane

HHT: 12-hydroxy-5,8,10-heptadecatrienoic acid

LT: leukotriene

Figure 3. The cyclooxygenase pathway of arachidonic acid metabolism leading to the biosynthesis of prostaglandins and thromboxanes.

AA: arachidonic acid; PGG_2: prostaglandin G_2; PGH_2: prostaglandin H_2; PGI_2: prostacyclin; TXA_2: thromboxane A_2; TXB_2: thromboxane B_2; PGE_2: prostaglandin E_2; PGD_2: prostaglandin D_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; HHT: 12-hydroxy-5,8,10-heptadecatrienoic acid; MDA: malondiadehyde.


AA
COOH
cyclooxygenase
PGG2
OOH
HHT
OH
PGH2
MDA
PGI2
6-oxoPGF1α
TXA2
TXB2
PGD2
PGE2
PGF2α

Lipoxygenase Pathway. In contrast to fatty acid cyclooxygenase, which is widely distributed, the distributions of lipoxygenases are more restricted. For instance, 5-lipoxygenase is mainly found in white cells and 12-lipoxygenase in platelets and a few other tissues (Samuelsson, 1982). The first step in the formation of leukotrienes is oxygenation of AA by 5-lipoxygenase to form a hydroperoxy acid (5-HPETE) (Figure 4). This reaction takes place in both rabbit and human polymorphonuclear leukocytes (PMNLs). 5-HPETE is dehydrated enzymatically into an unstable intermediate leukotriene A_4 (LTA_4) which is converted enzymatically by hydrolysis into leukotriene B_4 (LTB_4)* and by addition of glutathione into leukotriene C_4 (LTC_4). LTA_4 can also be converted nonenzymatically by hydrolysis into two isomers of LTB_4** (Radmark, et al., 1980; Corey, et al., 1980), which are biologically far less active than LTB_4 (Bray et al., 1981; Smith, 1981). An additional isomer of LTB_4***, isolated from swine leukocytes, has been recently identified and has much lower biological activity than LTB_4 (Borgeat et al., 1981). LTC_4 is metabolized to the corresponding cysteinylglycyl derivative (LTD_4) and cysteinyl derivative (LTE_4). LTE_4 can be converted into LTF_4 by addition of a γ-glutamyl residue (Samuelsson, 1982). The 5-HPETE can also be converted enzymatically into 5-HETE (Figure 2 and Figure 4).

The main lipoxygenase pathway metabolite of AA isolated from human platelets and guinea pig lung is 12-HETE, which is converted enzymatically by degradation from 12-HPETE (Hamberg and Samuelsson, 1974).

* LTB_4:5(S),12(R)-dihydroxy-6,8,10,14 - (Z.E.E.Z.)- eicosatetraenoic acid.
** These two isomers of LTB_4 are
5(S), 12(R)-dihydroxy - 6,8,10,14 - (E.E.E.Z.) - eicosatetraenoic acid
5(S), 12(S)-dihydroxy - 6,8,10,14 - (E.E.E.Z.) - eicosatetraenoic acid
*** 5(S), 12(S)-dihydroxy - 6,8,10,14 - (E.Z.E.Z.) - eicosatetraenoic acid

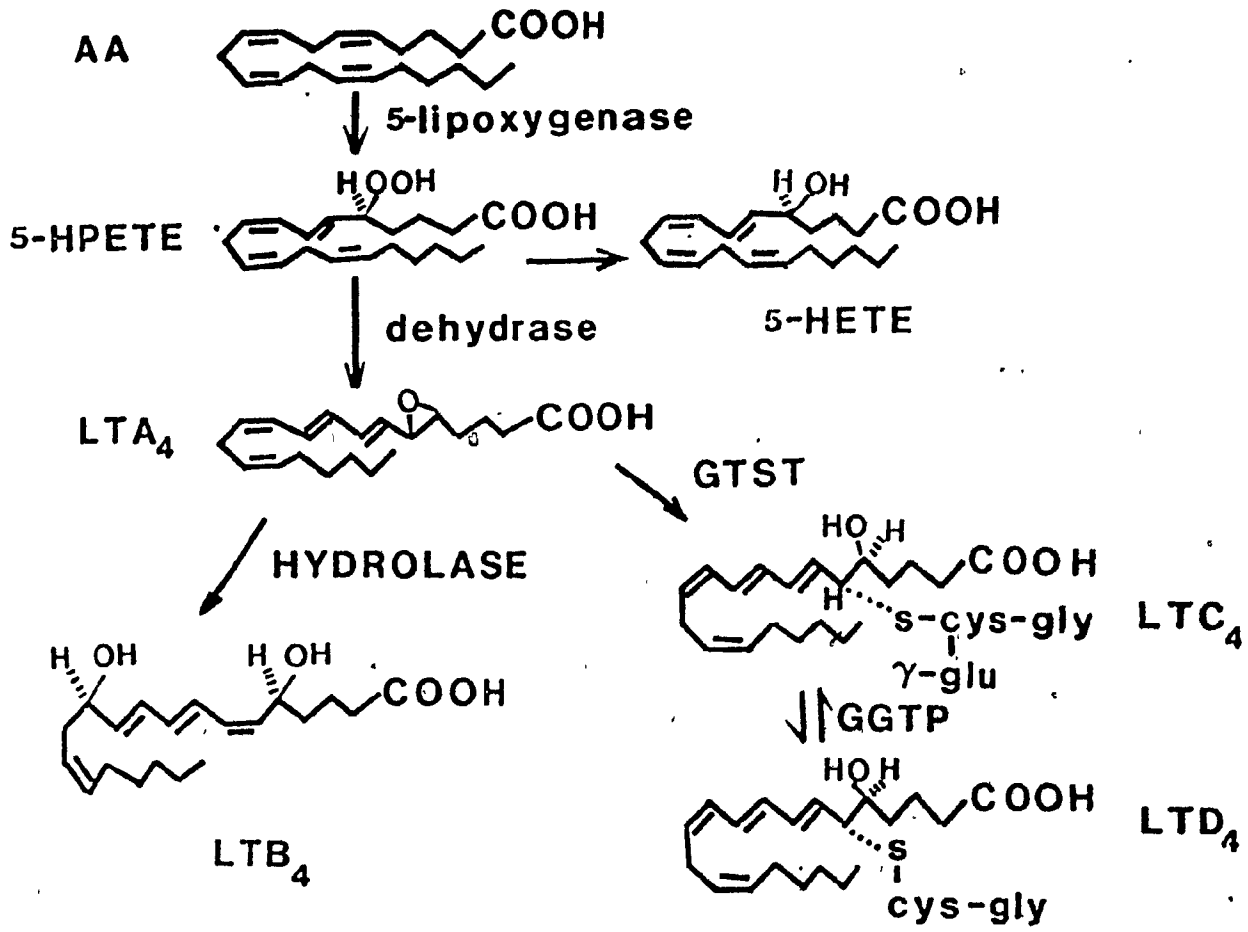


Figure 4. The 5-lipoxygenase pathway of arachidonic acid metabolism leading to the biosynthesis of leukotrienes

5-HPETE: 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE: 5-hydroxy-6,8,10,14-eicosatetraenoic acid; LTA_4: leukotriene A_4; LTB_4: leukotriene B_4; LTC_4: leukotriene C_4; LTD_4: leukotriene D_4; GTST: glutathione-S-transferase; GGTP: γ-glutamyl transpeptidase.

2.2.1.2. The Inflammatory Effects of Prostaglandins and Leukotrienes

It is generally accepted that AA-derived products, prostaglandins and leukotrienes, are involved in inflammation. They have been detected in numerous inflammatory conditions in both humans and experimental animals. They could originate from the inflamed tissues, local blood vessels or invading leukocytes (Vane, 1976; Williams and Peck, 1977; Higgs et al., 1979b, 1981; Moncada and Vane, 1979b; Kuehl and Egan, 1980; Klickstein et al., 1980; Weissman, 1982; Hasada et al., 1982). The main actions of AA-derived products in inflammation are vasodilatation, edema, pain (hyperalgesia) and leukocyte migration (chemokinesis and chemotaxis) (Samuelsson, 1983). The cyclooxygenase products and lipoxygenase products have a synergistic effect in inducing inflammation (Moncada and Vane, 1979; Smith, 1982; Samuelsson, 1982; Rackham and Ford-Hutchinson, 1983).

Prostaglandins of the E series in nanogram amounts cause erythema (Solomon et al., 1968) and vasodilatation (Williams and Peck, 1977; Dusting et al., 1978). There are two features of the vascular effects of PGs not shared by other putative mediators of inflammation - a long lasting vasodilator action and the capacity to counteract the vasoconstrictor effects of substances such as norepinephrine and angiotensin. PGI_2 and its degradation product, 6-oxo $PGF_{1\alpha}$, also induce erythema when injected into the rabbit skin and PGI_2 is a very potent vasodilator (Higgs et al., 1977, 1978, 1979a; Moncada and Vane, 1979; Williams and Peck, 1977).

PGs, like other putative mediators of inflammation, increase vascular permeability in the postcapillary and collecting venules. They can potentiate carrageenan-induced paw edema in rats (Higgs et al., 1978; Komoriya et al., 1978; Murota et al., 1979), increase vascular permeability (Murota and Morita,

1978), and enhance the vascular permeability induced by other agents such as bradykinin, histamine and 5-HT (Komoriya et al., 1978; Williams and Morley, 1977; Williams and Peck, 1977; Peck and Williams, 1978; Williams, 1979).

LTC_4 and LTD_4 promote plasma exudation from postcapillary venules (Hedqvist and Dahlen, 1983). When injected intradermally, as little as 100 fmole of either compound can induce plasma leakage. Higher doses produce dose-dependent increases in spot diameter (Drazen et al., 1980; Hedqvist et al., 1980). When applied topically to the cheek pouch, LTC_4 and LTD_4 elicit a direct, dose-dependent contraction of arterioles, which is followed by a dose-dependent leakage of macromolecules (Dahlen et al., 1981). LTB_4 itself has little or no effect on vascular permeability (Higgs et al., 1981; Lewis, 1981b; Morley et al., 1981; Wedmore and Williams, 1981; Dahlen et al., 1981), but has a synergistic effect with PGs in increasing plasma exudation (Wedmore and Williams, 1981; Higgs et al., 1981; Smith, 1981; Bray et al., 1981a; Morley et al., 1981); this is of importance in the formation of edema (Samuelsson, 1982; Rackham and Ford-Hutchinson, 1983). In addition, LTB_4 is equipotent with PGE_2 and PGI_2 in potentiating bradykinin-induced plasma exudation (Higgs et al., 1981).

Leukocytes, especially polymorphonuclear leukocytes (PMNLs), play an important role in the inflammatory process. The migration and accumulation of leukocytes is important in the development of inflammatory reactions (Weissmann, et al., 1979; Spisari and Traniello, 1979). Leukocytes respond to inflammatory stimuli and collect at the site where inflammation is initiated, owing to their mobility and their chemotactic behaviour. Leukocytes contribute to tissue damage and exacerbate inflammation by at least two distinct mechanisms, i.e., by phagocytic attack, which substantially increases PG release from PMN leukocytes (Higgs and Youlton, 1972; Higgs et al., 1975, 1983;

Zurier, 1976) and leads to the elaboration of other classes of mediators (Weissmann et al., 1979; Zurier et al., 1973), and by secretion of large amounts of lysosomal hydrolases and neutral proteinases (Baggiolini et al., 1979; Weissmann et al., 1979). It was found that the PG levels at inflammatory sites are closely related to leukocyte infiltration (Di Rosa and Willoughby, 1971). Moreover, the complement-derived peptide C5a and the synthetic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), affect vascular permeability indirectly through the rapid involvement of PMN leukocytes at the inflammatory site (Smith, 1982).

There is now a substantial body of evidence showing that LTB_4 possesses cytotactic activity. LTB_4 causes the aggregation in vitro of a number of leukocyte cell types that include human, rat and rabbit polymorphonuclear leukocytes, monocytes, and macrophages. The ED_{50} values for the aggregating activity of LTB_4 are between 0.5 and 1.0 ng/ml for human and rat PMNL suspensions (Ford-Hutchinson et al., 1980; 1981). Using human peripheral PMNLs as the test cells, LTB_4 shows maximal chemokinetic activity at concentrations ranging from 10 to 30 ng/ml. It has also been proven that despite differences in assay methods LTB_4 is a potent chemotaxin in vitro (Palmer et al., 1980; Smith, 1982). The concentration of LTB_4 that produces a maximal stimulation of the directed migration of human peripheral leukocytes is from 0.3 to 30 ng/ml (Smith, 1982). So its potency as a chemotaxin, chemokinesin, and aggregating agent in vitro is equivalent to that of the established cytotaxins, C5a and the synthetic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) (Ford-Hutchinson et al., 1980; Bray et al., 1981b,c; Smith, 1981, 1982; Palmer and Yeats, 1981; Cummingham et al., 1980). Moreover, a further similarity between LTB_4 and the other cytotaxins in vitro is its ability to cause increased release of lysosomal enzymes from leukocytes (Palmer et al., 1981; Goetzl and Pickett, 1980; Rae

and Smith, 1981). Single doses of LTB_4, ranging from 25 to 500 ng, cause the accumulation of neutrophils at local sites of administration in a number of animal species and man. An intraperitoneal injection of LTB_4 in the guinea pig or introduction of LTB_4 into the aqueous humor of the rabbit eye produces a significant increase in the total number of white cells in the peritoneal fluid or the aqueous humor (Bhattacherjee et al., 1981; Smith et al., 1980). Intradermal injection of LTB_4 causes a dose-dependent increase in the number of leukocytes in the dermis of the rabbit (Bray et al., 1981d; Carr et al., 1981). By using the intestinal mesentery of the rabbit or the cheek pouch of the hamster as a model, it was consistently found that LTB_4 caused leukocytes to adhere to, and pass through the walls of small venules (Bray et al., 1981b). The cytotactic activity of LTB_4 in vivo appears to be the basis of a further effect of the leukotriene on vascular permeability (Bray et al., 1981a). The mono-HETEs, e.g. 5-HETE and 12-HETE are also chemotactic agents, but they are at least 100 times less potent than LTB_4 (Ford-Hutchinson et al., 1980; Higgs et al., 1981; Bray et al., 1981b; Lewis, 1981a). Some cyclooxygenase pathway products, for instance, HHT and TXB_2 are also found to possess chemotactic effect, but these are also far less potent than LTB_4 (Boot et al., 1976; Kitchen et al., 1978; Goetzl and Gorman, 1978).

Induction of hyperalgesia is a typical effect of low concentrations of PGs. For example, minute amounts of PGE, given intradermally to man cause a long-lasting hyperalgesia (Ferreira, 1972). PGs lower the pain threshold to mechanical stimulation and enhance nociceptive actions of bradykinin, substance P, 5-HT and histamine in man (Ferreira, 1972) and other species (Rosenthale et al., 1972; Ferreira et al., 1973; Moncada et al., 1975; Juan and Lembeck, 1976; Willis and Cornelsen, 1973; Ferreira et al., 1978; Staszewska-Barczak et al., 1976; Ferreira and Nakamura, 1979). The effects of PGs of the E series are delayed and long lasting while those of PGI_2 are immediate and short

lasting.

Fever is often associated with inflammatory processes, since fever, pain and inflammation are all closely related as part of the body's defence mechanisms to injury and infection. PGE_1 and PGE_2 injected directly into the thermoregulatory area of the anterior hypothalamus promote heat gain and inhibit heat loss mechanisms (Milton and Wendlandt, 1970; Feldberg and Saxena, 1971; Milton and Wendlandt, 1971; Milton, 1982). Foreign and endogenous pyrogens, which produce fever, increase PGE_2 levels in the CSF. The concentration of PGE_2 in CSF found during fever would be sufficient to produce a rise in deep body temperature if it were applied to the region of the anterior hypothalamus (Feldberg and Gupta, 1973; Feldberg et al., 1973; Dey et al., 1975; Harvey et al., 1975). During bacterial pyrogen fever a circulating pyrogenic material of endogenous origin is found in the plasma, which when transferred to a recipient animal, can produce both fever and a rise in PGE_2 levels in the CSF. When administered during pyrogen fever, antipyretic drugs which inhibit cyclooxygenase also inhibit the rise in PGE_2 in the CSF at the same time as they produce antipyresis (Feldberg et al., 1973; Dey et al., 1975). On the basis of the foregoing evidence, it has been suggested that PGE_2 is an endogenous modulator of fever (Milton, 1982).

2.2.1.3. Inhibition of the Biosynthesis of Prostaglandins by Salicylates

Since the report by Vane in 1971, it has been abundantly confirmed that aspirin-like drugs inhibit prostaglandin synthetase in numerous species (including man) and in many biologic preparations, ranging from cell-free microsomal preparations of the synthetase itself to whole organs or organisms. Furthermore, the concentration of aspirin-like drugs achieved after therapeutic dosage is adequate to inhibit prostaglandin biosynthesis, and this is substantiated

by measurements in humans (Ferreira and Vane, 1974; Samuelsson, 1974; Higgs, 1974; Moncada and Vane, 1979a). There is also a striking correlation between the antienzyme activity of the drug and its anti-inflammatory activity (Collier and Flower, 1971; Smith and Willis, 1971; Ham et al., 1972; Tomlinson et al., 1972; Hamberg, 1972; Horton et al., 1973; Moncada and Vane, 1979a). The evidence, therefore, is overwhelming that aspirin-like drugs inhibit the biosynthesis of prostaglandins in all animal species so far tested and in humans and that this effect is achieved with therapeutic doses.

There are multiple steps in the conversion of arachidonic acid to prostaglandins. The first enzyme - cyclooxygenase - is inhibited by aspirin-like drugs, so that the generation of PG endoperoxides (PGG_2 and PGH_2) and all of their products are reduced or abolished. The mechanism of inhibition of PG synthetase by aspirin-like drugs is complex and might differ for various drugs. Aspirin itself acetylates a serine at the active site of the enzyme (Roth et al., 1975; Roth and Siok, 1978). In contrast to aspirin, salicylic acid has no acetylating capacity and is a much weaker inhibitor of cyclooxygenase in vitro, but can be as active as aspirin in vivo in reducing the synthesis of PGs and in inhibiting carrageenan-induced edema (Hamberg, 1972; Willis et al., 1972; Higgs et al., 1976; Smith et al., 1979). Salicylic acid must do so by quite a different mechanism. Since aspirin is rapidly hydrolyzed to salicylic acid in vivo, evidently the acetylated and non-acetylated species act as pharmacologically distinct entities, and aspirin probably has a dual mechanism of action (Flower et al., 1980; Smith et al., 1979).

At concentrations which inhibit cyclooxygenase, aspirin-like drugs do not inhibit lipoxygenase (Hamberg and Samuelsson, 1974). This may explain why aspirin has relatively weak effects on leukocyte migration or potentiates lipoxygenase activity in vitro, possibly through a diversion of substrate (Higgs

et al., 1979b). However, some nonsteroidal anti-inflammatory drugs, including aspirin and sodium salicylate, have been found to inhibit the peroxidase that transforms 12-hydroperoxy eicosatetraenoic acid (12-HPETE) to the 12-hydroxy fatty acid (12-HETE) (Siegel et al., 1979) and act to scavenge the free oxygen-derived radicals (Kuehl et al., 1979).

In conclusion, it is clear from the foregoing discussion that arachidonic acid metabolites play an important role in various aspects of the inflammatory response and that aspirin-like drugs exert most of their effects through the inhibition of the cyclooxygenase-dependent metabolism of arachidonic acid. However, inflammation is a complex process and it is likely that other direct or indirect actions of aspirin-like drugs contribute to their anti-inflammatory activity.

2.2.2. Effects on Lysosomal Membranes

Soon after the discovery of lysosomes, it became clear that lysosomal enzymes are important mediators of the inflammatory process (Weissmann, 1967, 1972; Ignarro, 1974; Baggiolini et al., 1979). Soluble extract of purified leukocyte lysosomes elicit cutaneous and joint inflammations and degrade the protein-mucopolysaccharide matrix of cartilage (Weissmann and Thomas, 1964). Activities of acid and neutral hydrolases are significantly elevated in inflamed tissues of animals as well as in synovia and synovial fluid from patients with rheumatoid arthritis (Ignarro and Slywka, 1972; Ignarro, 1974; Weissmann, 1972; Baggiolini et al., 1979). Adjuvant polyarthritis in rats is characterized by inflammation first at the site of inoculation and then in most of the extremities. Associated with this immunologic disease is the development of increased liver lysosome fragility, elevated plasma levels of lysosomal enzymes and elevated amounts of acid hydrolases in the affected extremities (Ignarro and Slywka, 1972; Piliero and Colombo, 1969; Collins and Lewis, 1971; Anderson, 1971; Walz et al., 1971). The close temporal relationship between liver lysosome fragility and elevated plasma hydrolases in adjuvant polyarthritis suggests that the discharge of lysosomal contents is a direct consequence of enhanced endocytic activity. These lysosomal enzymes then cause injury to cartilage and other tissues and increase the degree of inflammation. During this process PGs are also released (Higgs et al., 1975). It has also been reported recently that LTB_4 and other cytotaxins cause increased release of lysosomal enzymes from leukocytes treated with cytochalasin B (Palmer and Yeats, 1981; Rae and Smith, 1981).

On the basis of reports that lysosomal enzymes are released during acute and chronic inflammation and that this release is inhibited by steroidal and nonsteroidal anti-inflammatory agents, it has been proposed that anti-inflammatory agents act by stabilizing lysosomal membranes (Weissmann,

1972; Ignarro and Colombo, 1972; Ignarro, 1974). However, not all the available data support such a conclusion.

Salicylates were found to decrease the release of lysosomal enzymes in vitro from liver cells isolated from rats (Miller and Smith, 1966; Tanaka and Iizuka, 1968; Ignarro, 1971b, 1973) or rabbits (Ignarro, 1971a) at concentrations of 10^{-4} to 10^{-3}M. Lysosomes from a more dense mitochondrial fraction were more susceptible to the membrane stabilizing action of drugs than were lysosomes isolated from a less dense mitochondrial fraction (Ignarro, 1971b, 1974; Tanaka and Iizuka, 1968). Aspirin was found to inhibit the release of lysosomal enzymes from guinea pig peritoneal polymorphonuclear leukocytes (Ignarro and Colombo, 1972) and human neutrophils (Ignarro, 1974) in vitro. However, several reports have produced contradictory results (Gryglewski, 1979). Steroidal and nonsteroidal anti-inflammatory agents including aspirin did not produce stabilization of rat liver lysosomes (Ennis et al., 1968) and did not inhibit thermal labilization of rat liver lysosomes (Brown and Schwartz, 1969).

Furthermore, salicylates in concentrations of 1 to 10 mM did not inhibit the release of lysosomal enzymes from low density lysosomes of rat liver (Harford and Smith, 1970; Robinson and Willcox, 1969). Salicylates were found to even enhance thermally-induced release of enzymes from rat liver lysosomes (Brown and Schwartz, 1969) and labilize lysosomal membranes of circulating rat and rabbit leukocytes (Ignarro, 1971a). The contradictory results could partly be due to differences in experimental conditions such as drug concentrations (Lewis, 1970), incubation medium (Ignarro, 1973; Harford and Smith, 1970), temperature (Ignarro, 1971b) and the method of isolating lysosomes (Ignarro, 1974).

There are only a few reports on the effects of drugs administered in vivo on lysosomal membrane stability. Ignarro (1972) reported that pretreatment of rats with steroidal and nonsteroidal anti-inflammatory agents including aspirin reduced the relative mechanical and osmotic fragility of liver lysosomes. Phenylbutazone and indomethacin but not aspirin attenuated the swelling and inhibited the release of liver and hind paw lysosomal enzymes in rats with adjuvant-induced polyarthritis (Ignarro and Slywka, 1972). In another study in rats the steroidal (dexamethasone) but not the nonsteroidal (phenylbutazone and indomethacin) agents inhibited thermally-induced release of lysosomal enzymes although all of these agents attenuated the acute inflammatory response (Pollock and Brown, 1971). In view of these contradictory data, stabilization of lysosomal membranes as a mode of anti-inflammatory activity of salicylates can not be excluded.

3. OBJECTIVES OF THE PRESENT PROJECT

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The main objectives of the present investigation were:

(1) To find out if the anti-inflammatory effects of salicylates, considered to be model nonsteroidal anti-inflammatory drugs, are altered by protein-calorie malnutrition (PCM). Studies were done on rats as the experimental model.

(2) Because PCM increased the anti-inflammatory activity of salicylates, the following possibilities were explored in an attempt to determine the mechanism of this change.

i) Could protein-calorie malnutrition cause any alteration in pharmacokinetics, metabolism and disposition of the drug?

ii) Is this change in the anti-inflammatory activity of salicylates caused by a modification of their action on lysosomes?

iii) Since salicylates act by inhibiting cyclooxygenase, could the metabolism of arachidonic acid be altered by PCM? In order to observe the effect of dietary protein intake on the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism, neutrophils, which have been found to possess both cyclooxygenase and 5-lipoxgenase activity (Borgeat and Samuelsson, 1979b; Siegel et al., 1980) were used as the model system. Some studies were also done with kidney medulla and spleen.

4. MATERIALS AND METHODS

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4.1. Animals and Dietary Regimens

All studies reported here were performed on male Sprague-Dawley rats purchased from Canadian Breeding Farms, St. Constant, Quebec. In preliminary studies it was found that weanling rats (50-60 g) were too small for the present studies, especially those dealing with the pharmacokinetics of salicylate; animals did not gain weight when fed a low protein diet and serial blood collection of even small volumes (0.1 ml) resulted in death of many animals. It was also found that when animals were fed a 10% protein diet (approximately one-half of the optimum), the effects of protein-deficiency were too small to permit clear cut differentiation of changes in drug effects. A reduction of dietary protein to 0.5% produced weight loss and a small percentage of animals died after a 2 to 4-week period.

All experiments described in this thesis were therefore done on rats which weighed between 100-125 g and were approximately 5 weeks old at the beginning of these studies. These animals were placed individually in wire-bottomed suspended cages in the McIntyre Animal Centre of McGill University. The Animal Centre had a temperature of 22°C, and a routine of 12 h light and 12 h dark. Control animals were fed ad libitum a 21% protein diet. Protein-calorie malnutrition (PCM) was induced by feeding ad libitum a 5% protein diet. A group of rats were fed a restricted quantity (10 g/rat/day) of the control 21% protein diet; these animals were designated as pair-fed controls because they were fed approximately the same quantity of food as was consumed by rats fed the 5% protein diet ad libitum. All animals had free access to tap water and unless stated otherwise, animals were maintained on the respective diets for a 3-week period. Also included in this study were a group of rats which first received a 5% protein diet for 3 weeks and then a

control diet (protein-repleted), both ad libitum.

The composition of the control 21% protein diet was as follows (g/kg): vitamin-free casein, 231; sucrose, 519; corn starch, 150; corn oil, 50; mineral mixture (Williams-Briggs), 40; vitamin mixture (Teklad), 10. The 5% protein diet contained 55 g/kg vitamin-free casein and 695 g/kg sucrose; all other constituents were identical to those in the control diet (21% protein). Both diets were isocaloric in composition and were purchased in a pellet form from Teklad Test Diets, Madison, Wisc.

4.2. Anti-inflammatory Effects

The anti-inflammatory effect of drugs was determined by measuring the inhibition of carrageenan-induced paw edema (Winter et al., 1962) and carrageenan-induced pleurisy (Vinegar et al., 1973; Katori et al., 1980). These experiments were always started at the same time of the day (9:00 to 10:00 a.m.) to minimize any effects of diurnal variations.

4.2.1. Suppression of Paw Edema

Following the intraperitoneal administration of the test drug or the vehicle, 0.1 ml of a 1% (w/v) suspension of carrageenan (made up in pyrogen-free water) was injected into the subplantar area of one paw; paw volumes were measured before the administration of the drug and 3 h following the injection of carrageenan by immersing the paw up to the level of lateral maleus in a mercury column connected to a Statham pressure transducer. The changes in the volume were recorded as pressure changes on a Grass polygraph. In preliminary experiments it was found that the maximum effect of carrageenan was manifested at 3 h and there was little variation in the volumes of the two paws of the same animal.

The inhibition of edema by the drug was calculated according to the formula [(ΔCPV-ΔTPV)/ΔCPV] x 100, where Δ CPV was the mean change in the paw volume of vehicle-treated rats and ΔTPV was the change in the paw volume of drug-treated individual rats (Winter et al., 1962).

4.2.2. Suppression of Pleurisy

Thirty minutes following the oral administration of aspirin or the vehicle, 0.2 ml of a 1% (w/v) suspension of carrageenan in pyrogen-free water was injected into the right pleural cavity of rats under ether anesthesia. Pleural fluid was harvested from exsanguinated rats 4 h following the injection of carrageenan. Preliminary experiments showed the volume of pleural exudate reached a plateau 3 to 5 h after the injection of carrageenan.

In order to collect the pleural exudate a longitudinal incision was made on each side of the mediastinum under ether anesthesia. The fluid was aspirated from the right and left thoracic cavities using a tuberculin syringe attached to a 16-gauge intubation needle. The exudate was transferred to a 15 ml conical centrifuge tube and the total volume measured.

The inhibition of pleurisy caused by a drug was calculated according the the formula [(CPV-TPV)/CPV] x 100 where CPV was the mean volume of the pleural exudate from vehicle-treated rats and TPV was the volume of the pleural exudate from drug-tested individual rats. Ten control or protein-deficient rats were used at each dose level.

4.3. Pharmacokinetic Studies

4.3.1. Plasma half-life ($t_{½}$), apparent volume of distribution (V_d), and Plasma Clearance (Cl_p)

Sodium salicylate was slowly administered into the tail vein at 2-, 10-, 100- and 200- mg/kg dose levels (volume of the injection was 2 ml/kg). Blood samples (100-200 µl) were collected from the tail artery (Varma, 1979) under ether anesthesia at 1, 2, 4, 6, 9, 12 and 24 h. Occasionally it was not possible to collect blood from the tail artery in which case it was collected by means of cardiac punctures.

For the calculations of kinetic parameters, plasma concentrations of salicylate (in salicylic acid equivalents) from individual rats were converted into natural log units and plotted against time according to the method of least squares; the correlation coefficients of these plots were greater than 0.95. The slope of the regression line yielded the elimination rate constant which equalled $0.693/t_{½}$. Preliminary studies showed that the distribution phase lasted for less than 30 minutes. It was found that from 1 to 24 h plasma salicylate declined monoexponentially according to a one-compartment open model. The 24 h sample yielded too low a value of salicylic acid at 2 and 10 mg/kg dose levels of salicylate and hence was not used for the computation of the pharmacokinetic parameters. However, the 24 h value was used in the case of the high dose of sodium salicylate (100 mg/kg). The apparent volume of distribution (V_d) was calculated by dividing the total dose administered by C_o, where C_o was the concentration of salicylic acid at time-zero and was obtained by extrapolating the regression line to zero time. Clearance (Cl_p) was calculated by multiplying the V_d by the elimination rate constant (Greenblatt and Koch-Weser, 1975a,b). Five to eight control and protein-deficient rats were used at each dose level of sodium salicylate.

4.3.2. Bioavailability

In order to determine the relative bioavailability, 10 mg/kg sodium salicylate was administered orally and intravenously to separate groups of control and protein-deficient rats. A crossover study was not attempted because it would have introduced variability as far as the duration on the specific diet and body weights were concerned. Blood samples were collected as stated above. The values of plasma salicylate at 24 h were below the level of detection at the 10 mg/kg dose level; hence the area under the plasma salicylate concentration curve (AUC) was computed by the trapezoidal method on the basis of values from 0 to 12 h. The bioavailability was calculated by dividing the AUC following the oral dose in each animal by the mean AUC following the intravenous dose in the group. Five to eight control or protein-deficient rats were used for each group.

4.3.3. Tissue Distribution

One or 3 h after an iv injection of salicylate, animals were decapitated and exsanguinated. Blood samples were collected; liver, kidneys, stomach, and brain were quickly excised, washed with ice-cold saline, blotted and weighed. Tissues were homogenized in 4 volumes of saline by means of a motor driven Potter-Elvehjem homogenizer. Salicylates were extracted and assayed as described later.

4.3.4. Kinetics of the Urinary Excretion

In order to determine the rate of the urinary excretion of salicylic acid and its metabolites, salicylate was injected intravenously and rats were placed individually in metabolic cages; 0-3, 3-6, 6-12 and 12-24 h urine samples were collected for the extraction and assay of salicylic acid and metabolites. Aliquots of each urine sample were incubated with β-glucuronidase (500 units/ml of urine) at 37°C and pH 5 for 15 h; the increase in the urinary salicylic acid

after incubation was treated to represent the concentration of salicyl glucuronides. Each group contained 6 animals.

The elimination rate constant (K_{el}) and elimination half-life of salicylic acid were derived from the slope of the plots of the logarithm of the rate of excretion of salicylic acid against time (midpoint of urine collection intervals) (Cummings et al., 1967). Because the drug was absorbed instantaneously following an iv injection, the entire plot was linear, and similar to the terminal portion of the curve following oral administration of drugs. The rate constant of the urinary excretion (K_{ex}) of salicylic acid and the rate constant of metabolite formation (K_{mf}) were calculated according to equations

$$K_{ex} = K_{el}\ D_{u\infty}\ /D_o$$

and

$$K_{mf} = K_{el}\ M_{u\infty}\ /D_o$$

where $D_{u\infty}$ and $M_{u\infty}$ were, respectively, the amount of unchanged drug and metabolite excreted at infinite time (in the present studies, 24 h by extrapolation), and D_o was the amount of drug administered. K_{ex} values for metabolites were derived by the "rate vs amount" method of Martin (1967) from the plot of $\Delta M_u/\Delta t$ (in mol/h) against the metabolite in the body (MB); MB was derived from the equation (Martin, 1967):

$$MB = rD_u - M_u,$$

where r was calculated from the experimental data according to the equation (Martin, 1967):

$$r = M_{u\infty}\ /D_{u\infty}$$

k_{ex} and K_{mf} of salicyluric acid in control rats could not be calculated because the plot of the rate of excretion of salicyluric acid against time in the case of these animals was not linear.

4.3.5. Serum Protein-Salicylate Binding

The amount of salicylate bound to serum proteins was determined by the ultrafiltration procedure (Farese et al., 1970) by means of Amicon Centriflow membrane cones (CF50A). In order to estimate the binding of salicylate to serum proteins under in vivo condition, 3 ml blood was collected into nonheparinized syringes by means of cardiac punctures 1 h following 3 different intravenous doses of salicylate. Blood was allowed to clot and serum was separated by centrifuging the sample at 2,000 rpm. For in vitro protein-salicylate binding studies, sodium salicylate was added to 2 ml aliquots of serum to give final concentrations of 0.18, 0.36 and 0.72 mM. The mixture was allowed to equilibrate for 1 h at 37°C. Aliquots of serum were transferred to the cone and centrifuged at 2400 rpm for 15 min in a Sorvall centrifuge (RC 2-B) at room temperature. In preliminary experiments it was found that the total amount of salicylate which was retained by the cones was negligible and the concentration of proteins in the ultrafiltrate was less than 0.06 mg/ml. The bound fraction was assumed to be the difference between the total serum concentration of salicylate and its concentration in the ultrafiltrate. The percentage binding (B) of salicylate to serum proteins was calculated according to the following formula:

$$B = \frac{C_T - C_F}{C_T} \times 100\%$$

where C_T = total drug concentration in the serum; C_F = drug concentration in the ultrafiltrate.

4.3.6. Mitochondrial Metabolism of Salicylate

Crude mitochondrial preparations of kidney and liver were made as described by Forman et al. (1971). Following decapitation of the animals, kidneys and liver were quickly removed and placed on chilled petri dishes. Subsequent operations were performed at 0-4°C. One gram of tissue was homogenized with 4 ml of ice-cold 0.13 M KCl in a Potter-Elvehjem

Teflon/glass homogenizer (1000 rpm, 10 up-down strokes). The cell wall debris and nuclei were removed by centrifugation at 500 x g for 10 min. The supernatant was centrifuged for 30 min at 28,000 x g. The pellet was suspended in 0.62 ml of 0.13 M KCl/4 mM 2-mercaptoethanol /1 mM EDTA adjusted to pH 7.8 with NH_4OH, and frozen at -30°C overnight. The preparation was thawed the following morning and 0.07 ml of glycerol was added to achieve a final concentration of 10% (v/v); the suspension was centrifuged at 75000 x g for 60 min at 4°C in a Beckman centrifuge (L2-65B). The supernatant was decanted into glass tubes to which were added 100 µmol of Tris-HCl buffer (pH 8), 5µ mol of disodium ATP, 5 µmol of $MgCl_2$, 0.5 µmol of CoA, 0.5 µmol of sodium salicylate, and 100 µ mol of neutralized glycine. The mixture (1 ml) was incubated at 38°C for 10 min; the reaction was terminated with 0.1 ml of 4 M $HClO_4$. Following centrifugation, aliquots of the supernatant fluid (0.2 ml) were assayed for salicylic acid and salicyluric acid. One gram of tissue yielded 6-8 mg of mitochondrial proteins.

4.3.7. Extraction and Assay of Salicylates

Salicylic acid and metabolites from plasma, urine (diluted 1:5) or tissue homogenates, generally 50-100 µl, were extracted in benzene/ethyl acetate (1:1, v/v) after acidification of the samples with one drop of 85% H_3PO_4 (Peng et al., 1978). The solvent was evaporated at 4°C under a stream of N_2 and the residue was dissolved in the mobile phase (30% acetonitrile in 0.05% H_3PO_4, v/v). Salicylic acid and metabolites were assayed by HPLC (Altex) as described by Peng et al. (1978). The chromatographic conditions were as follows: 3.9 x 100-mm reverse-phase column (µBondapak-C18, Waters Associates, Milford, Mass.); a mobile phase of 30% acetonitrile in 0.05% H_3PO_4 (pH 2.5) at a flow rate of 1 ml/min at room temperature. The absorbance was measured at 237 nm in a Hitachi Model 100-40 spectrophotometer which was interfaced with an integrator-plotter (Hewlett-Packard Model 3380A). The quantities of salicylic

acid and its metabolites were determined by comparing their peak areas with that of the internal standard (phthalic acid). However, blank urine gave a peak at approximately the same retention time as the internal standard (Figure 8); hence the quantitation of urinary salicylates was made on the basis of standard curves for authentic compounds. The separation of different metabolites was satisfactory and there existed a linear relationship (r=0.99) between the concentration of salicylic acid, gentisic acid and salicyluric acid (6-50 µg/ml) and their peak areas (Figure 9). The retention times of different substances were as follows: phthalic acid, 4.2 min; gentisic acid, 4.8 min; salicyluric acid, 5.6 min; salicylic acid, 9.2 min.

4.4. Lysosome Stability Studies

4.4.1. Measurement of Lysosome Stability

Stabilization or labilization of liver lysosomes was determined by a modification of the methods of Deter and de Dure (1967) and Ignarro (1972). Animals were decapitated and exsanguinated 3 h after the intraperitoneal injection of the drug or the vehicle. The large lobe of liver was excised quickly, rinsed and minced in ice-cold 0.25 M sucrose-0.05 M Tris acetate (pH 7.4). Two portions of the liver each weighing 0.7 g were processed. One portion was homogenized in 6.3 ml of ice-cold 0.07 M sucrose-0.05 M Tris acetate (pH 7.4) in a Potter-Elvehjem glass homogenizer in an ice-bath. The homogenization was done by executing exactly 30 up-down strokes with a Teflon pestle driven at 600 rpm by a motor. The second portion of the liver was homogenized in 6.3 ml of 0.1% (v/v) Triton X-100-0.05 M Tris acetate (pH 7.4) by executing exactly 60 up-down strokes with the pestle driven at 2000 rpm. In preliminary experiments, a manually operated Dounce homogenizer as recommended by Ignarro (1972) was used, but was found too time consuming. A motor driven Potter-Elvehjem homogenizer was tried at different speeds and up-down strokes; 30 up-down strokes at 600 rpm and 60 up-down strokes at 2000 rpm were found satisfactory for the determination of the free and total

enzyme activity, respectively, (see results) and yielded results comparable to those reported by Ignarro (1972). The homogenates were kept at 0-4°C for 1 h and then centrifuged at 27,000 x g for 20 min at 4°C. The enzyme activity in the supernatant derived from the homogenate in hypotonic sucrose buffer represented free enzyme activity and that derived from the homogenate in Triton X-100 represented the total enzyme activity. In most experiments, lysosomal membrane stability was determined by measuring total and free β-glucuronidase activity, a marker enzyme frequently used for this purpose (Ignarro, 1971a,b; Platt and Gross-Fengels, 1979; Symons et al., 1969; Welman, 1979). Some studies were also done with arylsulfatase as the marker enzyme along with β-glucuronidase activity. The influence of dietary protein deficiency on total acid phosphatase activity was also measured; however, no attempt was made to use this as a marker enzyme to ascertain lysosomal membrane integrity since different marker enzymes are reported to yield comparable values (Ignarro, 1971a,b, 1972).

The effect of drugs on lysosomal membrane stability was determined by measuring β-glucuronidase as the marker enzyme. Results were expressed as changes in the ratios of free to total enzyme activity as well as according to the following formula:

$$[(R_c - R_t)/R_c] \times 100$$

where R_c was the mean of the ratios of free to total β-glucuronidase activity of all the vehicle-treated animals and R_t was the ratio of the free to total enzyme activity of drug-treated individual animals, so that the greater the R_c the greater was the membrane fragility and the smaller the R_t the greater was the lysosome membrane stabilizing effect of the drug.

4.4.2 Enzyme Assays

The activity of β-glucuronidase (EC 3.2.2.31, β-D-glucuronide-glucuronohydrolase) was assayed by measuring the formation of phenolphthalein from phenolphthalein glucuronide as described by Ignarro (1971b). To glass test tubes containing 1 ml of 0.3M citrate buffer (pH 4.8) were added 1 ml of freshly prepared substrate (1.5 mg of phenolphthalein glucuronide), 0.9 ml of distilled water and 0.1 ml of 27,000 x g supernatant; after incubation for 25 min at 37°C, the reaction was stopped by the addition of 0.5 ml of 2.2 M glycine-10 N sodium hydroxide (pH 12). Phenolphthalein was measured on a Beckman Acta III spectrophotometer at 540 nm; the enzyme activity of the sample was expressed as units where 1 U was defined as the activity of the standard enzyme releasing 3.14 nmol (1 μg) of phenolphthalein glucuronide per hour at 37°C and pH 5.

The activity of arylsulfatase (EC 3.1.6.1, arylsulfate sulphohydrolase) was assayed as described by Roy (1960) using dipotassium 2-hydroxy-5-nitrophenyl sulfate as the substrate. The 27,000 x g supernatant (0.1 ml) and 0.2 ml of distilled water were added to 0.6 ml of 0.125 M acetate buffer (pH 5) containing 0.93 mg of the substrate. The mixture was incubated at 37°C for 1 h, at which time 3 ml of 0.1 M trichloroacetic acid was added to precipitate the proteins. After centrifugation, 1.5 ml of the supernatant was pipetted into 2.5 ml of 2.5 N NaOH and the absorbance of the resulting solution was measured at 515 nm on a Beckman Acta III spectrophotometer. The enzyme activity of the sample was expressed as units where 1 U was defined as the activity of the standard enzyme releasing 1 μmol of p-nitrocatechol from the substrate per hour at 37°C and pH 5.

Acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) was assayed by a modification of the method of Ignarro

(1971b). To 1 ml of 0.3 M citrate buffer (pH 4.8) was added 0.1 ml of 27,000 x g supernatant and 0.9 ml of distilled water. After incubation of this mixture for 10 min, 1 ml of freshly prepared substrate (7.5 mg of sodium p-nitrophenylphosphate) was added and the incubation continued for another 10 min. The reaction was stopped by the addition of 0.2 ml of 4 N sodium hydroxide and the formation of p-nitrophenol was measured at 405 nm on a Beckman Acta III spectrophotometer. The enzymatic activity of the sample was expressed in terms of units; 1 U was defined as the activity of the standard enzyme releasing 1 µmol of p-nitrophenol per min at 37°C and pH 4.8.

4.4.3. Measurement of Plasma Concentrations of Oxyphenbutazone and Dexamethasone

Oxyphenbutazone was extracted in cyclohexane-ether (1:1) from 0.1 ml of plasma. Following the evaporation of cyclohexane-ether, the residue was redissolved in the solvent (0.002% acetic acid and 23% tetrahydrofuran in n-hexane) which was used as the mobile phase for subsequent analysis by high-pressure liquid chromatography (Altex) as described by Pound and Sears (1975). The internal standard was the 2,4 dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde.

Dexamethasone was measured by radioimmunoassay. Highly specific antisera against dexamethasone-3- carboxymethyloxime- bovine serum albumin (BSA) conjugate, which did not appreciably cross react (1%) with endogenous steroids or known dexamethasone metabolites was provided by Dr. S. Solomon, Endocrine Laboratory, Royal Victoria Hospital, McGill University. Radioimmunoassay was done by a modification of the procedure described by Hichens and Hogan (1974). Phosphate buffer containing 0.1% BSA (pH 7.4) was used to dissolve dexamethasone standards and tissue extracts as well as dilute

plasma samples (500- to 1,000-fold). To each assay tube was added 100 µl of standard solution, diluted plasma sample or tissue extracts, 100 µl of ^3H dexamethasone tracer (approximately 10,000 cpm) in phosphate buffer and 100 µl of antisera (1:2,500 dilution). The assay tubes were incubated for 2 hr at 4°C. In order to separate the bound and free steroids, dextran-coated charcoal 0.7 ml of a 1.25% (w/v) suspension was added and after a 10-min incubation the tubes were recentrifuged for 15 min at 4°C and 2,500 rpm. The supernatant was decanted into vials containing 10 ml of Scintiverse (Fisher) and counted in an Intertechnique scintillation counter. The standard curve was plotted on a Hewlett-Packard 9831A desktop computer according to a weighted Rodbard model program.

4.5 Metabolism of Arachidonic Acid

4.5.1. Preparation of Suspensions of Rat Pleural Neutrophils

Carrageenan (2 mg in 0.2 ml of pyrogen-free water) was injected into the right pleural cavity of control and protein-deficient rats under ether anesthesia. After 4 hours the rats were decapitated and the pleural fluid was harvested as described above. Neutrophils were isolated from the pleural exudates by centrifugation at 200 x g for 10 min at 4°C. The pellet was resuspended in 17 mM Tris-HCl buffer, pH 7.2, containing 0.75% NH_4Cl to lyse contaminating erythrocytes (Boyle, 1968), followed by centrifugation at 200 x g for 5 min. The neutrophils were washed once with Dulbecco's phosphate-buffered saline, pH 7.4, and resuspended in the same buffer at a concentration of 30 x 10^6 cells/ml. Observations on smears (Giemsa stain) showed that neutrophils accounted for more than 95% of the total leukocyte content of the purified preparation. The contaminants were monocytes (2-3%) and lymphocytes (1-2%). Eosinophils and basophils were less than 1%. Neither red cells nor platelets were detected. The viability of the cells as determined by trypan blue exclusion (Boyse et al., 1964) was greater than 98%.

4.5.2. Incubation and Extraction Procedures

Neutrophils (3×10^7 cells in 1 ml) were incubated with A23187 (final concentration, 20 μM), [1-^{14}C] arachidonic acid (200,000 cpm) and, in some cases, unlabelled arachidonic acid for 5 min at 37°C. After termination of the incubations with 2 volumes of ethanol, PGB_2 500 ng and, in some cases [3.3.4.4- ^{2}H] PGE_2, [3,3,4,4- ^{2}H] $PGF_{2\alpha}$ and [5,6,8,9,11,12,14,15- ^{2}H] TXB_2 (200 ng) were added as internal standards. Water was added to give a final concentration of ca. 5% ethanol and the mixtures were centrifuged for 10 min at 5,000 x g. The supernatant was acidified to pH 3 with 1N HCl and passed through a cartridge of octadecylsilylsilica (SEP-PAK C_{18} cartridge, Waters Associates, Milford, MA). The cartridge was eluted successively with 5% aqueous ethanol (20 ml), water (20 ml), petroleum ether (20 ml) and methyl formate (10 ml) (Powell, 1982). The methyl formate fraction was evaporated to dryness under a stream of nitrogen and the residue was dissolved in methanol (40 μl).

4.5.3. Separation of Prostaglandins (PGs) and Leukotrienes (LTs)

Samples were analyzed by reversed-phase HPLC on a C_{18} μBondapak column (0.39 x 30 cm, Waters Associates) using a linear gradient over 70 min between water/methanol/acetic acid (40:60:0.01, v/v) and water/methanol/acetic acid (20:80:0.01, v/v). The flow rate was 1.5 ml/min. A variable wavelength ultraviolet detector was used to monitor absorbance due to LTB_4 at 280 nm and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) at 232 nm. A Berthold radioactivity monitor was used to detect the fractions containing TXB_2, PGE_2 and $PGF_{2\alpha}$ which were not separated under these conditions (Figure 20). The fraction containing prostaglandins and TXB_2 was concentrated to dryness and rechromatographed on a column (0.46 x 30 cm) of 5 μm silica (Partisil) purchased from Alltech Associates, using a linear gradient of 20%-80%

toluene/ethyl acetate/acetonitrile/ methanol/ acetic acid (30:40:30:2:0.5, v/v) in hexane/ toluene/acetic acid (50:50:0:5, v/v) over a period of 60 min. The flow rate was 2 ml/min.

4.5.4. Biosynthesis of Prostaglandins by Rat Kidney Medulla

Rat kidney medullae were homogenized in 0.05 M Tris-HCl, pH 7.5 (9 volumes/g tissue) using a Potter-Elvehjem homogenizer. The homogenates (1 ml) were incubated for 10 min at 37°C with [1-^{14}C] arachidonic acid (0.2 μCi, 37 nmol). Incubations were terminated by the addition of ethanol (2 volumes) and [3,3,4,4-^{2}H] prostaglandin E_2 (200 ng) and [3,3,4,4-^{2}H] prostaglandin $F_{2\alpha}$ (200 ng) were added. The mixtures were extracted using octadecylsilyl silica as described above and prostaglandins E_2 and $F_{2\alpha}$ were purified by thin-layer chromatography on silicic acid with diethyl ether: methanol:acetic acid (100:6:1) as the mobile phase.

4.5.5. Biosynthesis of Cyclooxygenase and lipoxygenase-derived Products by Rat Spleen

Rat spleen was homogenized in 0.05 M Tris-HCl, containing 0.02M EDTA-2Na, pH 7.5 (1 g of tissue + 9 volume of buffer) in a Potter-Elvehjem homogenizer. The homogenates (1 ml) were incubated with [1-^{14}C] arachidonic acid (0.2 μCi) and in some cases unlabelled arachidonic acid for 10 min at 37°C. Incubations were terminated by the addition of ethanol (2 volumes) and a small amount (4×10^5 cpm) of [9β-^{3}H] $PGF_{2\alpha}$ (in order to monitor recovery) was added. The mixtures were extracted using octadecylsilyl silica as described above and the products separated by HPLC on a silica column with 4% Benzene/ethyl acetate/acetonitrile/methanol/acetic acid (30:40:30:2:0.5, v/v) for the first 10 min, followed by a linear gradient over 40 min between 4% and 100% benzene/ethyl acetate/acetonitrile/ methanol/acetic acid (30:40:30:2:0.5, v/v) in hexane/benzene/acetic acid (50:50:0.5, v/v). A Berthold radioactivity

detector was used and the fractions were collected and the radioacfivity determined in a Packard liquid scintillation counter (Tri-Carb Model).

4.5:6. Quantitation of Prostaglandins and Leukotrienes

LTB_4, 5-HETE and HHT were quantitated on the basis of ultraviolet absorbance by comparing the areas of their peaks with that of the internal standard, PGB_2. The extinction coefficients used for PGB_2, LTB_4 and 5-HETE were 26,800, 39,500 and 30,500, respectively (Borgeat and Samuelsson, 1979a).

TXB_2, PGE_2, $PGF_{2\alpha}$ and 6-oxo$PGF_{1\alpha}$ were quantitated by gas chromatography-mass spectrometry using selected ion monitoring on a LKB-9000 instrument (Green et al., 1978). Prior to analysis, TXB_2 and $PGF_{2\alpha}$ were converted to their methyl ester (diazomethane, room temperature, 15 min), trimethylsilyl ether (N-methyl-N-trimethyl- silyltrifluoroacetamide, room temperature, 30 min) derivatives. PGE_2 and 6-oxo$PGF_{1\alpha}$ were converted to their methyl ester, trimethylsilyl ether, 0-methyloxime (methoxylamine hydrochloride (1 mg) in pyridine (0.1 ml), room temperature, 16 h) derivatives. The ions monitored were m/z 256 and 260 (TXB_2 and its octadeuterated analog); 508 and 512 (PGE_2 and its tetradeuterated analog); 423 and 427 ($PGF_{2\alpha}$ and its tetradeuterated analog) and 598 and 602 (6-oxo$PGF_{1\alpha}$ and its tetradeuterated analog) (Figure 23).

4.6. Other Biochemical Determinations

Total plasma proteins, albumin, globulins, mitochondrial proteins as well as some tissue proteins were determined by the method of Lowry et al. (1951) as modified by Miller (1959). Plasma globulins were separated by precipitation with HCl-ethanol, and albumin was separated by precipitation with 0.2M sodium acetate (Fernandez et al., 1966). Bovine serum albumin was used as a standard for the assay of total proteins and albumin; bovine serum γ-globulins were used as standard for the assay of globulins. DNA content was measured by the

diphenylamine method (Burton, 1956) with bovine thymus DNA as the standard.

4.7. Chemicals and Drugs

Chemical/drug	Source
A23187	Calbiochem-Behring Corp. (La Jolla, CA.)
Acetonitrile	Fisher Scientific Co. (Montreal, Que.)
Acetylsalicylic acid (Aspirin)	Sigma Chemical Co. (St. Louis, MO.)
Acid phosphatase (0.68 U/mg, type II)	Sigma Chemical Co.
Adenosine 5-triphosphate disodium salt	Sigma Chemical Co.
Arachidonic acid	Nu Chek Prep Inc. (Elysian, MN.)
[1-^{14}C] Arachidonic acid (55 Ci/mol)	New England Nuclear (Boston, MA.)
Arylsulfatase (45U/mg, type VII)	Sigma Chemical Co.
Benzene	Fisher, (Montreal, Que.)
Bovine serum albumin	Sigma Chemical Co.
Bovine serum γ-globulins	Sigma Chemical Co.
Coenzyme A sodium salt	Sigma Chemical Co.
λ-Carrageenan	Sigma Chemical Co.
Dexamethasone phosphate	Merck Frosst Lab. (Montreal, Que.)
[6,7-^{3}H] Dexamethasone (40.1 Ci/mmol)	New England Nuclear (Boston, MA.)
Ethyl acetate	Fisher (Montreal, Que.)
Gentisic acid (2,5-dihydroxybenzoic acid)	Eastman Chemicals (Rochester, NY.)
β-Glucuronidase (Glucurase, 5000 U/ml, bovine liver)	Sigma Chemical Co.
Indomethacin	Sigma Chemical Co.
Methyl formate	Fisher Scientific (Montreal, Que.)
p-nitrocatechol sulfate dipotassium salt	Sigma Chemical Co.

p-nitrophenyl phosphate ditris salt	Sigma Chemical Co.
Oxyphenbutazone	Ciba-Geigy Corp. (Summit, NJ.)
Phenolphthalein mono-β-glucuronic acid sodium salt	Sigma Chemical Co.
Phthalic acid	BDH (Montreal, Que.)
Prostaglandin E_2 (PGE_2)	Upjohn Co. (Kalamazoo, MI.)
[3,3,4,4-^{2}H]PGE_2	Upjohn Co.
Prostaglandin $F_{2\alpha}$	Upjohn Co.
[3,3,4,4-^{2}H]$PGF_{2\alpha}$	Upjohn Co.
Salicylic acid	BDH (Montreal, Que.)
Salicyluric acid (o-hydroxyhippuric acid)	Sigma Chemical Co.
Sodium salicylate	BDH (Montreal, Que.)

PGB_2 was prepared by treatment of PGE_2 with 0.05N NaOH for 30 min. [9β-^{3}H] -$PGF_{2\alpha}$ was synthesized from PGE_2 with sodium [^{3}H] borohydride. [5,6,8,9,11,12,14,15-^{2}H]- TXB_2 was prepared biosynthetically from [5,6,8,9,11,12,14,15-^{2}H]-arachidonic acid as described by Hamberg and Samuelsson et al. (1974).

Only high purity grade chemicals were used for the chromatography. Unlabeled arachidonic acid and [1-^{14}C] arachidonic acid were purified by normal phase HPLC just prior to use. Drugs were freshly prepared. Sodium salicylate was dissolved in distilled water; acetylsalicylic acid (aspirin), oxyphenbutazone and indomethacin were dissolved in a minimum volume of 0.1 N NaOH and diluted to the desired concentration with phosphate buffer (pH 7.4).

For the oral administration of drugs, animals were starved overnight and for 1 h after the administration of the drug; water was freely available.

4.8. Statistics

Statistical significance between two sample means was determined by Student's t-test for unpaired data. For comparing more than two sample means one-way analysis of variance followed by a multiple comparison according to Duncan's multiple range test extended by Kramer (Milton and Tsokos, 1983) was done. A probability of less than 0.05 was assumed to denote a significant difference. Unless stated otherwise, data are presented as mean±SE

5. RESULTS

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5.1. General Effects of Protein Deficiency

The most obvious and consistent effect of protein-calorie malnutrition (PCM) was a decrease in body weight gain (Table 1). The average daily food consumption of control animals was significantly greater than that of protein-deficient animals. However, the food consumption per day per kilogram body weight did not differ in the two groups of rats. A dietary protein deficiency led to a significant decrease in total plasma protein, albumin and globulins, hepatic total and microsomal proteins; however, hepatic DNA, blood leukocytes and urinary pH did not change. On the other hand, a restricted supply of the control diet (pair-fed controls, 10g/day/rat) caused a decrease in only body weight gain and liver weight but not in liver and plasma proteins. A small proportion of rats fed a low protein diet lost hair; there was no apparent decrease in their motor activity and there was no mortality.

5.2. Influences of Protein Deficiency on the Anti-inflammatory Effects of Salicylates

5.2.1. Suppression of Paw Edema

The increase in the paw edema volume 3 hours following an injection of carrageenan into the subplantar region was 59.7±1.8% in control rats (21% protein diet ad libitum, n=50), 55±3.2% in pair-fed controls (a restricted supply of 21% protein diet, n=11) and 61.4±2.3% in protein-deficient rats (5% protein diet ad libitum, n=54). The increase in the paw volume of control rats was not different from those of either protein-deficient rats or of pair-fed animals. Maximum edema was observed 3 h following the carrageenan injection. This period was selected for determining the effects of drugs. Both sodium salicylate and aspirin produced significantly greater inhibition of paw edema in protein-deficient than in control animals fed ad libitum or pair-fed rats (Table 2; Figures 5, 6). There was no difference in the paw edema suppressant

Table 1

Influence of dietary protein on certain physiological and biochemical parameters in male rats

Parameters	Dietary Protein (3-Week Period)		
	21% ad lib. (control)	5% ad lib. (PCM)	21%(10g/day) (pair-fed)
	Mean ± S.E. (n = 8-240)		
Initial body weight (g)	115 ± 2.0	114 ± 2.0	114 ± 1.9
Final body weight (g)	249 ± 5.3	117 ± 3.3[a,b]	211 ± 5.3[a]
Food consumed (g/day/rat)	18.2 ± 1.3	9.8 ± 1.1[a]	10 ± 0[a]
Protein intake (g/day/rat)	3.9 ± 0.4	0.5 ± 0.1[a,b]	2.1 ± 0[a]
Caloric intake (kcal/day/rat)	73.1 ± 6.7	39 ± 4.0[a]	40 ± 0[a]
Plasma proteins (g/dl)	7.4 ± 0.13	5.8 ± 0.13[a,b]	7.5 ± 0.27
Plasma albumin (g/dl)	2.6 ± 0.02	2.3 ± 0.09[a,b]	2.7 ± 0.08
Plasma globulins (g/dl)	3.6 ± 0.1	3.3 ± 0.1[a,b]	3.6 ± 0.06
Liver weight (g)	12.8 ± 0.41	5.9 ± 0.28[a,b]	9.7 ± 0.42[a]
Liver protein (mg/g)	206 ± 3.4	162 ± 7.3[a,b]	216 ± 8.6
Liver microsomal protein (mg/g)	14.8 ± 0.5	11.6 ± 7.3[a]	not done
DNA (mg/g liver)	3.4 ± 0.2	2.9 ± 0.2	not done
DNA (% liver protein)	1.5 ± 0.1	1.8 ± 0.1	not done
Blood leukocytes (10^6/ml)	17.3 ± 0.5	19.1 ± 1.1	not done
Urinary pH	6.4 ± 0.3	6.4 ± 0.2	not done

[a] Significantly different ($p < 0.05$) from the corresponding control (21% protein diet ad libitum group) values.

[b] Significantly different ($p < 0.05$) from the corresponding pair-fed control (21% protein diet 10g/day/rat group) values.

Table 2

Effect of salicylates on the carrageenan-induced paw edema in control, pair-fed and protein-deficient rats

Agents	Dose (mg/kg)	Route	Inhibition of paw edema (%)		
			21% protein ad lib.	5% protein ad lib.	21% protein 10 g/day
			Mean ± S.E. (n=9-16)		
Na Salicylate	50	i.p.	-4.0 ± 3.3*	28.0 ± 5.9	4.0 ± 2.8*
Na Salicylate	200	i.p.	42.8 ± 6.1*	63.0 ± 4.7	38.1 ± 7.4*
Aspirin	50	p.o.	16.2 ± 8.2	25.1 ± 9.8	not done
Aspirin	100	p.o.	22.7 ± 6.6*	49.6 ± 5.9	not done
Aspirin	200	p.o.	45.8 ± 5.9*	68.1 ± 5.7	not done

The increase in the paw volume of vehicle-treated control (n=50), pair-fed (n=11) and protein deficient (n=54) rats was 59.7 ± 1.8%, 55 ± 3.2% and 61.4 ± 2.3%, respectively.

*Significantly different ($p < 0.05$) from the corresponding values of protein-deficient rats (5% protein diet ad libitum group).

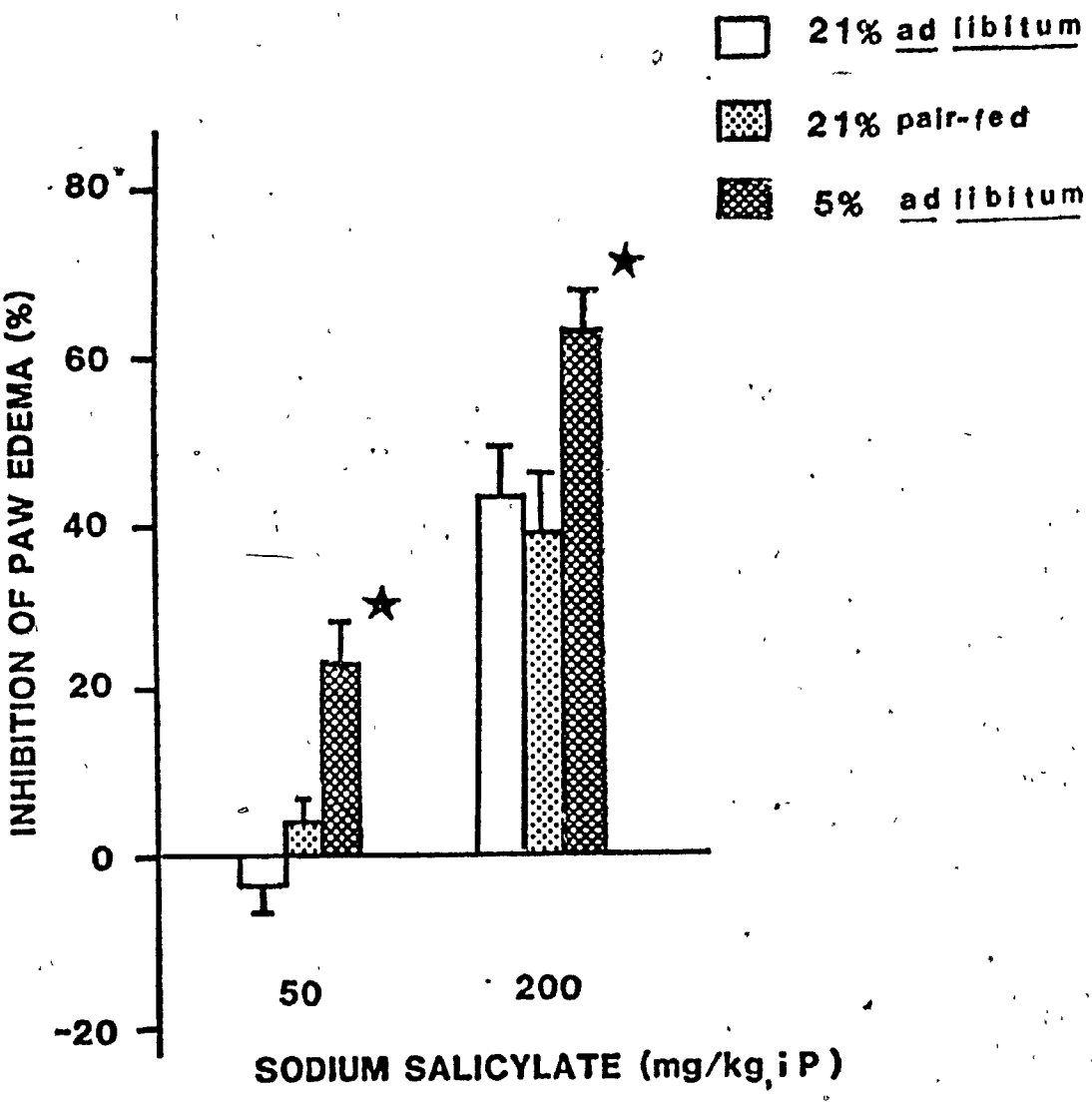


Figure 5. Inhibition of carrageenan-induced paw edema by sodium salicylate in male rats. Animals were fed ad libitum either a 21% (control) or 5% (protein-deficient) protein diet or a 21% protein diet was fed in restricted quantities (10 g/day/rat) (pair-fed control). Mean±S.E. of 9-16 animals at each dose level. Asterisks denote differences ($P<0.05$) from the values in both controls fed ad libitum and pair-fed controls.

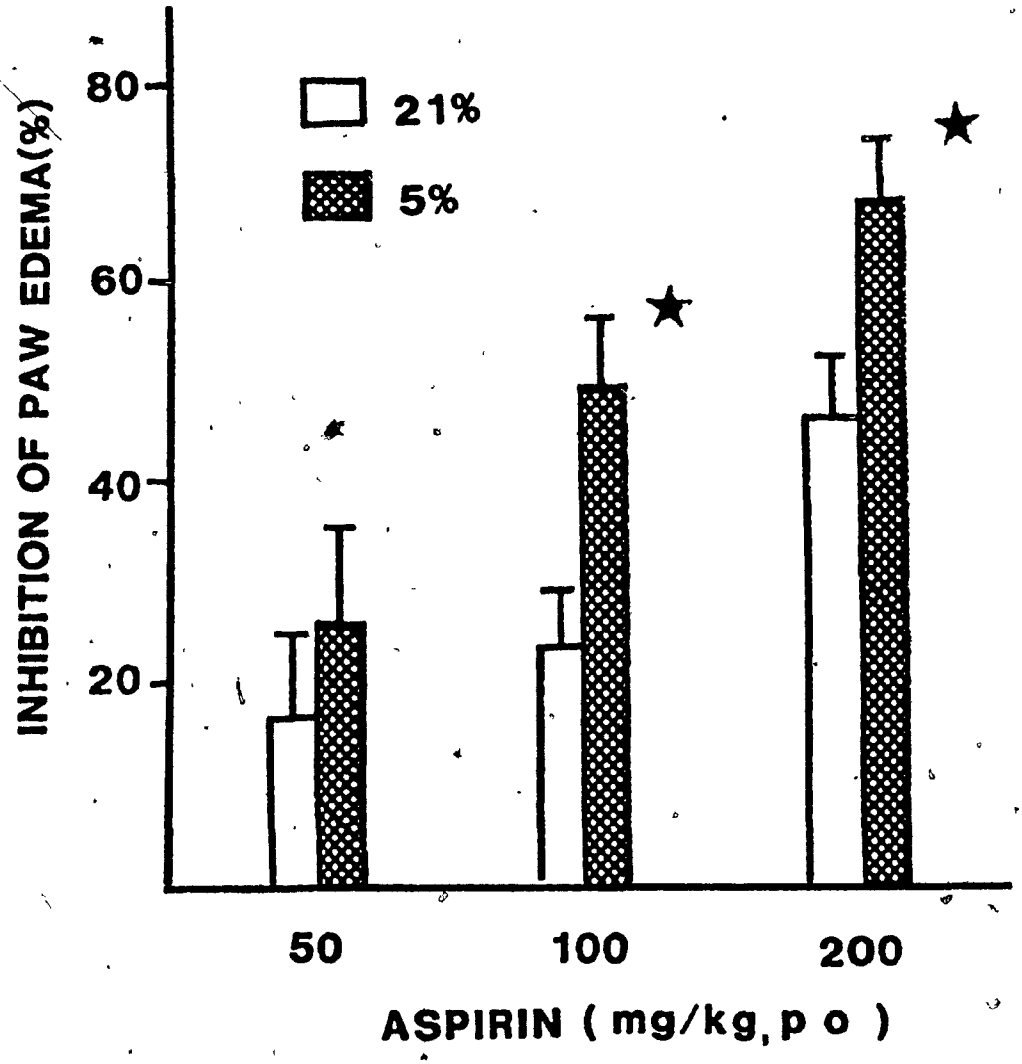


Figure 6. Inhibition of carrageenan-induced paw edema by aspirin in male rats. Animals were fed a 21% (control) or a 5% (protein-deficient) protein diet for 3 weeks. Each value is the mean±S.E. of 10 animals. Asterisks denote differences ($P<0.05$) from the adjacent control values.

activity of salicylate in control and pair-fed rats (Figure 5).

5.2.2. Suppression of Pleurisy

The effect of aspirin on carrageenan-induced pleurisy is shown in Figure 7 and Table 3. The inhibition of pleural exudate at three dose levels studied was significantly greater in animals with protein-calorie malnutrition than in control rats. The total volume of pleural exudate per rat was 1.5±0.1 ml in control animals (n=27), which was higher ($p<0.001$) than the volume of exudate (0.9±0.1 ml) in protein-deficient rats (n=27). However, the volume of pleural exudate on the basis of body weight was significantly greater ($p< 0.001$) in protein-deficient than in control rats; the values (ml/kg, mean±S.E.) for control and protein-deficient rats were 6.1±0.3 and 9.3±0.5, respectively. The leukocyte concentration in the pleural exudate of control rats (n=27) was 78±4.4 x 10^6/ml, and significantly higher ($p<0.01$) than the concentration (51±6.6 x 10^6/ml) in the exudate of protein-deficient rats (n=27); blood leukocyte counts in the two groups of rats did not differ significantly (Table 1).

5.3. Measurement of Salicylate and its Metabolites by HPLC

The HPLC method used in this study for the measurement of salicylic acid and its metabolites in biological tissues and fluids was sensitive and accurate. Salicylic acid, salicyluric acid and gentisic acid could simultaneously be assayed accurately at concentrations as low as 1 μg/ml (100 ng/0.1 ml plasma or extracts). Figure 8 shows typical chromatograms of salicylic acid and its metabolites in plasma, liver, kidney and urine. The retention times for salicylic acid, salicyluric acid, gentisic acid and phthalic acid (internal standard) were 9.2, 5.6, 4.8 and 4.2 minutes respectively. The chromatograms of plasma, urine and tissue blanks showed no peaks that could interfere with salicylic acid.

Table 3

Effect of aspirin on the carrageenan-induced pleurisy in control and protein-deficient rats

Oral dose (mg/kg)	Inhibition of pleural exudate (%)	
	21% protein ad lib.	5% protein ad lib.
	Mean ± S.E. (n=5-9)	
50	18.91 ± 8.10	49.98 ± 5.58*
100	35.98 ± 5.41	61.12 ± 7.86*
200	50.37 ± 6.68	75.38 ± 5.11*

The total volume of pleural exudate per rat was 1.5 ± 0.1 ml in vehicle-treated control (n=27) and 0.9 ± 0.1 ml in vehicle-treated protein-deficient rats (n=27).

* Significantly different ($p < 0.05$) from the corresponding control values.

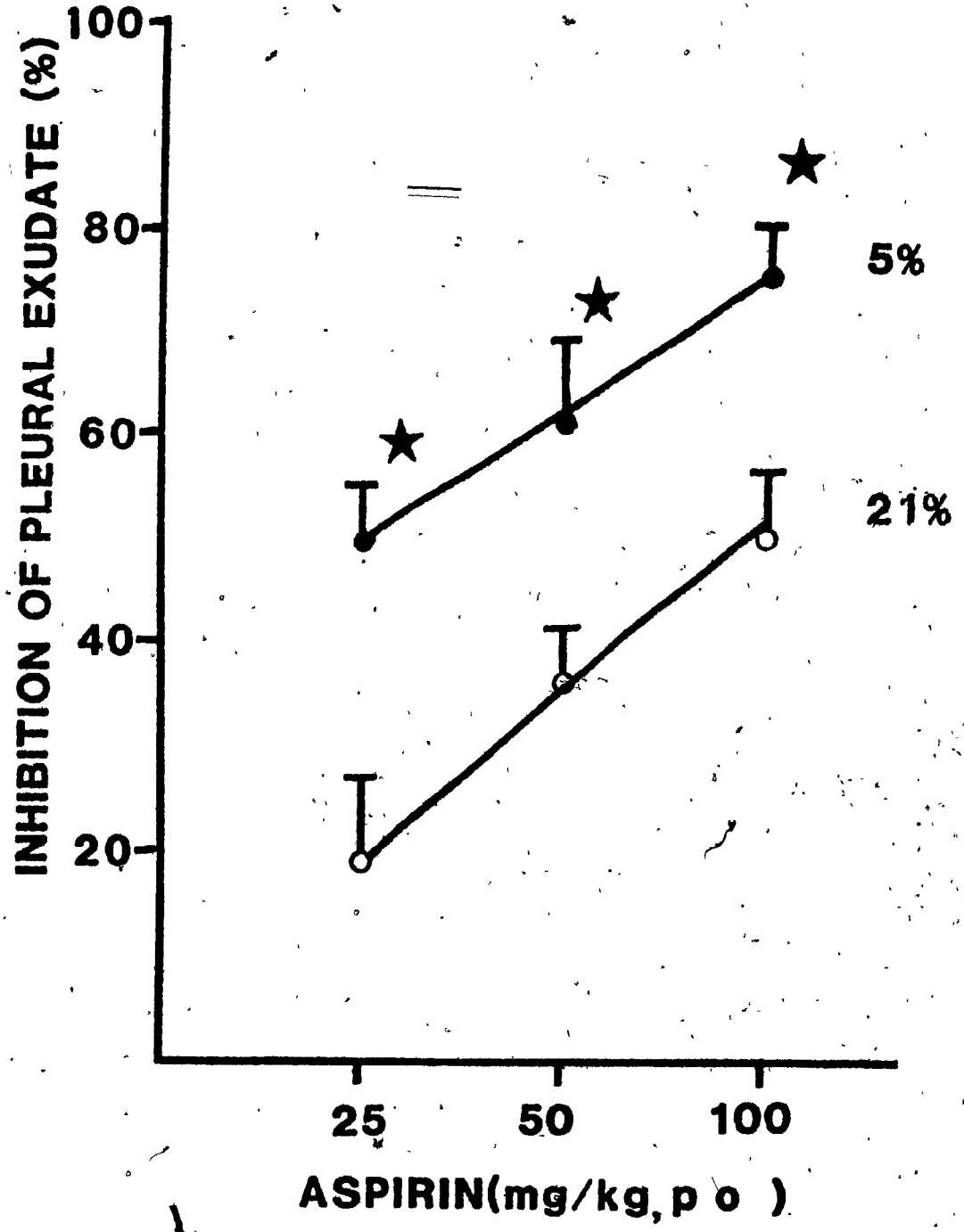


Figure 7. Inhibition of carrageenan-induced pleurisy by aspirin. Rats were fed ad libitum a 21% (control) or a 5% (protein-deficient) protein diet for 3 weeks. Each value is the mean±S.E. of 5-9 rats. Asterisks denote differences ($P<0.05$) from the control values.

Figure 8. High-pressure liquid chromatograms of salicylic acid and its metabolites in plasma, urine and tissue extracts. Salicylic acid and its metabolites were separated on a reversed-phase column (C_{18} μBondapak, Waters) with 30% acetonitrile in 0.05% H_3PO_4 at a flow rate of 1 ml/min. SA: salicylic acid; SU: salicyluric acid; GA: gentisic acid; IS: internal standard (phthalic acid).

a. spiked plasma; b. plasma sample; c. liver extract; d. kidney extract; e. blank urine; f. urine sample.

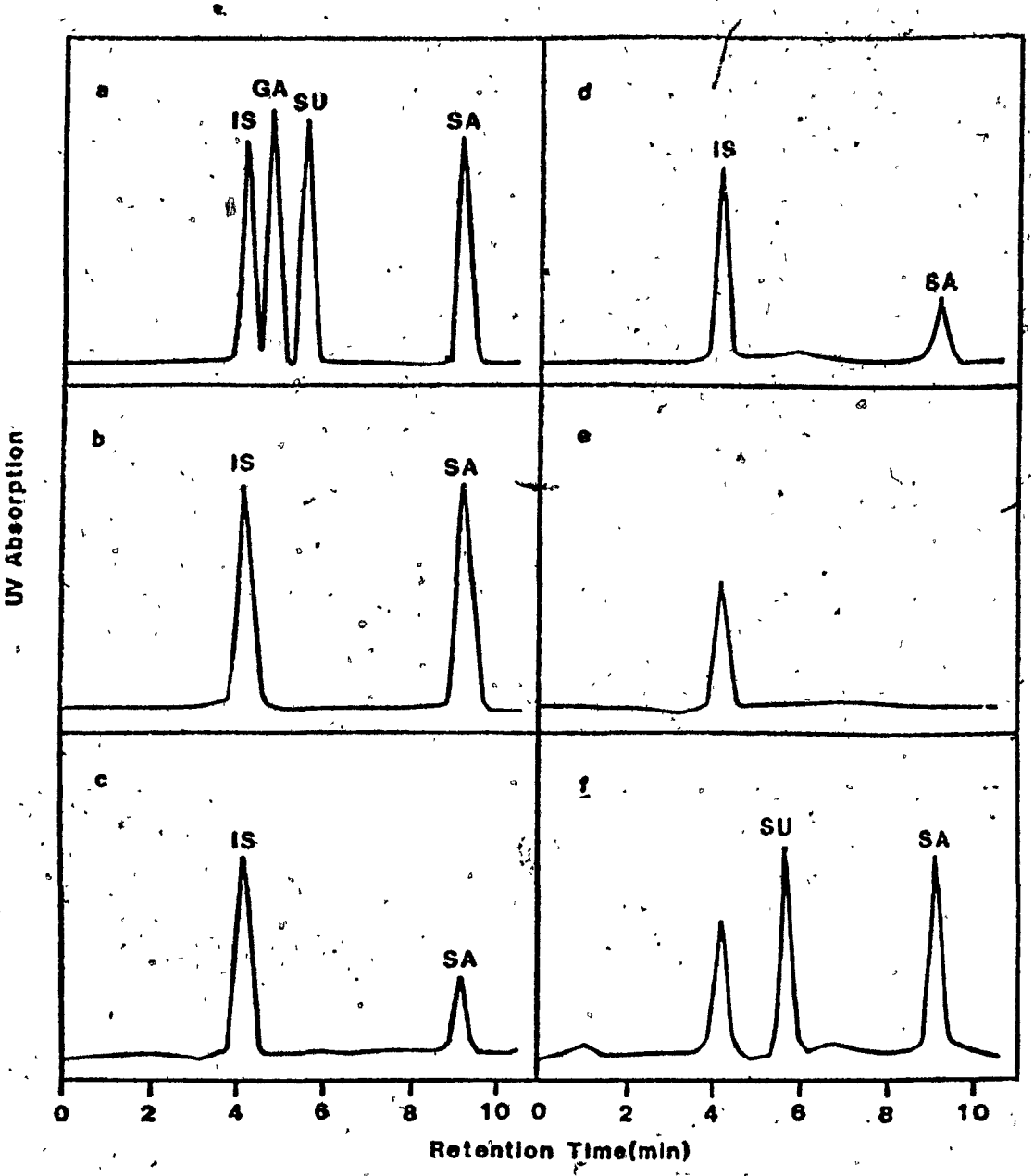

a
GA
SU
IS
SA
d
IS
SA
b
IS
SA
e
c
IS
SA
f
SU
SA
UV Absorption
0
2
4
6
8
10
0
2
4
6
8
10
Retention Time(min)

Table 4 illustrates the linearity of the calibration curves for salicylic acid and salicyluric acid in plasma in concentrations ranging from 6.25 to 50 µg/ml. The values of peak area ratio/weight ratio were fairly consistant for salicylic acid and salicyluric acid at all concentrations studied, indicating good linearity. The precision and reproducibility of the assay are summarized in Table 5. The coefficients of variation of these results were less than 4% at all concentrations studied.

Blank urine gave a peak at approximately the same retention time as the internal standard (Figure 8). Therefore the quantitation of urinary salicylates was made on the basis of standard curves to authentic compounds. The separation of different metabolites was satisfactory and there existed a linear relationship (r=0.99) between the concentration of salicylic acid, gentisic acid and salicyluric acid (6.25-50 μg/ml) and their peak areas (Figure 9).

Table 4

Linearity of calibration curves for salicylic acid (SA) and salicyluric acid (SU) by HPLC.

Internal standard (μg/ml)	Concentration of SA and SU (μg/ml)	Peak area ratio[a]		Peak area ratio/weight ratio[b]	
		SA[c]	SU[d]	SA	SU
		Mean ± S.E.			
25	6.25	0.53 ± 0.01	0.48 ± 0.01	2.08 ± 0.04	1.91 ± 0.02
25	12.5	1.06 ± 0.01	0.92 ± 0.01	2.13 ± 0.03	1.87 ± 0.01
25	25.0	2.03 ± 0.03	1.80 ± 0.02	2.03 ± 0.03	1.80 ± 0.02
25	50.0	3.96 ± 0.05	3.60 ± 0.04	1.98 ± 0.02	1.80 ± 0.01

[a] Area of drug/area of internal standard.

[b] Weight of drug/weight of internal standard.

[c] Linear regression line for salicylic acid: $Y=0.078x + 0.066$, $r=0.99$

[c] Linear regression line for salicyluric acid: $Y=0.069x + 0.059$, $r=0.99$

4-6 samples at each concentration.

Table 5

Assay precision and reproducibility of salicylic acid and salicyluric acid by HPLC

Compound	Concentration added to plasma (µg/ml)	Concentration found (µg/ml)		CV (%)
		Mean ± SD	Range	
Salicylic acid	6.25	6.15 ± 0.10	5.98 - 6.25	1.62
	12.5	12.20 ± 0.27	11.80 - 12.40	2.21
	25.0	24.88 ± 0.09	23.20 - 25.50	3.61
	50.0	49.50 ± 1.28	47.60 - 51.10	2.60
Salicyluric acid	6.25	6.24 ± 0.22	5.95 - 6.56	3.61
	12.5	11.98 ± 0.24	11.80 - 12.30	2.00
	25.0	24.15 ± 0.76	23.10 - 25.10	3.16
	50.0	49.60 ± 1.21	47.90 - 51.20	2.44

n=4

$$CV\ (\%) = \frac{SD}{mean} \times 100$$

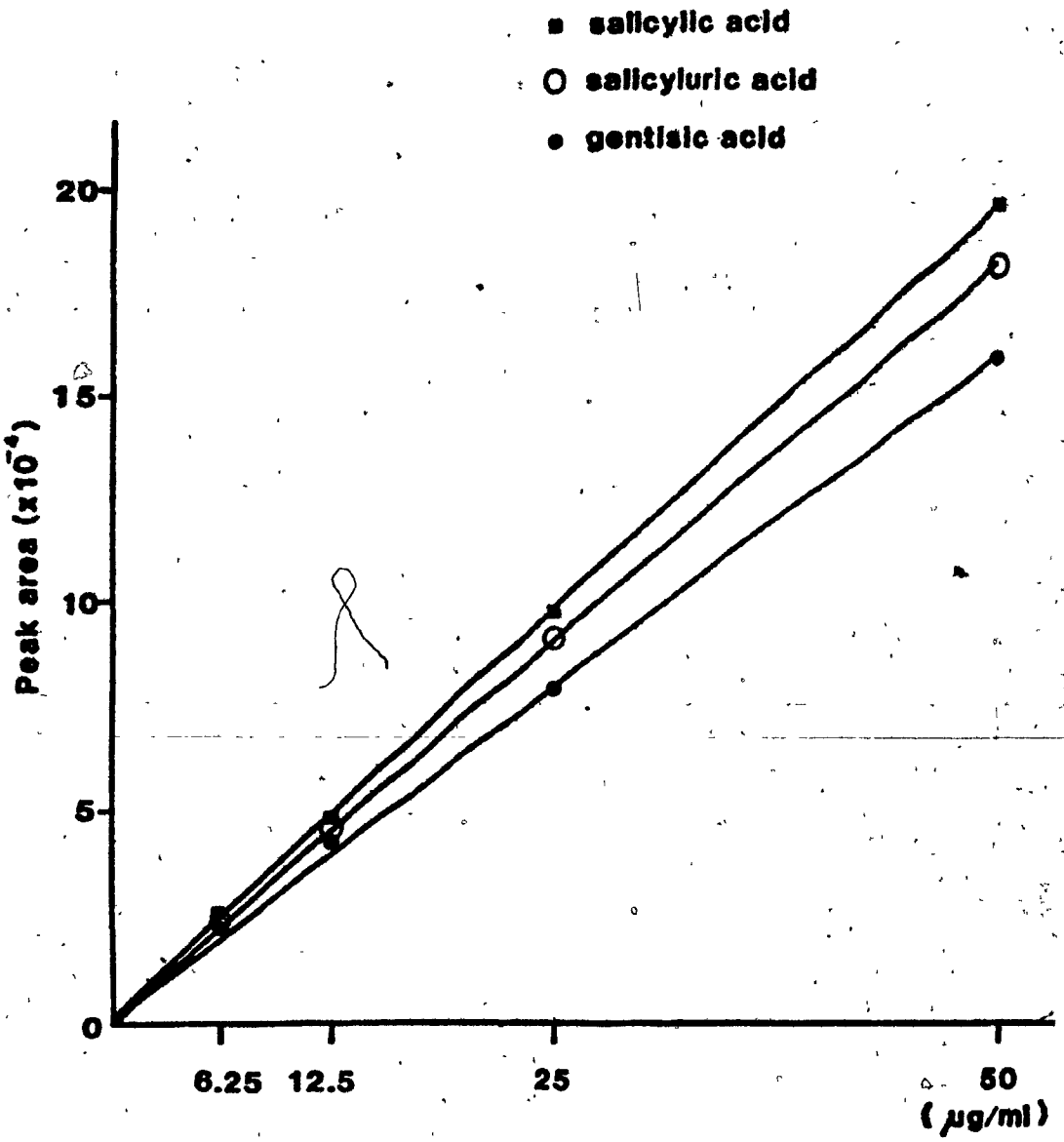


Figure 9. Standard curves of peak areas of salicylic acid, salicyluric acid and gentisic acid extracted from 0.1 ml urine (1:5 diluted) spiked with different concentrations of three substances. A linear relationship was obtained with a correlation coefficient of 0.99.
Salicylic acid: Y=0.40X-0.24; r=0.99
Salicyluric acid: Y=0.37X-0.12; r=0.99
Gentisic acid: Y=0.32X+0.21; r=0.99

5.4. Pharmacokinetics of Salicylate in Control and Protein-deficient Male Rats

5.4.1. Plasma half life ($t_{½}$), Apparent Volume of Distribution (V_d), and Plasma Clearance (Clp)

Semilogarithmic plots of plasma salicylic acid concentrations as a function of time after the administration of sodium salicylate are shown in Figures 10 and 11. Dose-dependent changes in plasma $t_{½}$, V_d and Cl_p were found in both control and protein-deficient rats (Figures 10, 11 and Table 6). At a 2 mg/kg dose level, $t_{½}$, V_d and Cl_p in control and protein-deficient animals did not differ significantly. However, at 10- and 100 mg/kg dose levels the plasma $t_{½}$ was shorter and Cl_p greater in protein deficient that in control rats; the V_d in the two groups of animals did not differ significantly. At a dose level of 200 mg/kg plasma concentrations of salicylate did not exhibit a linear decay up to 10 h (Figure 11). However, plasma levels of salicylate at 200 mg/kg dose were again lower in protein-deficient than in control rats. There was no significant difference in the plasma $t_{½}$, V_d and Cl_p of pair-fed controls and controls fed ad libitum (Table 6). When rats maintained on a low-protein diet for three weeks were fed a control diet, all the pharmacokinetic parameters returned to control levels in two weeks (Table 6).

5.4.2. Bioavailability

After oral administration, peak plasma concentrations were achieved in approximately 30 minutes in both the control and protein-deficient groups of rats; also, in both groups of animals the absorption of salicylate was rather complete. There was no significant difference in the relative bioavailability of sodium salicylate in the two groups of rats (Table 6).

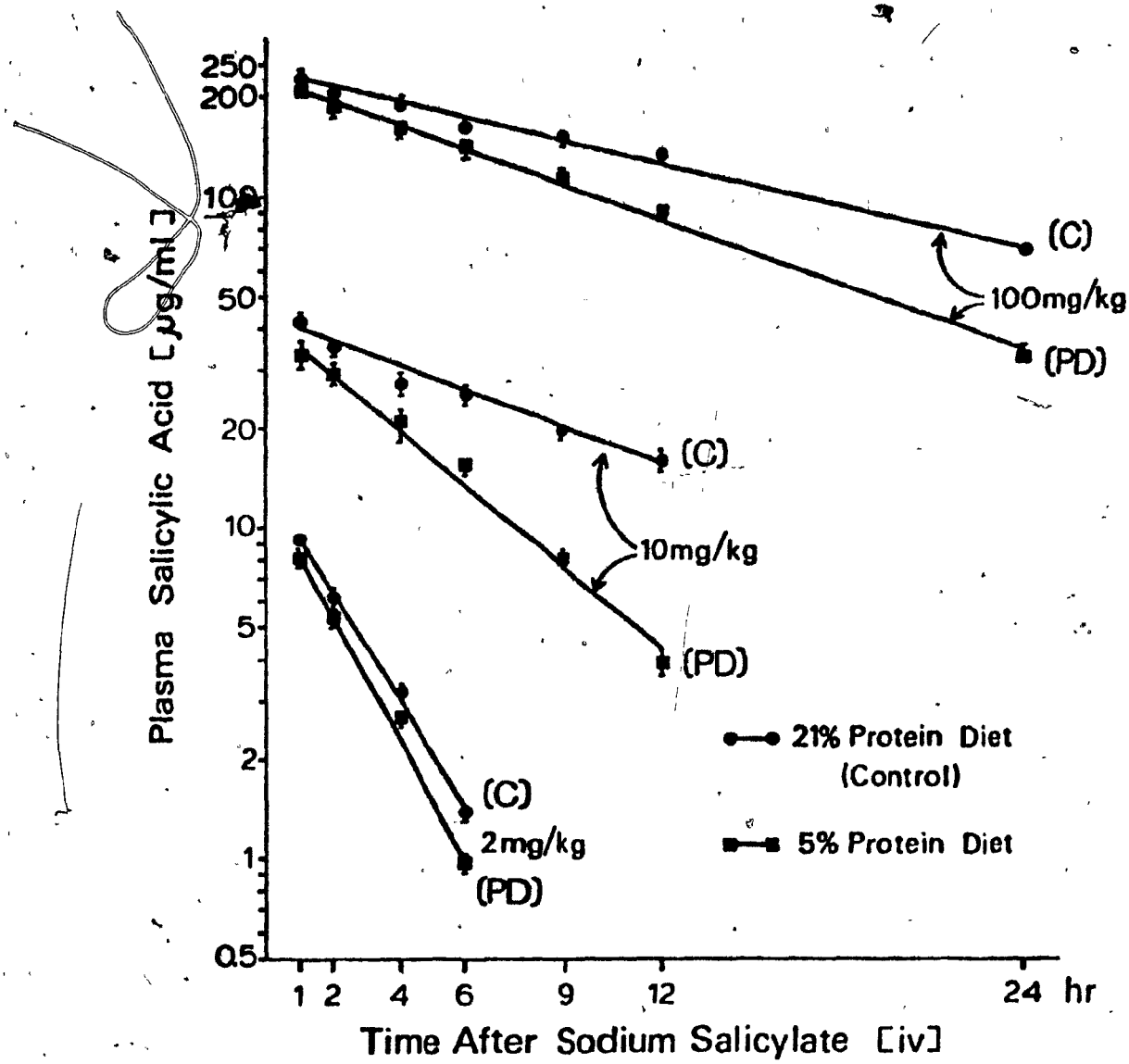


Figure 10. Semilogrithmic plots of plasma salicylic acid concentrations as a function of time following iv administration of 2-,10- and 100 mg/kg sodium salicylate in control and protein-deficient male rats. Each data point is the mean±S.E. of 5-8 experiments.

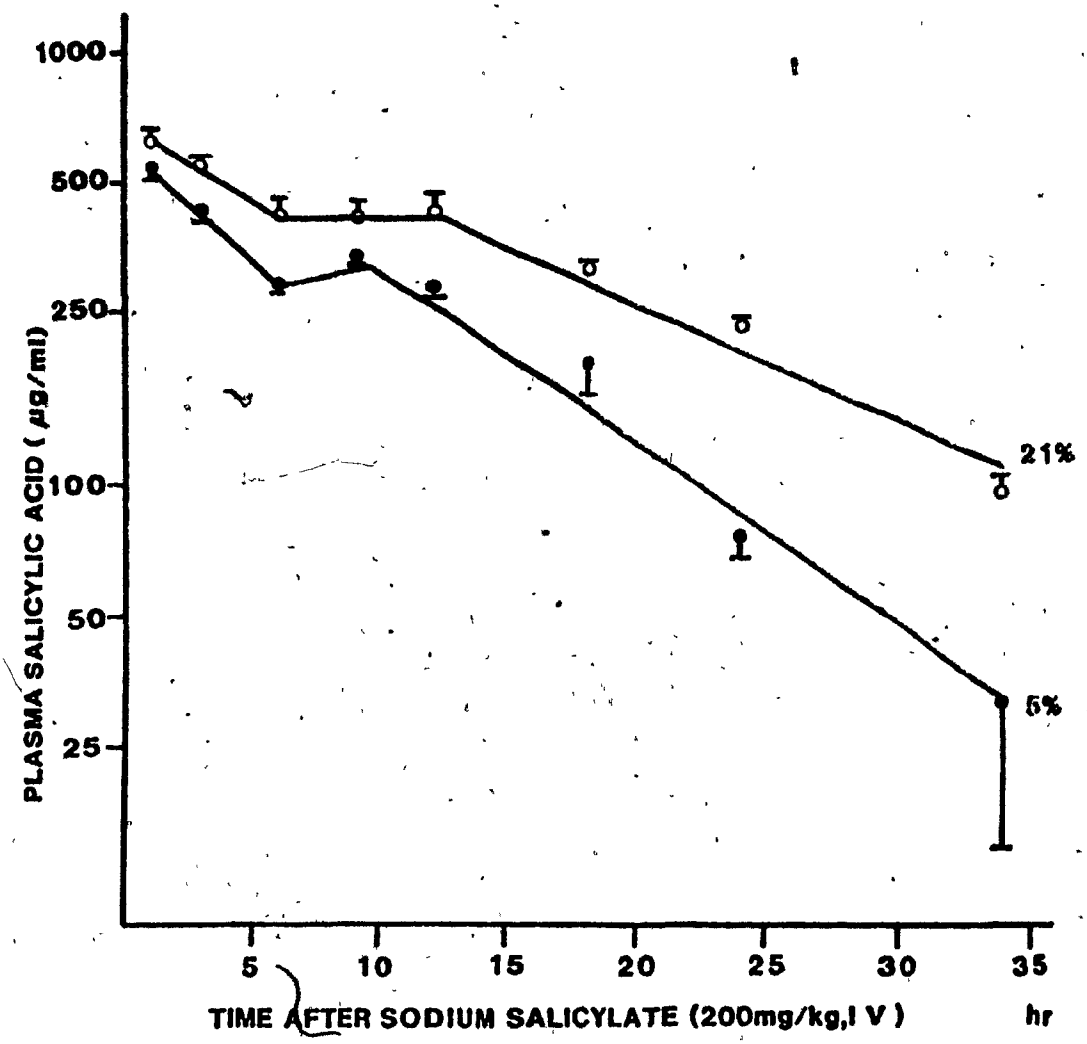


Figure 11. Semilogrithmic plots of plasma salicylic acid concentrations as a function of time following iv administration of sodium salicylate 200 mg/kg in control (21% protein diet) and protein-deficient (5% protein diet) male rats. Each datd point is the mean±S.E. of 5 experiments.

Table 6

Influence of different dietary regimens on the pharmacokinetics of salicylate in male rats

Parameters	Sodium Salicylate (mg/kg)	Dietary Protein (3-week period)			
		21% ad lib.	5% ad lib.	21%(10g/day)*	Protein-Repleted**
Plasma $t_{\frac{1}{2}}$(h)	2 iv	1.6±0.2	1.5±0.2	ND	ND
	10 iv	7.3±0.7[a]	3.3±0.2[a,b,c]	5.1±0.8[c]	9.3±2.1
	100 iv	12.2±0.6[a]	8.6±0.5[a,b]	ND	ND
V_d (ml/kg)	2 iv	131±7	133±13	ND	ND
	10 iv	225±20[a]	212±13[a]	205±15	235±20
	100 iy	425±28[a]	439±18[a]	ND	ND
Cl_p (ml/kg/h)	2 iv	58±5	64±4	ND	ND
	10 iv	22±1[a]	45±3[a,b,c]	32±6	21±5
	100 iv	24±2	36±2[a,b]	ND	ND
$AUC_{0-12\,hr}$	10 iv	338±28	229±15[b]	ND	ND
	10 po	310±7	200±14[b]	ND	ND
AUC_{po}/AUC_{iv}	10	0.92±0.02	0.88±0.06	ND	ND

Data represent mean±SE for 5 to 8 experiments; ND: not determined.

* Pair-fed to 5% protein diet ad lib. group of rats.

** A 5% protein diet for 3 weeks followed by a 21% protein diet for 2 weeks, ad lib.

a Significantly different ($p<0.05$) from the value immediately above.

b Significantly different ($p<0.05$) from the corresponding control value on the left.

c Significantly different ($p<0.05$) from the value in protein-repleted group.

5.4.3. Urinary Excretion of Salicylic Acid and its Metabolites

The urinary excretion of salicylic acid and its metabolites was determined at a 10 mg/kg dose of sodium salicylate. The cumulative excretion of salicyluric acid was significantly greater in protein-deficient than in control rats at 3, 6, 12 and 24 hours after the drug (Figure 12C). However, there was no difference in the excretion rate of salicyl glucuronides at any time during the 24 hour period (Figure 12A), and the excretion of unchanged salicylic acid was greater in protein-deficient than in control rats only within the first 3 h after the administration of the drug (Figure 12B). The cumulative excretion of all three fractions combined was higher in protein-deficient than in control rats at 6 and 12 h after drug administration (Figure 12D). Only in the control animals was the excretion of salicyluric acid found to proceed at a constant rate (Figure 12C, lower curve). Gentisic acid was detected only in a few urinary samples; when present its concentration was < 5% of the total and was not included in the calculation of the overall excretion rate of salicylates.

The kinetics of the urinary excretion of salicylic acid and its metabolites are presented in Table 7. The elimination half-life of salicylate was significantly shorter in protein-deficient than in control rats. There was no significant difference in K_{ex} and K_{mf} of salicyl glucuronides in the two groups of animals. K_{ex} and K_{mf} of salicyluric acid for the control rats could not be calculated because the plots of the logarithm of the rate of excretion against time were not linear ($r=0.21$) and no linear terminal portion of the curve could be selected from the four available data points; such a plot was linear ($r=0.98$) in the case of protein-deficient rats and hence K_{ex} and K_{mf} could be calculated (Table 7).

Figure 12. Influence of protein deficiency on the cumulative urinary excretion of salicylic acid and its metabolites in male rats. Asterisks denote significant differences ($P<0.05$) from the values in control rats at equivalent time periods; each value is the mean±S.E. of 6 experiments.

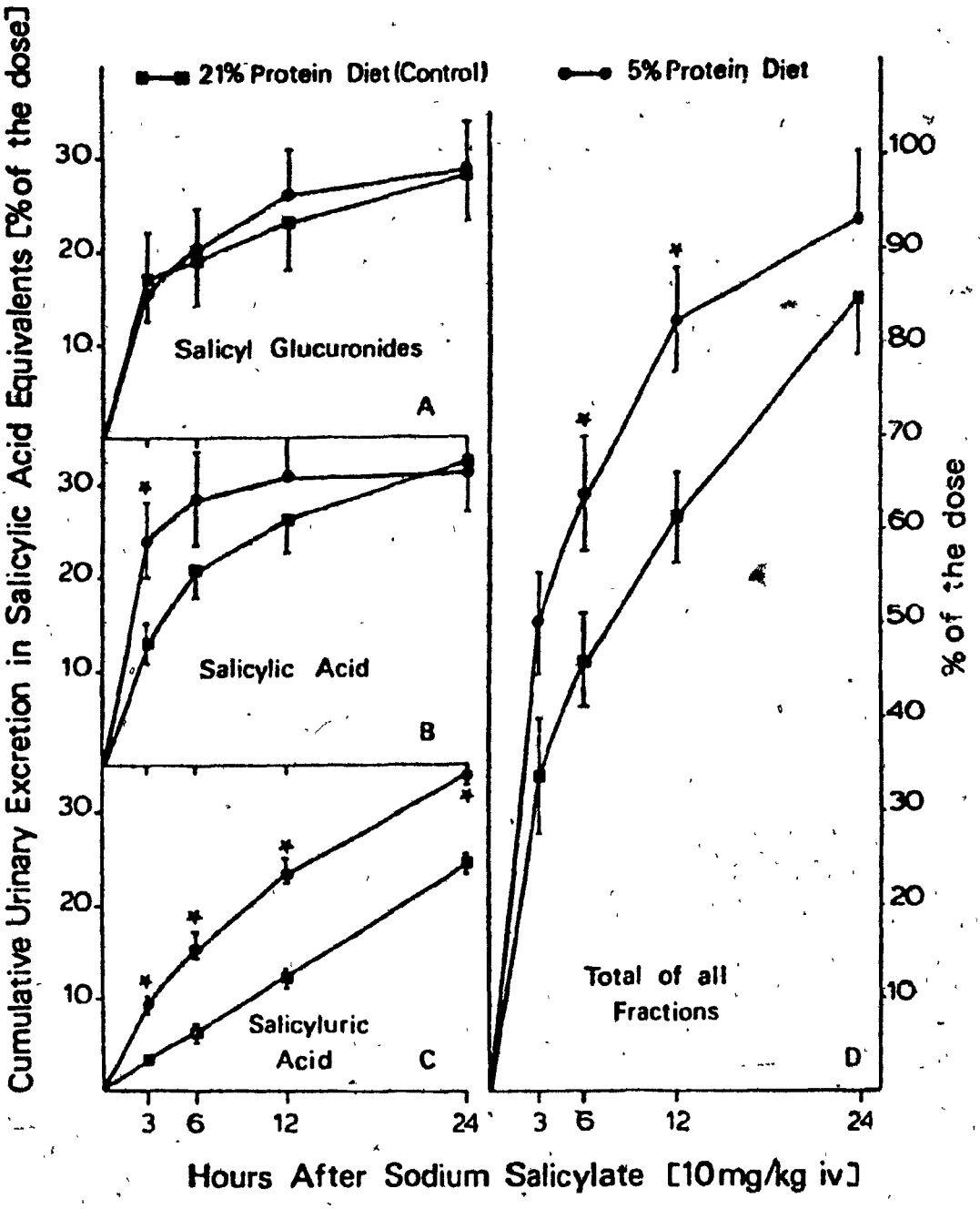

21% Protein Diet (Control)
5% Protein Diet
Cumulative Urinary Excretion in Salicylic Acid Equivalents [% of the dose]
% of the dose
Salicyl Glucuronides
A
Salicylic Acid
B
Salicyluric Acid
C
Total of all Fractions
D
30
20
10
100
90
80
70
60
50
40
30
20
10
3
6
12
24
Hours After Sodium Salicylate [10 mg/kg iv]

Table 7

Kinetics of urinary excretion of salicylic acid and its metabolites in control and protein-deficient male rats

Substances[a]	Parameters[b]	Control	Protein-deficient	P
		Mean ± S.E.		
Salicylic acid	K_{el} (h^{-1})	0.13 ± 0.01	0.38 ± 0.07	< 0.01
	$t_{\frac{1}{2}}$ (h)	5.9 ± 0.5	2.0 ± 0.3	< 0.001
Salicyluric acid	K_{mf} (h^{-1})	c	0.06 ± 0.002	
Salicyl glucuronides	K_{mf} (h^{-1})	0.04 ± 0.02	0.06 ± 0.02	> 0.05
Salicyluric acid	K_{ex} (h^{-1})	c	0.11 ± 0.003	
Salicyl glucuronides	K_{ex} (h^{-1})	0.12 ± 0.03	0.13 ± 0.04	> 0.05

[a] Substances recovered in the urine following sodium salicylate (10 mg/kg i.v.)

[b] K_{el}: elimination rate constant;

K_{mf}: metabolite formation rate constant;

K_{ex}: excretion rate constant

[c] Could not be calculated from the available data (see methods)

The urinary pH of control (6.4±0.30) and protein-deficient (6.4±0.21) rats were identical. The urinary protein concentrations (mg/ml) of control and protein-deficient rats were 2.7±0.18 and 2.3±0.2, respectively; the difference was not significant.

5.4.4. Protein-salicylate binding

There was no difference in the binding of salicylate to serum proteins of control and protein-deficient rats at the three dose levels studied (Table 8). When sodium salicylate was added to the serum in vitro, its binding to serum proteins of control rats was signficantly greater than to that of protein-deficient animals at all three concentrations (Table 9).

5.4.5. Mitochondrial Metabolism of Salicylate

Under the experimental conditions of the present study, incubation of salicylate with liver mitochondrial preparations did not yield any detectable metabolite. However, incubation of salicylate with kidney mitochondrial preparations consistently resulted in the formation of salicyluric acid; no other metabolite was detected. The yield of salicyluric acid in kidney mitochondrial preparations from protein-deficient rats was significantly greater than in control preparations, both in terms of wet weight as well as mitochondrial proteins (Table 10).

5.4.6. Tissue Distribution

Sodium salicylate (200 mg/kg) was injected intraperitoneally into control and protein-deficient rats followed by subplantar injection of carrageenan into one paw (see method). The inhibition of paw edema was measured and this was followed by removal of blood and soft tissues of both inflamed and noninflamed paws for the assay of salicylate. The inhibition of paw edema was greater and plasma salicylate concentration lower in protein-deficient than in

Table 8

In vivo binding of salicylate to serum proteins from control and protein-deficient rats

Dose of sodium salicylate[a]	Control rat serum[b]		Protein-deficient rat serum[c]	
(mg/kg iv)	Total salicylic acid (µg/ml)	% Bound	Total salicylic acid (µg/ml)	% Bound
	Mean ± S.E.			
1	3.51 ± 0.47	100	2.99 ± 0.18	100
10	42.83 ± 2.81	87.7 ± 3.4	37.5 ± 2.26	90.4 ± 1.5
100	243.9 ± 10.80	67.9 ± 3.1	211.1 ± 14.88	66.7 ± 2.3

[a] Blood sample was collected 1h following the drug; n=4, at each dose level.

[b] Rats were fed *ad libitum* a 21% protein diet for 3 weeks; serum proteins and albumin concentrations (mean ± SE, g/dl) were 7.49 ± 0.11 and 3.05 ± 0.15, respectively (n=12).

[c] Rats were fed *ad libitum* a 5% protein diet for 3 weeks; serum proteins and albumin concentrations (mean ± SE, g/dl) were 6.05 ± 0.18 and 2.34 ± 0.05, respectively (n=12).

Table 9

In vitro binding of salicylate to serum proteins from control and protein-deficient rats

Salicylic acid concentration	Control rat serum[a]	Protein-deficient rat serum[b]	P
mM	% bound, Mean ± S.E. (n=6)		
0.18	89.4 ± 0.8	84.8 ± 1.1	< 0.02
0.36	89.9 ± 0.1	84.9 ± 1.5	< 0.02
0.72	83.8 ± 2.2[c]	73.8 ± 3.2[c]	< 0.05

[a] Rats were fed ad lib. a 21% protein diet for 3 weeks; serum protein and albumin concentrations (mean ± SE, g/dl) were 8.2 ± 0.17 and 3.2 ± 0.09, respectively (n=18).

[b] Rats were fed ad lib. a 5% protein diet for 3 weeks; serum protein and albumin concentrations (mean ± SE, g/dl) were 6.10 ± 0.15 and 2.14 ± 0.06 respectively (n=18).

[c] Significantly different ($p < 0.05$) from the top values in the column.

Table 10

Metabolism of salicylate by rat kidney mitochondrial preparations

Group	Salicyluric Acid Formed	
	nmol/g tissue	nmol/mg mitochondrial protein
Control	12.0 ± 0.83	1.7 ± 0.09
Protein-deficient	16.2 ± 0.72	2.5 ± 0.11
	$p < 0.005$	$p < 0.001$

Salicyluric acid was the only detectable metabolite. Values are means ± SE; N = 9 for each group.

control rats. However, there was no difference in salicylate concentration in soft tissues of inflamed or noninflamed paws from the two groups of animals (Figure 13).

Salicylic acid concentrations in kidneys, liver, and plasma at 1 and 3 h, and in brain and stomach at 1 or 3 h following iv administration of 10 mg/kg sodium salicylate are presented in Table 11; with the exception of the value in the liver at 3 h after drug administration, there was no significant difference in salicylic acid concentrations in tissues of control and protein-deficient rats. However, the ratios of kidney to plasma concentrations of salicylic acid at 1 and 3 h after the drug were significantly higher in protein-deficient than in control animals.

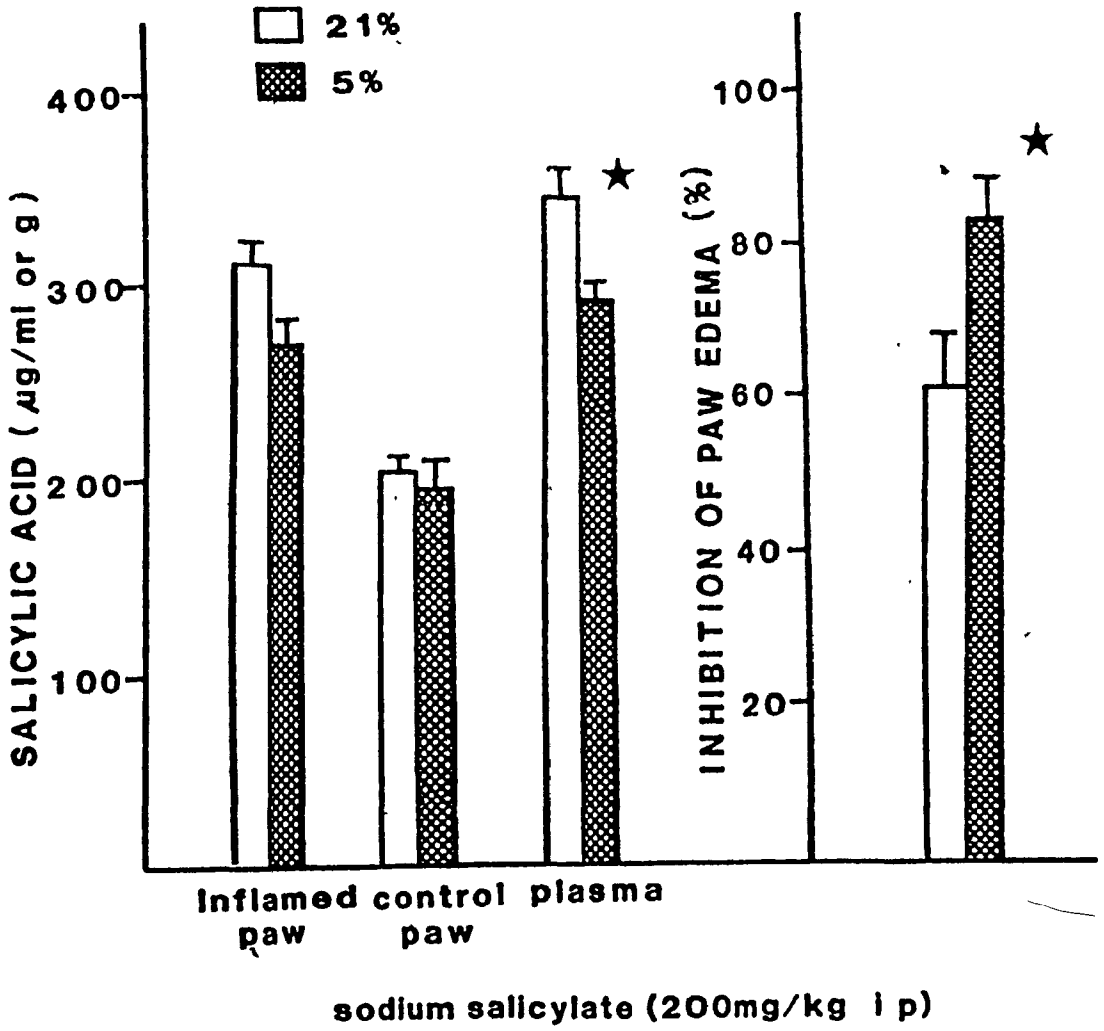


Figure 13. Influence of protein-calorie malnutrition (5% protein diet) on the paw edema suppressant activity of sodium salicylate and on its concentrations in the paw and plasma three hours after the administration of the drug in male rats. Each value is the mean±S.E. of 5 experiments; asterisks denote differences ($P<0.05$) from the adjacent control values.

Table 11

Tissue distribution and tissue/plasma concentration ratios of salicylate in control and protein-deficient male rats

Tissue	t[a]	Value when Dietary Protein ad lib. (3-Week Period) was			
		21%	5%	21%	5%
	h	µg/g or ml[b]		ratio[c]	
Kidney	1	21 ± 2.2	26 ± 0.2	0.64 ± 0.02	0.82 ± 0.08[d]
Kidney	3	7.6 ± 1.3	7.7 ± 0.5	0.24 ± 0.02	0.31 ± 0.02[d]
Liver	1	7.4 ± 0.8	5.9 ± 1	0.26 ± 0.03	0.18 ± 0.04
Liver	3	2.2 ± 0.2	1.6 ± 0.1[d]	0.07 ± 0.02	0.06 ± 0.01
Brain	1	0.9 ± 0.4	0.7 ± 0.3	0.02 ± 0.01	0.02 ± 0.01
Stomach	3	1.3 ± 0.3	0.7 ± 0.4	0.04 ± 0.01	0.03 ± 0.01
Plasma	1	34 ± 2.7	31 ± 3.2		
Plasma	3	32 ± 2.4	25 ± 2.0		

[a] Time after a dose of 10 mg of sodium salicylate per kg iv.

[b] Tissue or plasma concentration of salicylic acid; means ± SE for five experiments

[c] Ratio of tissue to plasma concentration of salicylic acid; means ± SE.

[d] Significantly different ($p < 0.05$) from control value on the immediate left.

5.5. Influence of Protein-calorie Malnutrition on Lysosome Stabilizing Activity of Salicylates

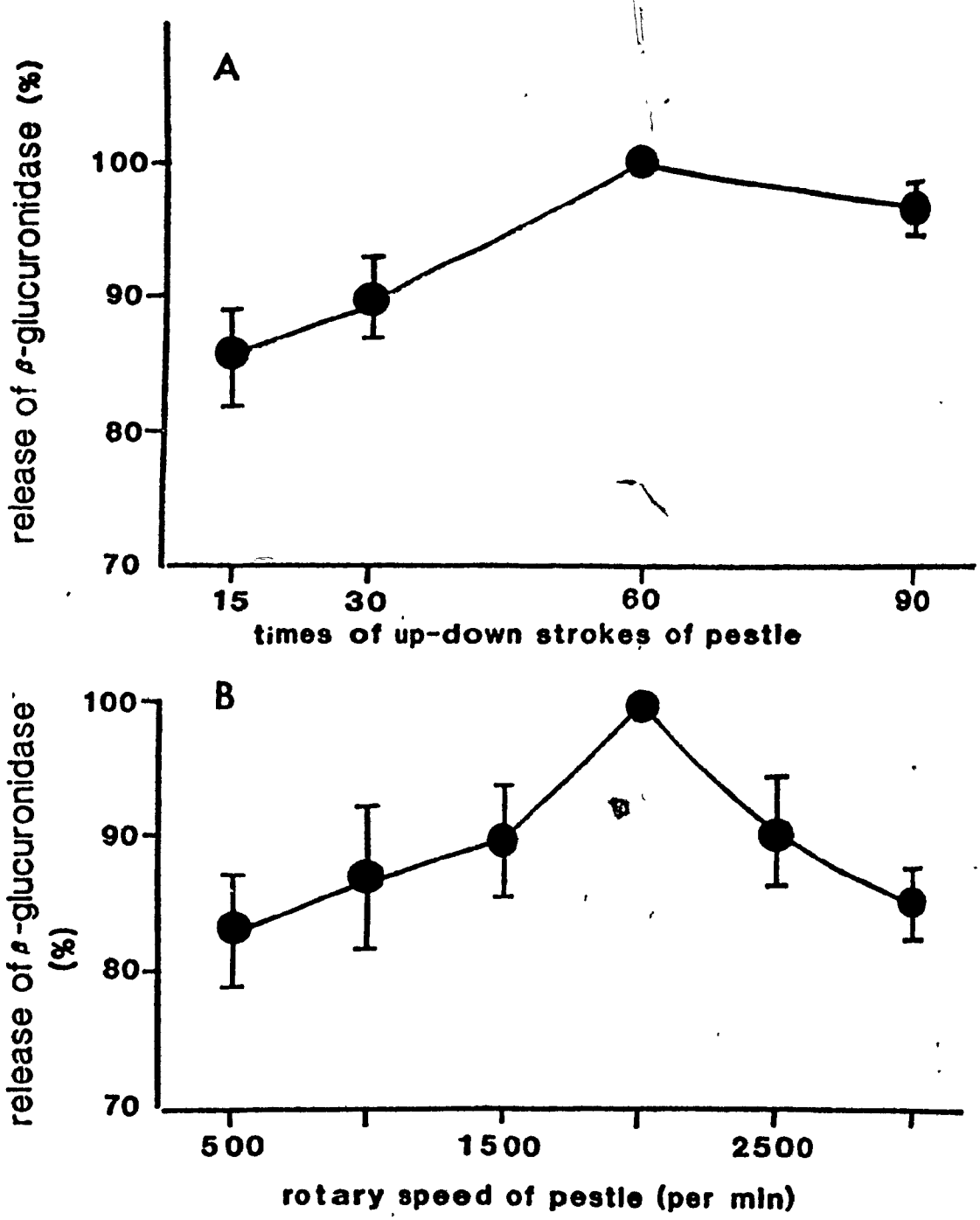
5.5.1. Establishment of Experimental Conditions

A motor-driven Potter-Elvehjem homogenizer instead of a manually-driven Dounce homogenizer was employed and different rotary speeds and up-down strokes were tried. As shown in Figure 14, release of total enzyme activity at 60 up-down strokes and a pestle speed of 2000 rpm was found satisfactory. When the liver was homogenized at 600 rpm and 30 up-down strokes in hypotonic sucrose buffer (0.07 M), approximately 20% of the total enzyme activity was released (Figure 15). This value was reproducible and comparable to that reported by Ignarro (1972). In the present study 30 up-down strokes at 600 rpm and 60 up-down strokes at 2000 rpm were used for the determination of free and total enzyme activities, respectively.

5.5.2. The Time-course of Dietary Protein Influence on Lysosomes

Protein-calorie malnutrition (a 5% protein diet, ad libitum) led to a significant increase in the ratio of free to total and a significant decrease in the total liver β-glucuronidase (Figure 16). A decrease in membrane stability (an increase in the ratio of free/total enzyme activity) was found as early as 3 days after the start of a low protein diet and reached a peak in 1 week (Figure 16A); total β-glucuronidase activity decreased significantly within 1 week and reached a minimum in approximately 2 weeks (Figure 16B); after which time there were no further significant changes in membrane stability or total β-glucuronidase activity. The ratio of free to total as well as the total β-glucuronidase activity of animals fed a 5% protein diet for 3 weeks returned to control levels within 7 days after the low protein diet was substituted with a normal 21% protein diet (Figure 16).

Figure 14. Effect of the number of up-down strokes (A) and of rotary speed of the pestle (B) on the release of total ß-glucuronidase from rat liver lysosomes in 0.1% Triton X-100–0.05 M Tris acetate (pH 7.4). A motor driven Potter-Elvehjem homogenizer was used. In panel A, the rotary speed was 2000 rpm. In panel B, the number of up-down strokes was 60. The highest values were assumed to represent 100% release of the enzyme. Each value is the mean±S.E. of 5-7 experiments.


A
release of β-glucuronidase (%)
100
90
80
70
15
30
60
90
times of up-down strokes of pestle
B
release of β-glucuronidase (%)
100
90
80
70
500
1500
2500
rotary speed of pestle (per min)

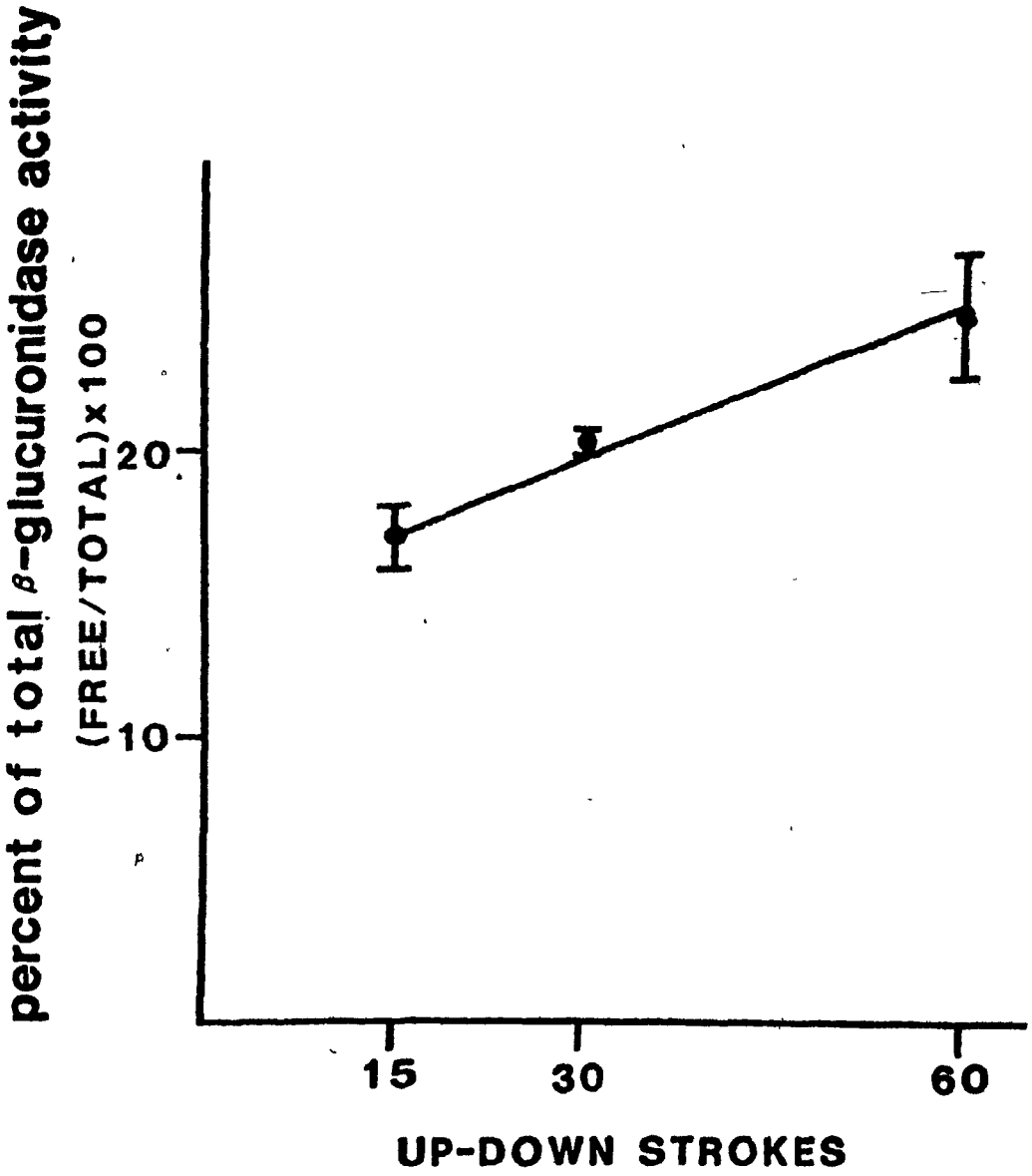
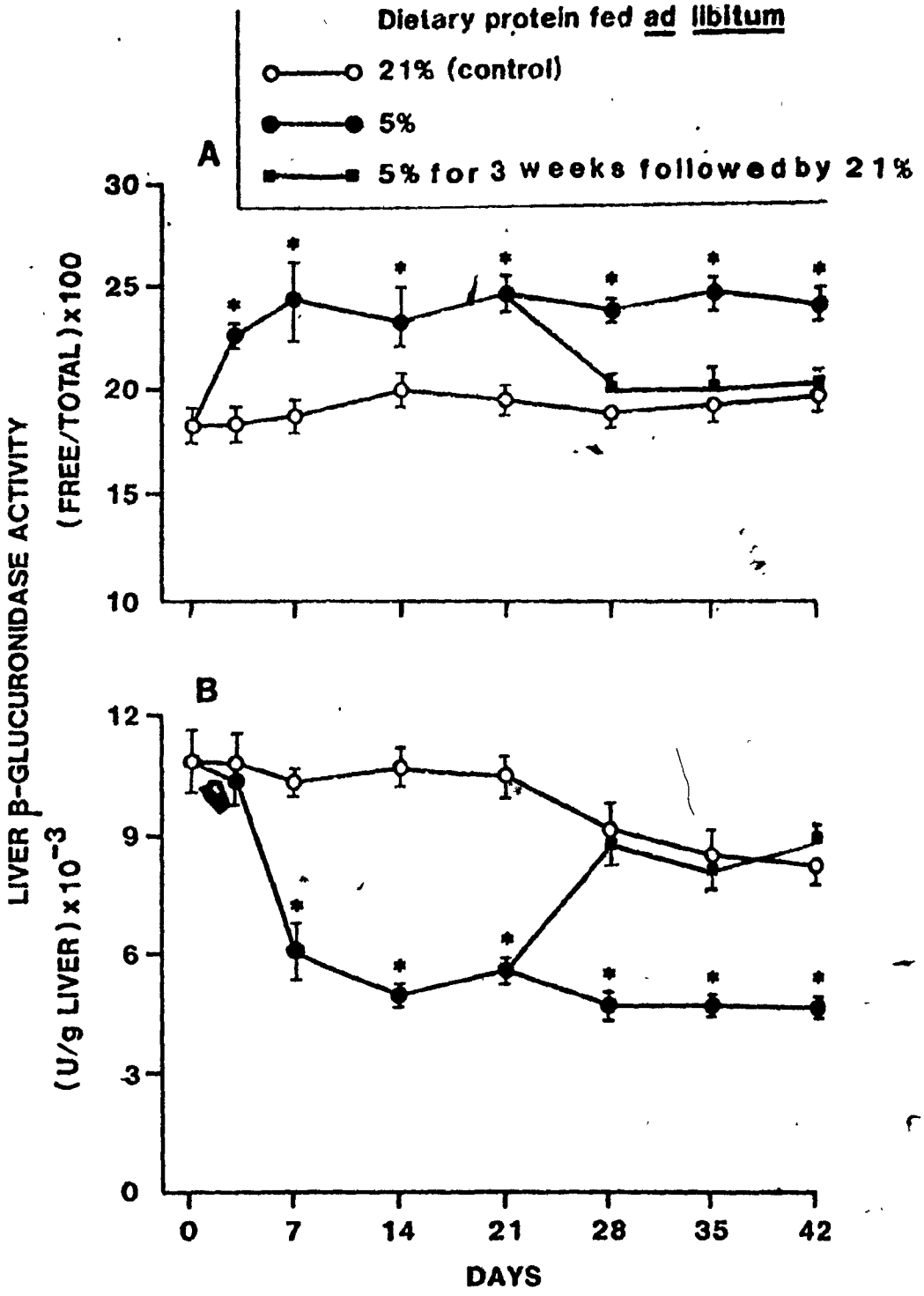


Figure 15. Effect of the number of up-down strokes on the release of β-glucuronidase from rat liver lysosomes in hypotonic sucrose buffer (0.07 M sucrose-0.05 M Tris acetate, pH 7.4) at a rotary speed of 600 rpm. The total release of the enzyme was achieved at rotary pestle speed of 2000 rpm and 60 up-down strokes in 0.1% Triton X-100-0.05 M Tris buffer (pH 7.4). Each value is the mean±S.E. (n=7-9)

Figure 16. Time-course of changes in membrane stability (panel A) and β-glucuronidase activity (panel B) of liver lysosomes as a function of the duration of dietary protein deprivation and replenishment in male rats. An increase in the free as a percentage of total enzyme activity implies a decrease in membrane stability (labilization). Each data point is the mean±S.E. of 5-10 animals. Whenever a value is significantly different ($p<0.05$) from the corresponding control value (21% protein diet group), it is shown by an asterisk.


Dietary protein fed ad libitum
21% (control)
5%
5% for 3 weeks followed by 21%
A
B
LIVER β-GLUCURONIDASE ACTIVITY
(FREE/TOTAL) x100
(U/g LIVER) x10⁻³
30
25
20
15
10
12
9
6
3
0
0
7
14
21
28
35
42
DAYS

5.5:3. Effects of a Low Protein Diet on Different Lysosomal Enzymes

The influence of a low protein diet on the three liver lysosomal enzymes measured in these experiments was not uniform (Figure 17); there was a significant decrease in β-glucuronidase activity and a significant increase in arylsulfatase activity. The pattern was similar whether the enzyme activity was expressed as a function of wet liver weight or supernatant protein. However, the β-glucuronidase activity in the protein-deficient rats was only 54% of the control when expressed in terms of wet liver weight and 72% when expressed in terms of milligrams of supernatant protein. The arylsulfatase activity in the protein-deficient animals per gram liver was 125% of the control; it was 160% of the control when expressed on the basis of per milligram protein. There was no change in acid phosphatase activity, which was 5.00±0.30 U/g liver or 0.06±0.002 U/mg protein in control (n=6) and 4.63±0.60 U/g liver or 0.07±0.006 U/mg protein in protein-deficient rats (n=6).

The ratios of both free to total β-glucuronidase activity and free to total arylsulfatase activity were significantly greater for protein-deficient animals than for controls. Free acid phosphatase activity was not measured.

5.5.4. Influence of Dietary Protein on Lysosomal Stabilizing and Paw Edema Inhibitory Effects and on the Plasma Concentration of Salicylate

As shown in Table 12 and Figure 18 (panel C) the effect of sodium salicylate on lysosomal membrane was biphasic, depending upon the dose; at a 50 mg/kg dose level sodium salicylate produced a stabilization of lysosomes both in control and protein-deficient rats. However, at a 200 mg/kg dose level, sodium salicylate produced a labilization of lysosomal membranes both in control and protein-deficient rats. A labilization of lysosomes was also

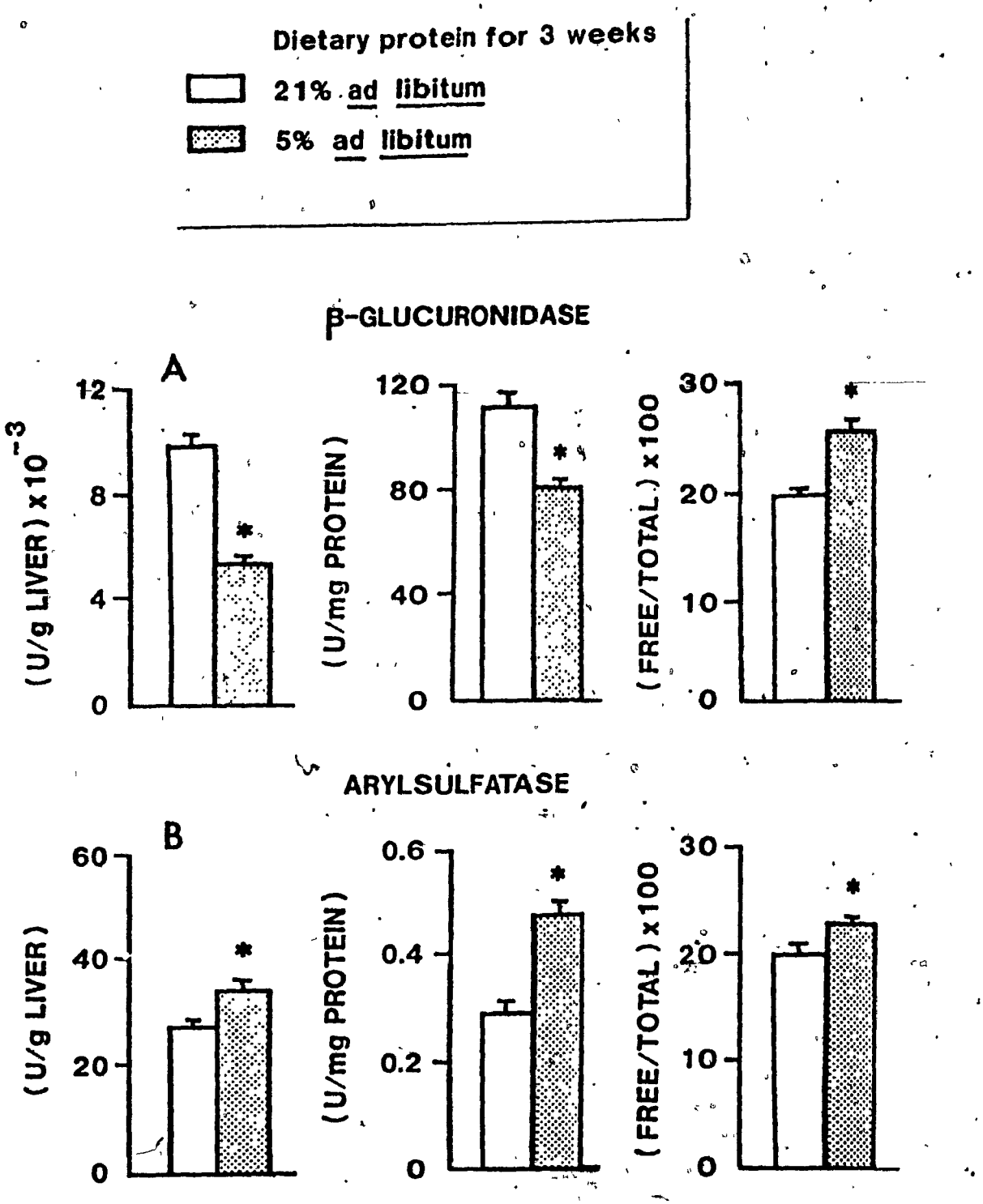


Figure 17. Influence of dietary protein deficiency on liver β-glucuronidase (panel A) and arylsulfatase (panel B) activities of male rats. Each value is the mean±S.E. of 6-36 animals. Asterisks denote significant differences ($p<0.05$) from the values in rats fed a control diet.

Table 12

Effects of nonsteroidal and steroidal anti-inflammatory agents on liver lysosomal membrane stability of control and protein-deficient male rats

Agent	Dose[a]	β-glucuronidase activity					
		21% protein ad lib.			5% protein ad lib.		
		Total	Free	F/T	Total	Free	F/T
	mg/kg	(U/g liver)x 10^{-3}		%	(U/g liver)x 10^{-3}		%
Saline		9.9±0.33[b]	1.9±0.06	20±0.4	5.4±0.29	1.4±0.06	26±0.9
Na-SA	50	9.4±0.28	1.6±0.12*	17±0.9*	5.2±0.17	1.2±0.07*	23±1.2*
Na-SA	200	9.5±0.19	2.3±0.16*	25±1.5	5.1±0.11	1.4±0.15	28±3.1
Aspirin	200	10.1±0.38	2.5±0.19*	25±1.4*	5.7±0.19	1.7±0.15	30±1.1*
OPBZ	50	10.4±0.77	1.7±0.16	16±0.8*	6.0±0.28	1.3±0.09	22±0.9*
IMC	10	10.8±0.45	1.8±0.19	16±1.4*	5.9±0.26	1.2±0.13	21±1.6*
DEXA	0.2	9.7±0.40	1.4±0.09*	15±0.9*	5.1±0.18	1.0±0.05*	20±0.6*

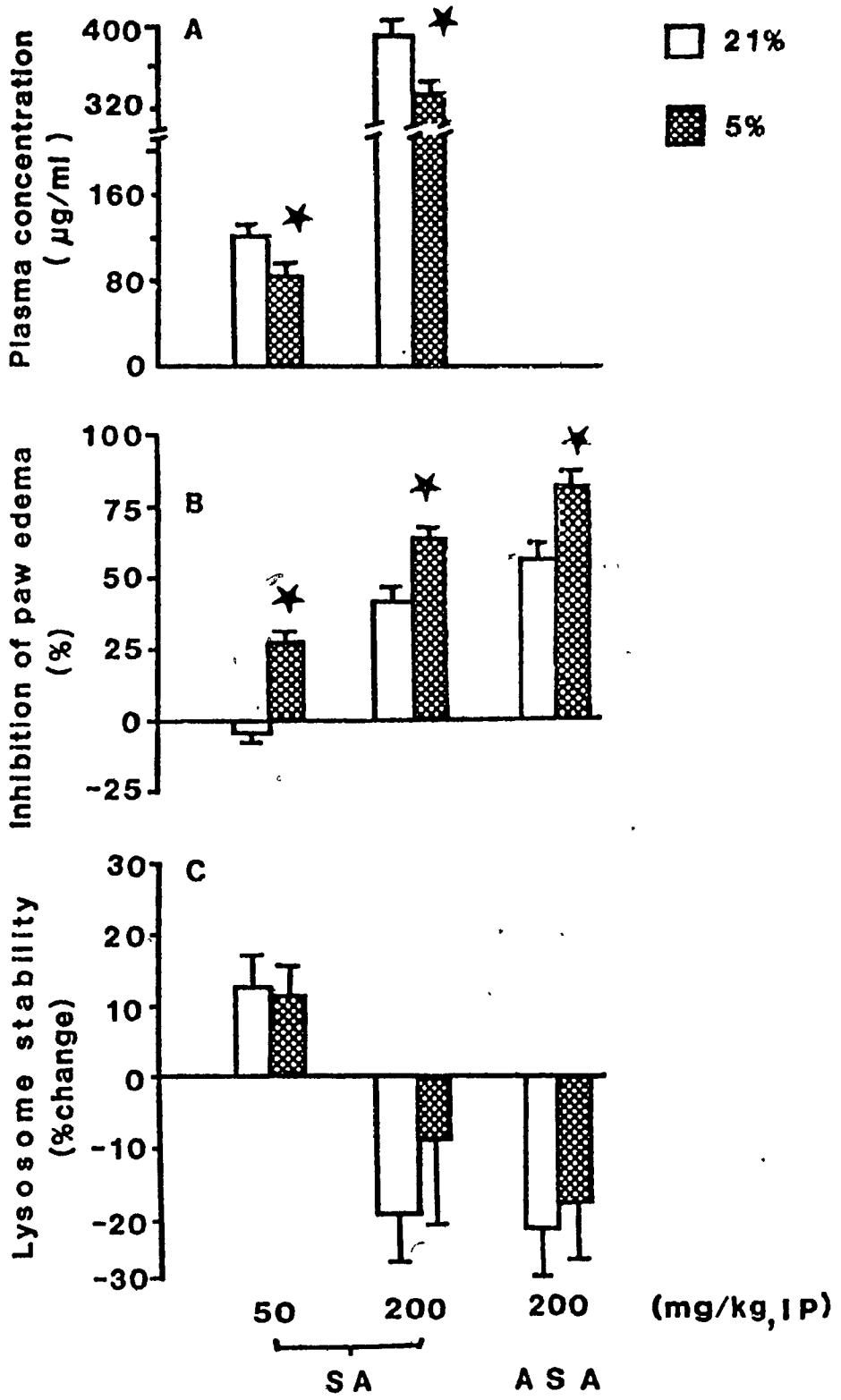
a Administered intraperitoneally approximately 3 h before killing the animals.

b Mean±SE of 9-27 animals.

* Significantly different ($p<0.05$) from the top value (saline-treated) in the same column.

Na-SA: sodium salicylate; OPBZ: oxyphenbutazone; IMC: indomethacin; DEXA: dexamethasone.

Figure 18. Relationship between paw edema suppressant and lysosome stabilizing activities of sodium salicylate (SA) and aspirin (ASA) in male rats fed ad libitum for 3 weeks a 21% (control) or a 5% (protein-deficient) protein diet. Each value is the mean±S.E. of 9-27 experiments. Asterisks denote differences ($p<0.05$) from the adjacent control values. Pharmacological effects were calculated as described in the Methods section. Plasma aspirin concentration was not measured.


A
400
320
160
80
0
Plasma concentration (μg/ml)
21%
5%
B
100
75
50
25
0
-25
Inhibition of paw edema (%)
C
30
20
10
0
-10
-20
-30
Lysosome stability (%change)
50
200
200
(mg/kg, IP)
SA
ASA

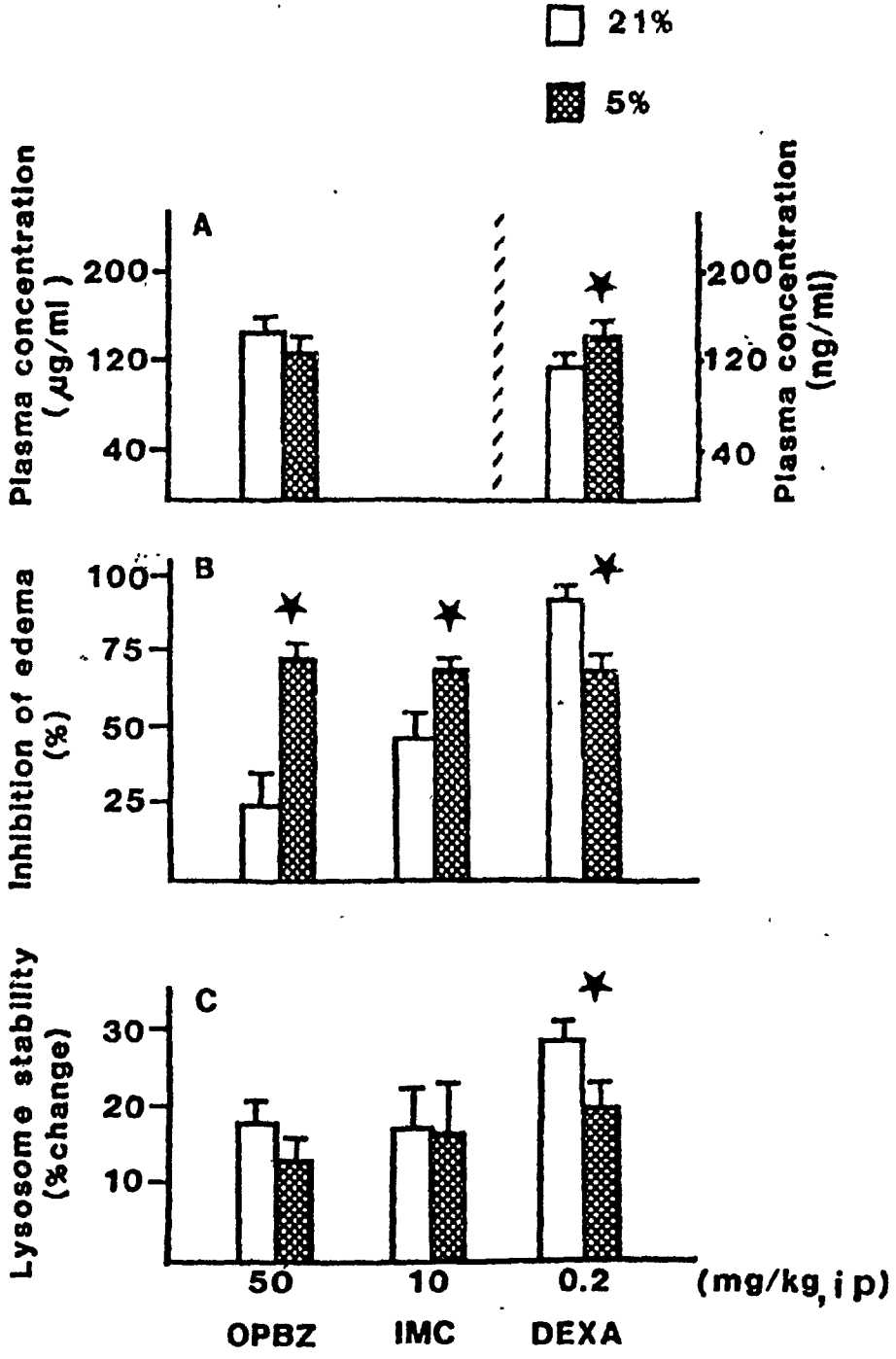
produced by aspirin at a dose of 200 mg/kg. The effects of sodium salicylate and aspirin on liver lysosomal membranes of control and protein-deficient rats did not significantly differ at the dose levels studied.

Sodium salicylate and aspirin produced greater inhibition of carrageenan-induced paw edema in protein-deficient rats than in controls (Figure 18, panel B). There was no consistent relationship between the lysosome-stabilizing and paw edema suppressant activities of both sodium salicylate and aspirin. The plasma concentration of salicylate was determined in samples collected immediately after the measurement of its effect on paw edema, i.e. 3 h after dosing (Figure 18, panel A); this also corresponded to the time of the measurement of lysosome stability. The concentration of salicylic acid was significantly higher in control than in protein-deficient rats. The difference in paw edema suppressant activity of salicylate in the two groups of rats was not related to differences in its plasma concentration. The plasma concentration of aspirin was not measured.

5.5.5. Influence of Dietary Protein on Lysosome Stabilizing and Paw Edema Inhibitory Effects and on the Plasma Concentration of Oxyphenbutazone, Indomethacin and Dexamethasone

Figure 19 and Table 12 show that oxyphenbutazone, indomethacin and dexamethasone caused a significant stabilization of lysosomal membrane and inhibition of carrageenan-induced paw edema both in control and in protein-deficient rats. The paw edema inhibitory effect of oxyphenbutazone and indomethacin were greater in protein-deficient than in control animals but there was no difference in the effects of these drugs on the stabilization of lysosomal membrane in the two groups of rats. In contrast to the nonsteroidal anti-inflammatory drugs, dexamethasone produced less paw edema inhibitory effect in protein-deficient rats than in controls and there appears to be a

Figure 19. Relationship between paw edema suppressant and lysosome stabilizing activities of oxyphebutazone (OPBZ), indomethacin (IMC) and dexamethasone(DEXA) in male rats fed *ad libitum* for 3 weeks a 21% (control) or a 5% (protein-deficient) protein diet. Each value is the mean±S.E. of 9-14 experiments. Asterisks denote differences ($p<0.05$) from the adjacent control values. Calculation of the pharmacological effects are described in the Methods section. Plasma indomethacin concentration was not measured.


21%
5%
A
Plasma concentration (μg/ml)
200
120
40
Plasma concentration (ng/ml)
200
120
40
B
Inhibition of edema (%)
100
75
50
25
C
Lysosome stability (%change)
30
20
10
50
10
0.2
(mg/kg, i p)
OPBZ
IMC
DEXA

consistent relationship between this effect and the effect of dexamethasone on lysosomal membrane stability (Figure 19).

Plasma concentration of dexamethasone was higher in protein-deficient than in control rats; the concentration of oxyphenbutazone in the two groups of animals did not differ significantly. Also the difference in the lysosome-stabilizing or paw edema suppressant activity of these drugs in the two groups of rats was not related to differences in their plasma concentrations (Figure 19, panel A). The plasma indomethacin concentration was not measured.

5.6. Effect of Protein-Calorie Malnutrition on the Metabolism of Arachidonic Acid

5.6.1. Metabolism of Arachidonic Acid by Rat Neutrophils

Rat neutrophils were incubated in the presence of A23187 and [1-^{14}C] arachidonic acid (2 μM) and the products analyzed by reversed-phase HPLC (Figure 20). Since prostaglandins and thromboxane B_2 were not well resolved under these conditions, the materials in these peaks (between 8 and 12 min., Figure 20) were rechromatographed on a silica column. Figure 21 shows a high pressure liquid radiochromatogram of the products of this fraction after incubation of rat neutrophils with a high concentration of arachidonic acid (100 μM) in the presense of A23187. All products were identified on the basis of their mass spectra and by comparison of their chromatographic properties with those of standards. The major products formed by lipoxygenases were 5-HETE and a dihydroxyeicosatetraenoic acid product. Rechromatography of the latter material (t_R,25.5 min, Figure 20) on a silica column (Borgeat, et al., 1981) gave a single peak. The mass spectrum of the trimethylsilyl ether derivative of the methyl ester of this substance was identical to that reported for LTB_4 (Figure 22). Little or no 5S, 12S-dihydroxy-(EZEZ)-6,8,10,14-eicosatetraenoic acid was present. At high substrate concentrations small amounts of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) were detected. The major cyclooxygenase products were 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2. Smaller quantities of prostaglandin E_2, prostaglandin $F_{2\alpha}$ and 6-oxoprostaglandin $F_{1\alpha}$ were also produced, especially in the presence of high substrate concentrations (Figure 21). There were no qualitative differences in the profile of arachidonic acid metabolites synthesized by neutrophils from control and protein-deficient rats.

Figure 20. High-pressure liquid chromatogram of products synthesized by rat pleural neutrophils from a control male rat. Neutrophils ($3x10^7$ cells in 1 ml) were incubated for 5 min at 37°C with A23187 (20 μM) and [1-^{14}C]arachidonic acid (2μM) and the products separated by HPLC on a reversed-phase column by means of a linear gradient over a 70 min period between water/methanol/acetic acid (40:60:0.01, v/v) and water/methanol/acetic acid (20:80:0.01,v/v) at a flow rate of 1.5 ml/min. The upper panel (A) shows the ultraviolet absorbance monitored initially at 280 nm and then at 232 nm after 27 min. The lower panel (B) shows the radioactivity, which was detected using a Berthold HPLC radioactivity monitor. The fractions in the shaded area were combined and rechromatographed by normal-phase HPLC (see Figure 21).

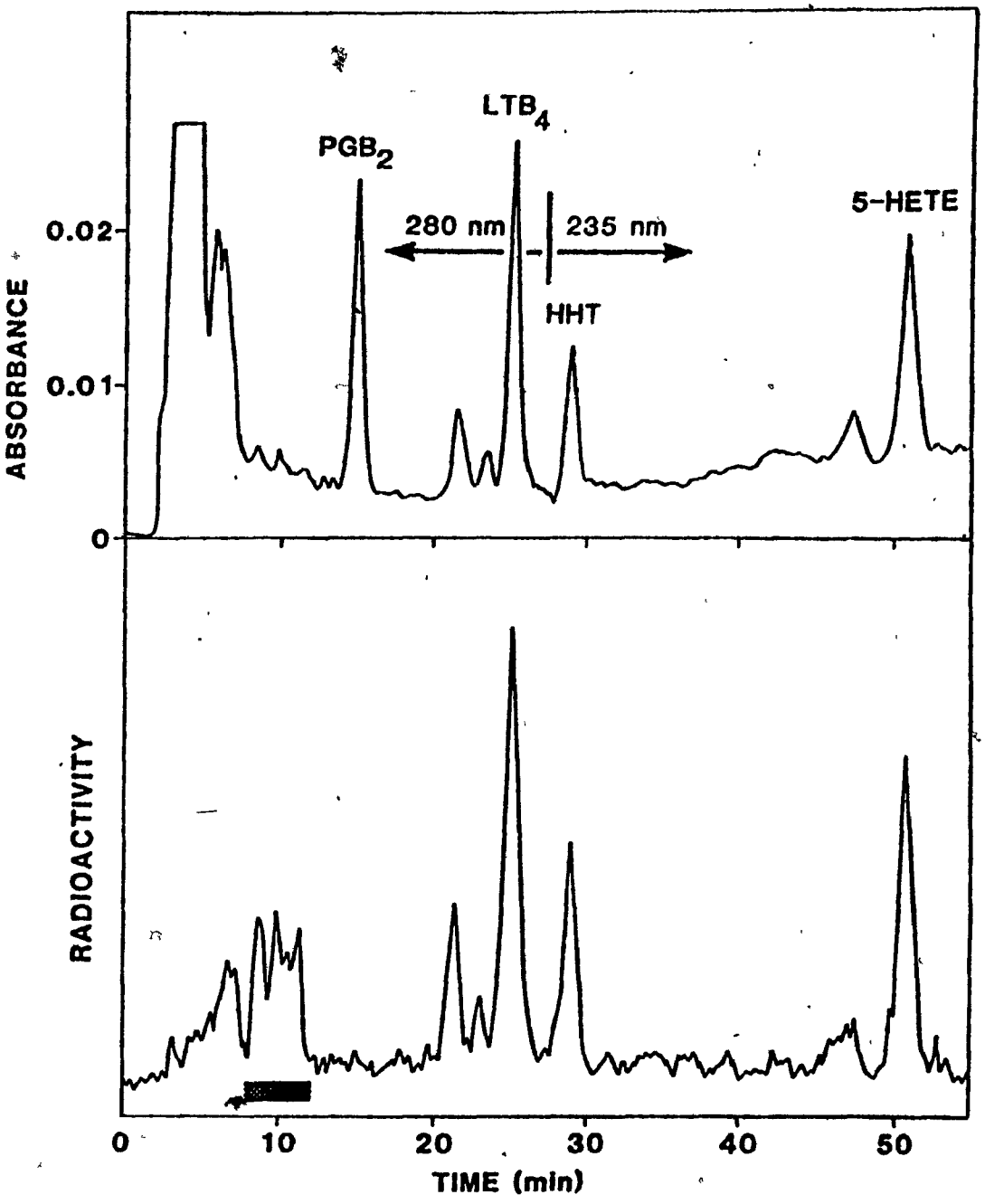
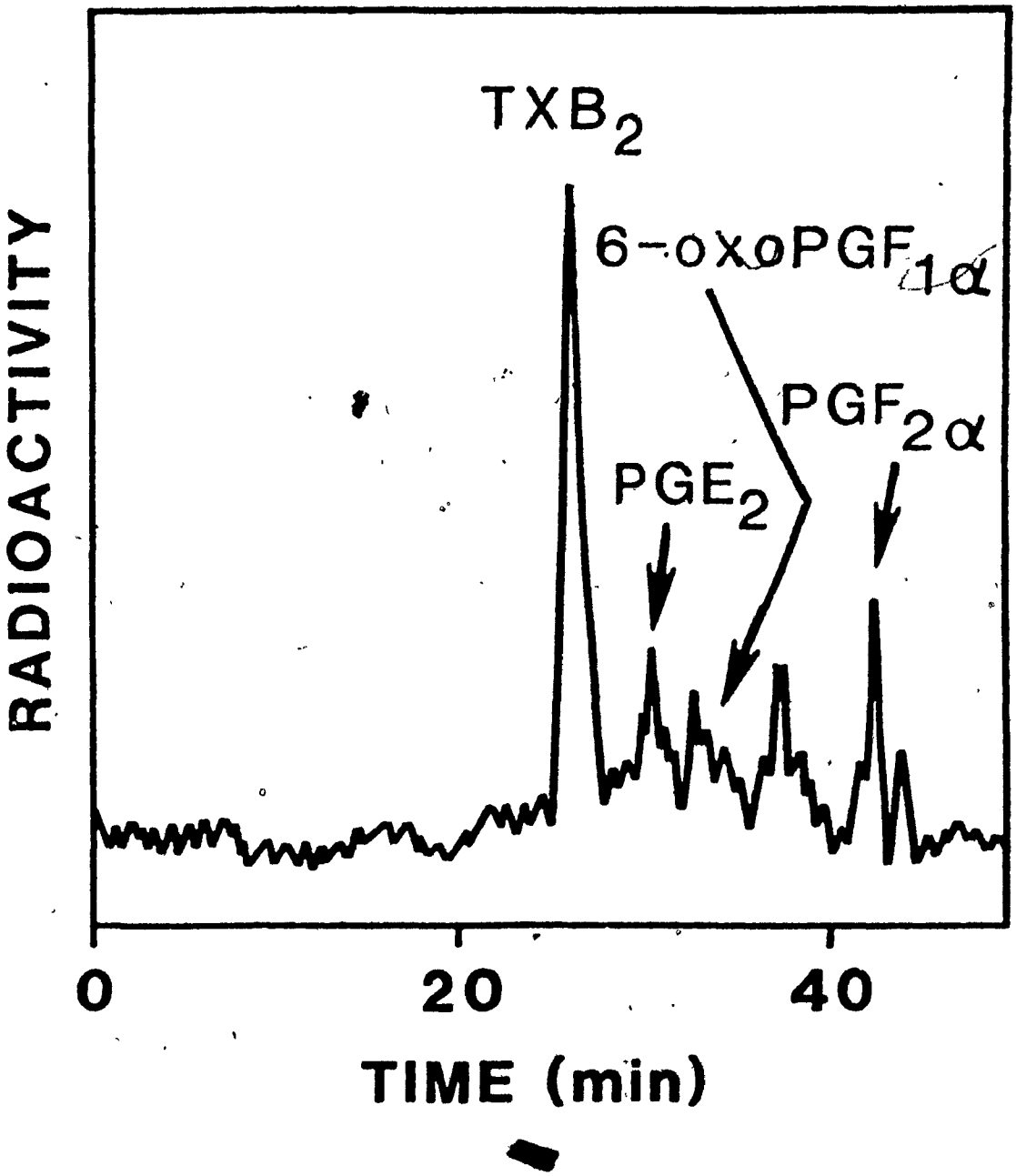

LTB4
PGB2
5-HETE
280 nm
235 nm
HHT
0.02
0.01
0
ABSORBANCE
RADIOACTIVITY
0
10
20
30
40
50
TIME (min)

Figure 21. High-pressure liquid radiochromatogram of the products in the prostaglandin and thromboxane fraction obtained after incubation of rat pleural neutrophils with [1-^{14}C]arachidonic acid (0.6 μCi,100 μM) for 5 min in the presence of A23187 (20 μM). The products were extracted and partially purified by reversed-phase HPLC (cf. Figure 20). The fraction eluting between 8 and 12 min was evaporated and rechromatographed on a column of silica using a linear gradient over 60 min between 20% and 80% toluene/ethyl acetate/acetonitrile/methanol/acetic acid (30:40:30:2:0.5,v/v) in hexane/toluene/acetic acid (50:50:0.5,v/v).


TXB2
6-oxoPGF1α
PGF2α
PGE2
RADIOACTIVITY
0
20
40
TIME (min)

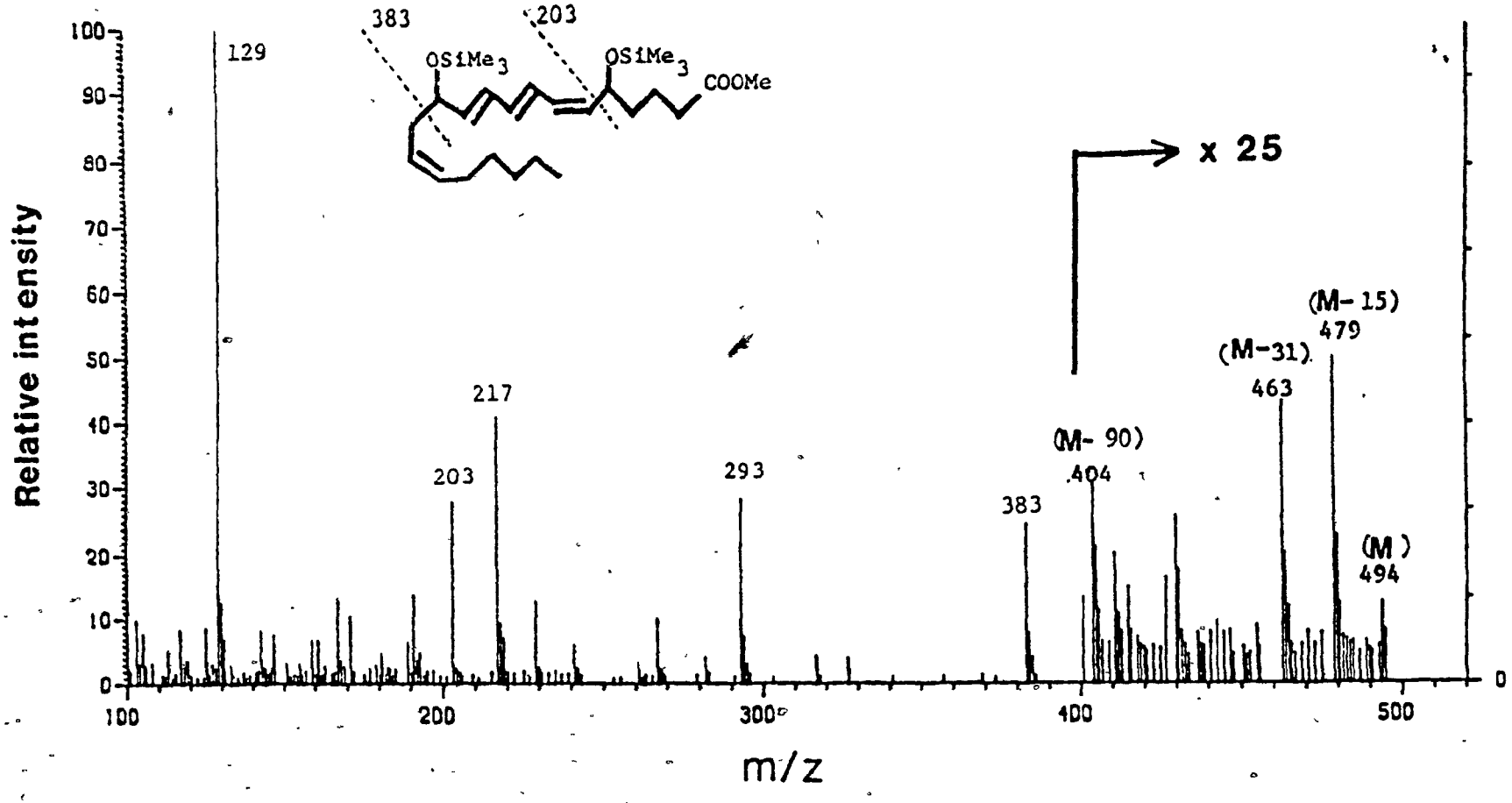


Figure 22. Mass spectrum of the trimethylsilyl ether derivative of the methyl ester of LTB_4 from rat pleural neutrophils. The fraction containing LTB_4 (see Figure 20) was concentrated to dryness and converted to its methyl ester (diazomethane, room temperature, 15 min), trimethylsilyl ether (N-methyl-N-trimethylsilyltrifluoroacetamide, room temperature, 30 min) derivative. The gas chromatography-mass spectrometry was done on an LKB-9000 instrument.

5.6.2. Effects of Protein-calorie Malnutrition on the Biosynthesis of Lipoxygenase and Cyclo-oxygenase-derived products by rat neutrophils

Figure 23 shows the standard curves for the quantitation of PGE_2, $PGF_{2\alpha}$, TXB_2 and 6-oxo$PGF_{1\alpha}$ by GC-MS using selected ion monitoring. There existed a good linear relationship between the concentrations of PGE_2, $PGF_{2\alpha}$, TXB_2 and 6-oxo$PGF_{1\alpha}$ (ranging from 20 ng to 500 ng) and the ratios of the peak heights of the selected ions for PGE_2, $PGF_{2\alpha}$, TXB_2 and 6-oxo$PGF_{1\alpha}$ to those of their corresponding deuterated analogs obtained from mixtures of a constant amount deuterated product and different amounts of its corresponding nondeuterated product (see method).

The major products formed by rat pleural neutrophils at low concentrations of arachidonic acid were leukotriene B_4, 5-HETE, HHT and thromboxane B_2 (Table 13); only small amounts of prostaglandins were formed under these conditions. Protein-calorie malnutrition caused a decrease in the formation by neutrophils of all the cyclooxygenase-derived products which were measured. Leukotriene B_4 synthesis was also decreased due to protein deficiency, but there was no change in the formation of 5-HETE.

Increasing the concentrations of arachidonic acid up to 50 μM had similar effects on the synthesis of leukotriene B_4, 5-HETE and thromboxane B_2 by neutrophils from control and protein-deficient rats (Figure 24). In all cases, the amounts of 5-HETE formed by neutrophils from the two groups of rats were identical, whereas the formation of the other products was considerably lower for protein-deficient rats. At the highest substrate concentration employed (100 μM), the formation of leukotriene B_4 and 5-HETE was inhibited in neutrophils from protein-deficient rats, but not in those from control rats.

Figure 23. Standard curves for the quantitation of PGE_2 (panel A), $PGF_{2\alpha}$ (panel B), TXB_2 (panel C) and 6-oxo$PGF_{1\alpha}$ (panel D) by GC-MS. Two hundred ng of tetradeuterated PGE_2, $PGF_{2\alpha}$ or 6-oxo$PGF_{1\alpha}$ and 300 ng of octadeuterated TXB_2 were mixed with varing amount of nondeuterated analogs. TXB_2 and $PGF_{2\alpha}$ were converted to their methyl ester, trimethylsilyl ether derivatives and PGE_2 and 6-oxo$PGF_{1\alpha}$ converted to their methyl ester, trimethylsilyl ether, o-methyloxime derivatives, and quantitated by GC-MS using selected ion monitoring on an LKB-9000 instrument. The ratios of peak heights for the ions from nondeuterated standards to those from their deuterated analogs were plotted against the concentrations of the non-deuterated products (25-500 ng).

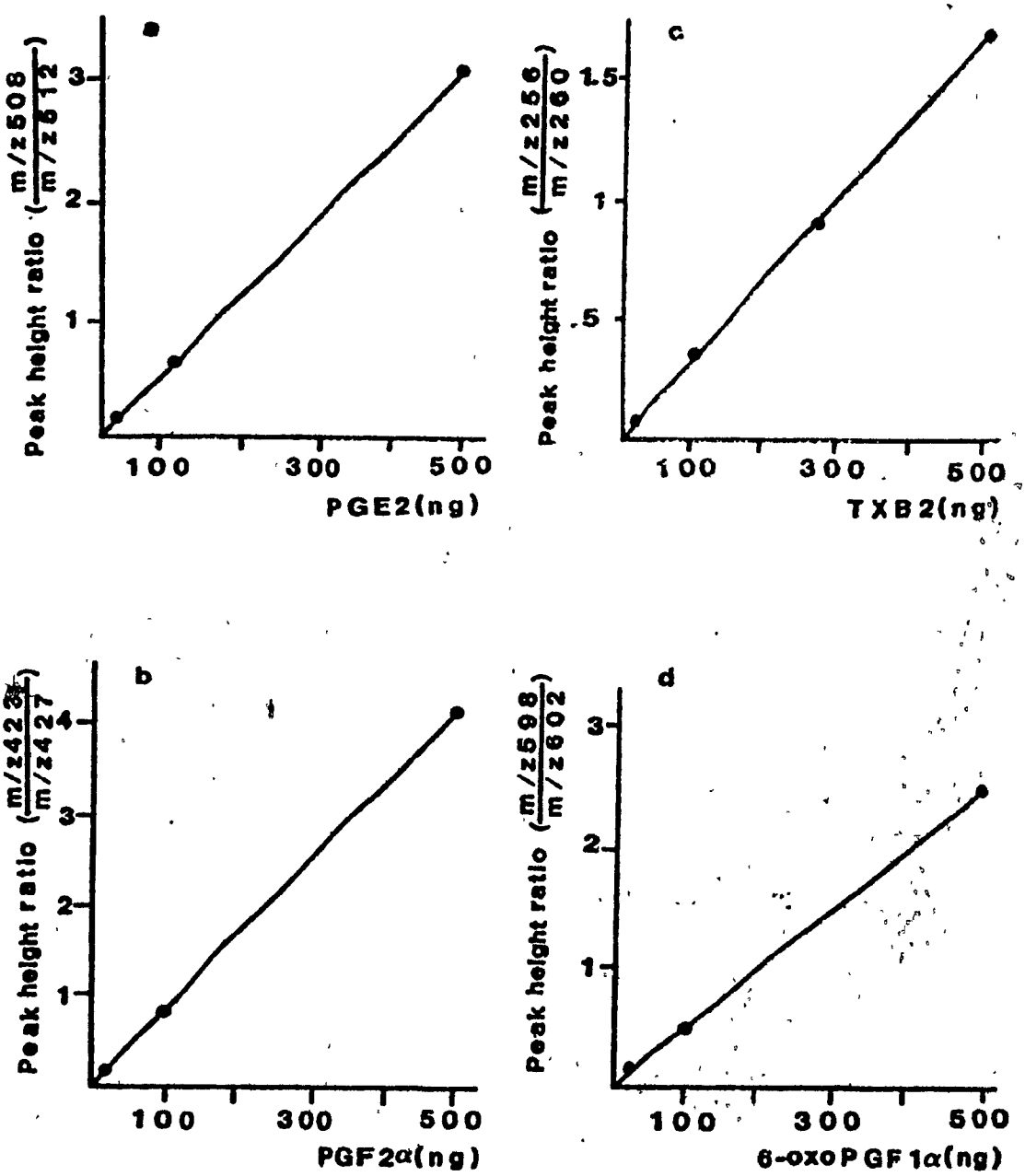

a
Peak height ratio (m/z508 / m/z512)
3
2
1
100
300
500
PGE2(ng)
c
Peak height ratio (m/z256 / m/z260)
1.5
1
.5
100
300
500
TXB2(ng)
b
Peak height ratio (m/z423 / m/z427)
4
3
2
1
100
300
500
PGF2α(ng)
d
Peak height ratio (m/z598 / m/z602)
3
2
1
100
300
500
6-oxoPGF1α(ng)

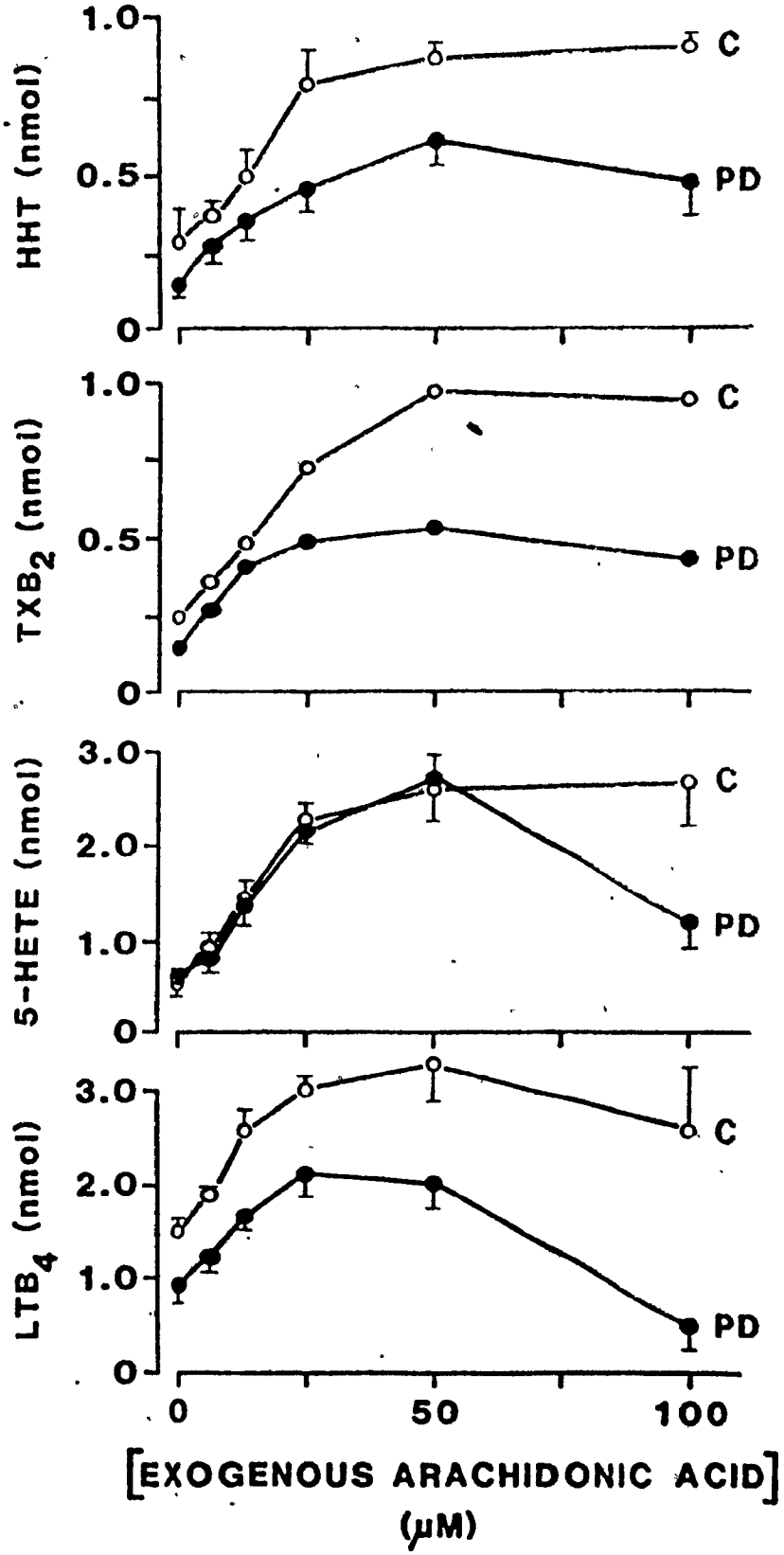
Table 13

Biosynthesis of leukotriene B_4, Thromboxane B_2, HHT, 5-HETE and prostaglandins E_2 and $F_{2\alpha}$ by pleural neutrophils from control and protein-deficient rats

Rats were fed ad libitum a 21% (control) or a 5% (deficient) protein diet for 3 weeks. Neutrophils (3×10^7 cells in 1 ml), isolated from the pleural exudate of rats with carrageenan-induced pleurisy, were incubated for 5 min at 37°C with A23187 (20 μM) and [1-^{14}C]arachidonic acid (2 μM). Metabolites of arachidonic acid were analyzed by HPLC or GC-MS as described in the text. The data are presented as means ± S.E., followed by the numbers of determinations in parentheses.

Product	Amount (pmol/ml)		
	Control	Protein deficient	P
Leukotriene B_4	1690 ± 70 (11)	1100 ± 60 (11)	< 0.001
5-HETE	860 ± 150 (13)	760 ± 130 (11)	> 0.05
Thromboxane B_2	260 ± 10 (5)	170 ± 10 (5)	< 0.001
HHT	440 ± 80 (11)	200 ± 40 (7)	< 0.02
Prostaglandin $F_{2\alpha}$	100 ± 10 (3)	20 ± 2 (3)	< 0.001
Prostaglandin E_2	3 ± 0.1 (3)	2 ± 0.1 (3)	< 0.001

Figure 24. Effect of increasing the concentration of exogenous arachidonic acid on the biosynthesis of LTB_4, TXB_2, HHT and 5-HETE by pleural neutrophils from control(C) and protein-deficient (PD) rats. Neutrophils ($3x10^7$ cells in 1 ml) were incubated with A23187 (20 μM) and unlabeled arachidonic acid (12.5-100 μM) for 5 min at 37°C. Each data point is the mean ±S.E. of 4-6 determinations, except for TXB_2, for which each point is based on 2 determinations.


HHT (nmol)
1.0
0.5
0
C
PD
TXB2 (nmol)
1.0
0.5
0
C
PD
5-HETE (nmol)
3.0
2.0
1.0
0
C
PD
LTB4 (nmol)
3.0
2.0
1.0
0
C
PD
0
50
100
[EXOGENOUS ARACHIDONIC ACID]
(μM)

5.6.3. Effects of Protein-calorie Malnutrition on the Biosynthesis of Prostaglandins by Rat Kidney Medulla

The major radioactive products detected in HPLC after incubation of a homogenate of rat kidney medulla with [1-^{14}C] arachidonic acid were prostaglandin E_2 and $F_{2\alpha}$. Smaller amounts of prostaglandin D_2, 6-oxo$PGF_{1\alpha}$, and TXB_2 were also formed (Figure 25). The amounts of PGE_2 and $PGF_{2\alpha}$ synthesized by kidney medulla from exogenous arachidonic acid (37 μM) were measured by GC-MS. Protein deficiency reduced the biosynthesis of PGE_2 and $PGF_{2\alpha}$ by this tissue by approximately 38% (Table 14).

5.6.4. Effects of Protein-calorie Malnutrition on the Biosynthesis of cyclooxygenase and lipoxygenase-dependent products by rat spleen

The major radioactive products detected by HPLC after incubation of a homogenate of spleen with [1-^{14}C] arachidonic acid were prostaglandin D_2 and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). A small amount of thromboxane B_2 was also formed (Figure 26). Protein deficiency decreased the conversion of arachidonic acid to PGD_2 by 39% and to 12-HETE by 65% (Table 15). Increasing the concentration of arachidonic acid from 5 μM to 250 μM had similar effects on the synthesis of PGD_2 and 12-HETE; the formation of both products was considerably decreased by protein deficiency (Figure 27).

5.6.5. Effects of Aspirin on Arachidonic Acid Metabolism by Neutrophils from Control and Protein-deficient rats

The effects of aspirin on the metabolism of [1-^{14}C] arachidonic acid (2 μM) by neutrophils is shown in Figure 28. The biosynthesis of HHT and other cyclooxygenase-dependent products in the prostaglandin/thromboxane region of the chromatogram (mainly thromboxane B_2; see Figures 20, 21) was inhibited by aspirin in a dose-dependent manner. The amounts of these products were

Figure 25. High-pressure liquid radiochromatogram of the products synthesized by rat kidney medulla. The homogenate of the tissue (0.1 g in 1 ml 0.05 M Tris buffer,pH 7.5) was incubated for 10 min at 37°C with [1-^{14}C]arachidonic acid (0.2 μCi, 37 nmol) and the products separated by HPLC on a column of silica with 4% benzene/ethyl acetate/acetonitrile/methanol/acetic acid (30:40: 30:2:0.5,v/v) in hexane/benzene/acetic acid (50:50:0.5,v/v) for the first 20 min, followed by a linear gradient over 40 min between 4% and 100% benzene/ethyl acetate/acetonitrile/methanol/ acetic acid (30:40:30:2:0.5, v/v) in hexane/benzene/acetic acid 50:50:0.5:, v/v).

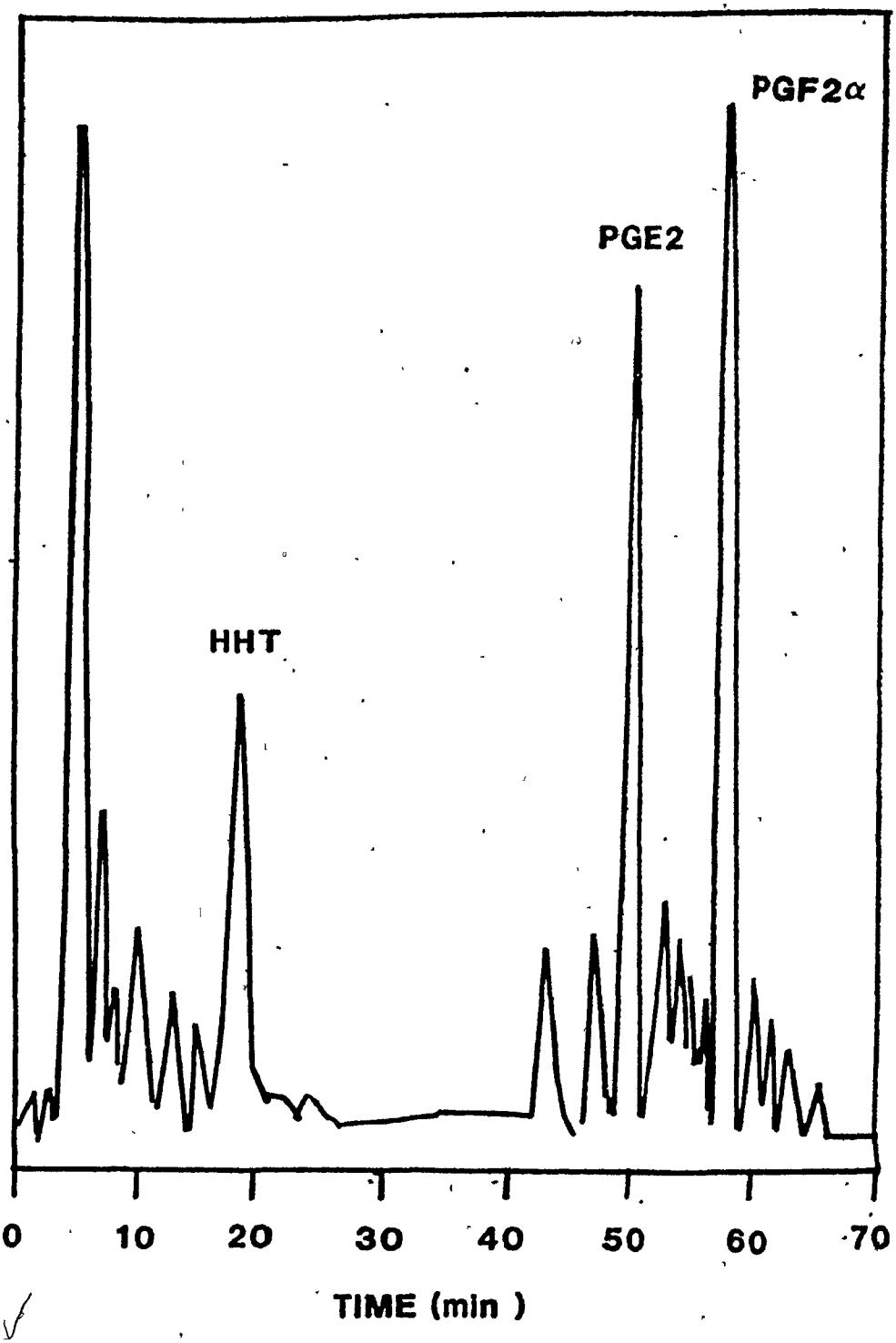

PGF2α
PGE2
HHT
0
10
20
30
40
50
60
70
TIME (min)

Table 14

Biosynthesis of prostaglandins E_2 and $F_{2\alpha}$ by homogenates of kidney medulla from control and protein-deficient rats

Homogenates of kidney medulla (0.1 g tissue in 0.9 ml 0.05 M Tris-HCl, pH 7.5) were incubated for 10 min at 37°C with [1-^{14}C]arachidonic acid (0.2 μCi, 37 nmol). The products were extracted, purified by thin-layer chromatography, and analysed by GC-MS. The values are means ± S.E., followed by the numbers of determinations in parentheses.

Product	Amount (nmol/g tissue)		
	Control	Protein deficient	P
Prostaglandin E_2	9.4 ± 1.1 (5)	5.9 ± 0.7 (5)	< 0.05
Prostaglandin $F_{2\alpha}$	28.1 ± 3.5 (5)	17.3 ± 2.3 (5)	< 0.025

Figure 26. High-pressure liquid radiochromatogram of products synthesized by spleen homogenate from a control rat. Spleen homogenate (0.2 g tissue in 1 ml 0.05 M Tris-HCl buffer, pH 7.5) was incubated for 10 min at 37°C with [1-^{14}C]arachidonic acid (4 μM) and the products separated by HPLC on a column of silica with 4% benzene/ethyl acetate/acetonitrile/methanol/acetic acid (30:40:30:2:0.5, v/v) in hexane/benzene/acetic acid (50:50:0.5, v/v) for the first 20 min, followed by a linear gradient over 40 min between 4% and 100% benzene/ethyl acetate/acetonitrile/methanol/acetic acid (30:40:30:2:0.5, v/v) in hexane/benzene/acetic acid (50:50:0.5, v/v).

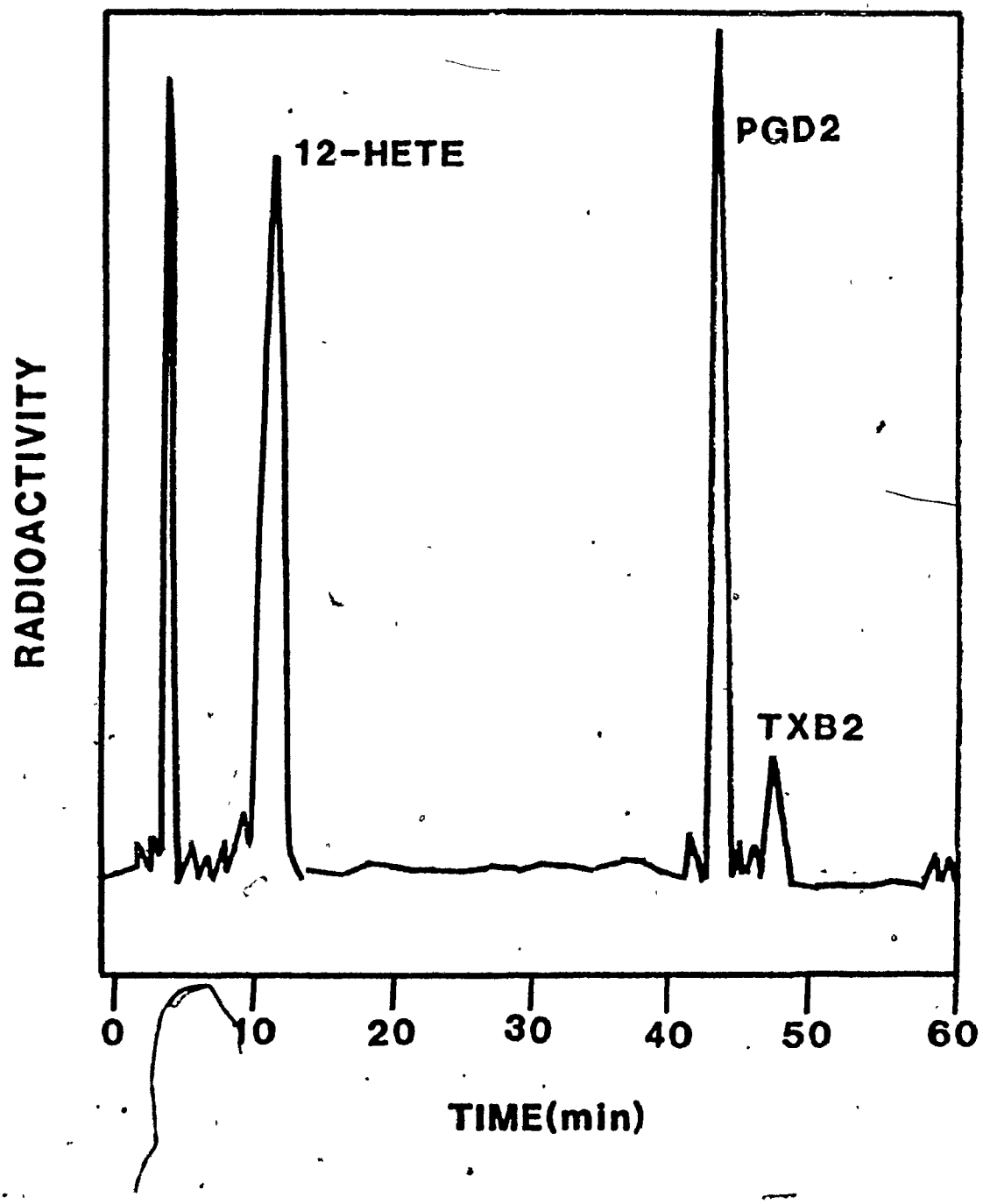

12-HETE
PGD2
TXB2
RADIOACTIVITY
0
10
20
30
40
50
60
TIME(min)

Table 15

Biosynthesis of PGD_2 and 12-HETE by homogenates of spleen from control and protein-deficient rats

Homogenates of spleen (0.15 g tissue in 1 ml 0.05M Tris-HCl buffer, containing 0.02M EDTA-2Na, pH 7.5) were incubated for 10 min at 37°C with [1-^{14}C]arachidonic acid (0.2 μCi) and unlabelled arachidonic acid (250 μM). The fractions corresponding to prostaglandin D_2 and 12-HETE were collected and radioactivity measured by liquid scintillation counting. The values are means ± S.E.

Product	n	control rats	protein-deficient rats
		cpm x 10^{-4}/g tissue	
PGD_2	13	7.62 ± 0.93	4.63 ± 0.58*
12-HETE	13	11.19 ± 1.40	3.83 ± 0.66*

*$P < 0.02$

Figure 27. Effect of increasing concentrations of exogenous arachidonic acid on the biosynthesis of PGD_2 and 12-HETE by spleen homogenates from rats fed ad libitum a 21% (control) and a 5% (protein-deficient) protein diet for 3 weeks. Spleen homogenates (0.15 g of tissue in 1 ml 0.05 M Tris-HCl containing 0.02 M EDTA-2Na, pH 7.5) were incubated with [1-^{14}C]arachidonic acid (0.2 μCi) and unlabeled arachidonic acid (5-250 μM) for 10 min at 37°C. The radioactivity of fractions corresponding to PGD_2 and 12-HETE was measured. The formation of PGD_2 and 12-HETE was calculated assuming an absence of endogenous substrate. Each data point is based on 2 determinations done in duplicate.

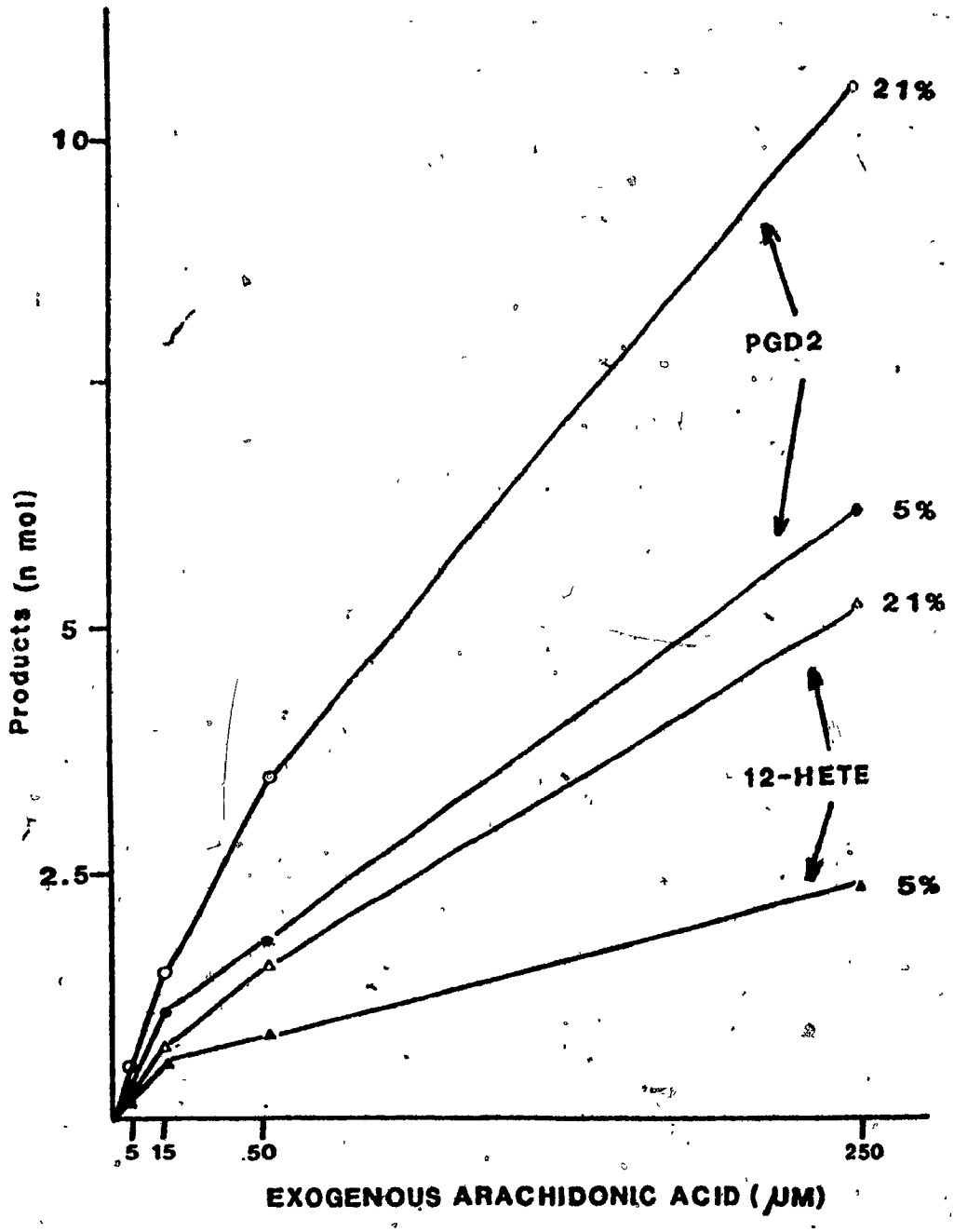
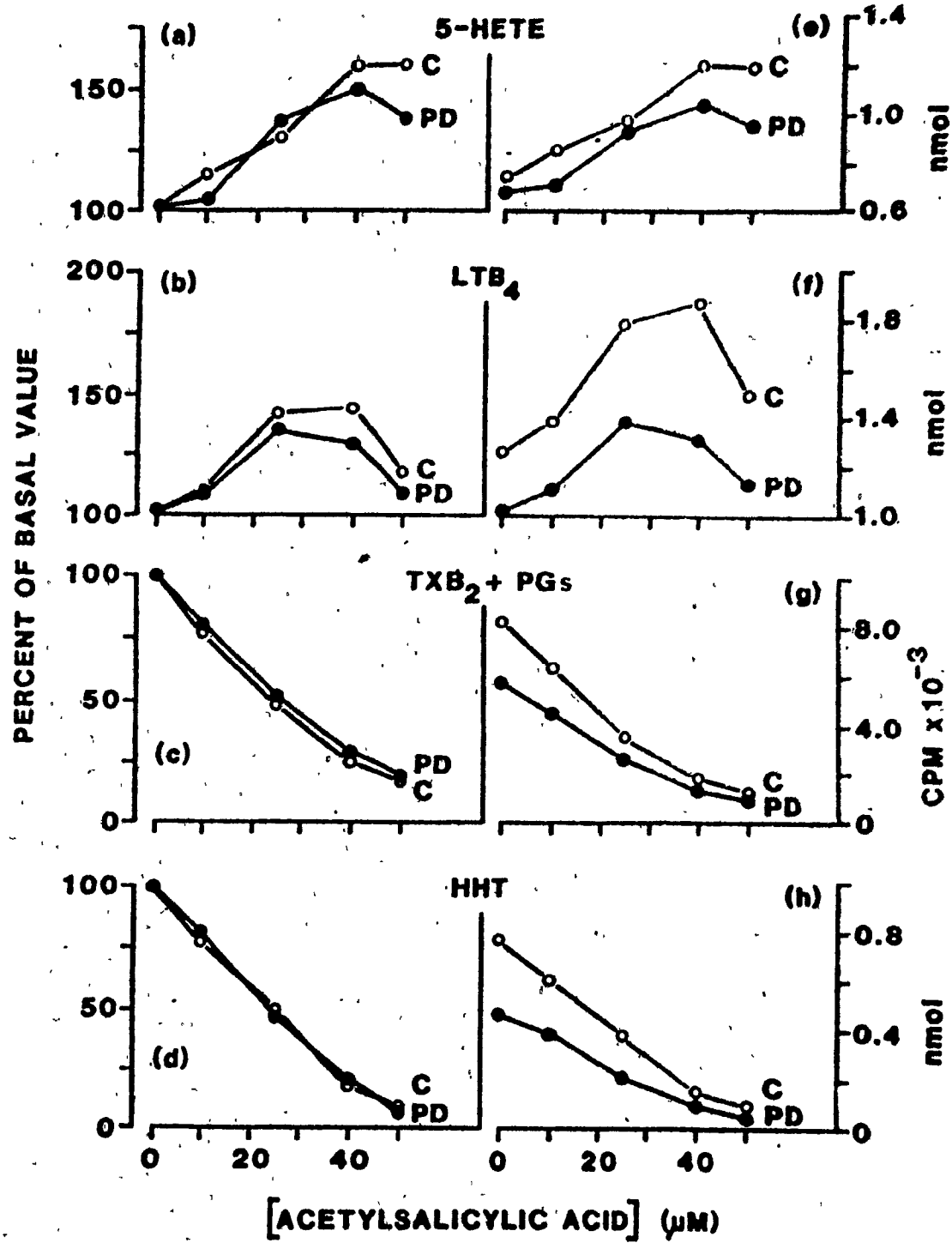

21%
10
PGD2
5%
21%
Products (n mol)
5
12-HETE
2.5
5%
.5
15
.50
250
EXOGENOUS ARACHIDONIC ACID (μM)

Figure 28. Effects of aspirin on arachidonic acid metabolism by pleural neutrophils from rats fed ad libitum a 21% (control) and a 5% (protein-deficient) protein diet for 3 weeks. Neutrophils ($3x10^7$ cells in 1 ml) were incubated for 10 min at 37°C with various concentrations of aspirin and then for an additional 5 min with A23187 (20 μM) and [1-^{14}C]arachidonic acid (2 μM). The products were extracted and analyzed by reversed-phase HPLC. LTB_4, HHT and 5-HETE were quantitated on the basis of ultraviolet absorbance. The radioactivity in the prostaglandin and thromboxane fraction (t_R 8-12 min, cf. Figure 20) was measured by liquid scintillation counting. The panels on the left (a,b,c and d) show the percent changes compared to the basal values obtained in the absence of aspirin, whereas those on the right (e,f,g and h) show the absolute values. Each value is the mean of 2 experiments done in duplicate. C: control; PD: protein-deficient.

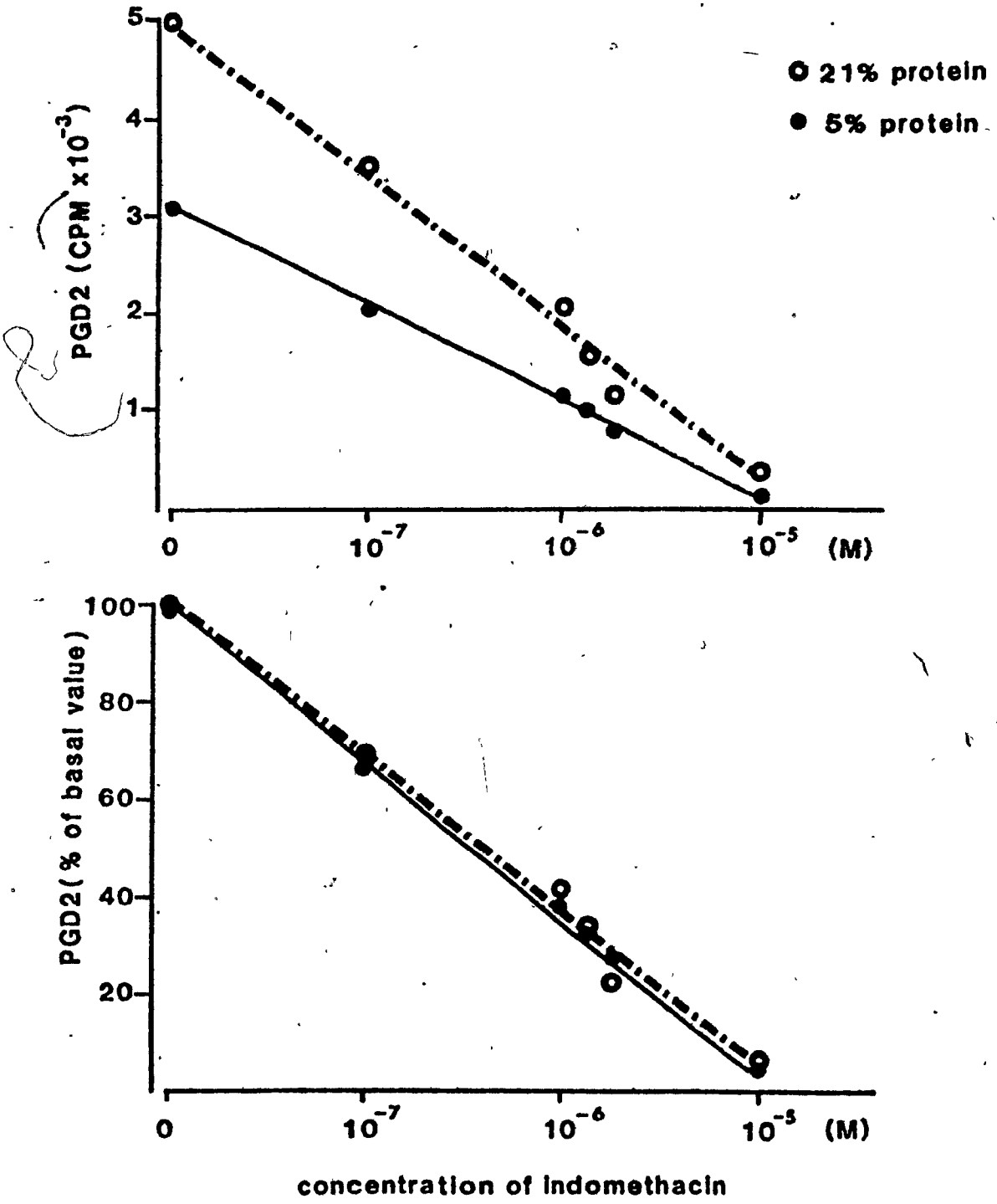

(a)
5-HETE
(e)
C
PD
150
100
1.4
1.0
0.6
nmol
(b)
LTB4
(f)
200
150
100
1.8
1.4
1.0
nmol
PERCENT OF BASAL VALUE
TXB2 + PGs
(g)
100
50
0
(c)
8.0
4.0
0
CPM x10-3
HHT
(h)
(d)
0.8
0.4
0
nmol
0
20
40
[ACETYLSALICYLIC ACID] (μM)

lower in the case of protein-deficient rats than in the case of controls at all concentrations of aspirin. The concentration of aspirin required to inhibit the synthesis of cyclooxygenase-dependent products by 50% of the maximum (IC_{50}), however, was similar in both groups of rats. In contrast to the inhibitory effect of aspirin on the formation of cyclooxygenase products, the formation of the lipoxygenase products, leukotriene B_4 and 5-HETE, was stimulated in neutrophils from both protein-deficient and control rats; this stimulatory effect was less pronounced in preparations from protein-deficient than in those from control rats.

5.6.6. Effect of Indomethacin on the Biosynthesis of Prostaglandin D_2 by Spleen Homogenates from Control and Protein-deficient Rats

Figure 29 shows the effect of indomethacin on the biosynthesis of PGD_2 from [1-^{14}C] arachidonic acid (50 μM) by spleen homogenates. The synthesis of PGD_2 was inhibited by indomethacin in a dose-dependent manner. As with aspirin, the formation of PGD_2 by spleen homogenates of protein-deficient rats was significantly lower than that of controls at all concentrations of indomethacin. At the higher concentrations of indomethacin (2.5×10^{-6}M and 1×10^{-5}M), the formation of 12-HETE increased by 5-10% in both control and protein-deficient rats.

Figure 29. Effects of indomethacin on the biosynthesis of prostaglandin D_2 by spleen homogenates from rats fed ad libitum a 21% (control) and a 5% (protein-deficient) protein diet for 3 weeks. Spleen homogenates (0.1 g tissue in 1 ml of 0.05 M Tris buffer containing 0.02 M EDTA-2Na, pH 7.5) were first incubated for 2 min at 37°C with various concentrations of indomethacin and then for an additional 10 min with [1-^{14}C]arachidonic acid (50 μM). A known amount of ^{3}H-$PGF_{2\alpha}$ was added (for checking recovery). The products were extracted and analyzed by normal phase HPLC (cf.Figure 26). The radioactivity in the PGD_2 fraction (t_R 42-45 min, cf.Figure 26) was measured by liquid scintillation counting. The bottom panel shows the percent changes compared to basal values obtained in the absence of indomethacin, whereas the top panel shows the absolute values. Each value is the mean of two experiments done in duplicate.


21% protein
5% protein
PGD2 (CPM x10^{-3})
5
4
3
2
1
0
10^{-7}
10^{-6}
10^{-5}
(M)
PGD2(% of basal value)
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
(M)
concentration of indomethacin

6. DISCUSSION

6. DISCUSSION

Although it has been recognized that dietary protein deficiency can modify biological activity, metabolism and pharmacokinetics of drugs (Boyd, 1972; Campbell and Hayes, 1974; Krishnaswamy, 1978; Varma, 1981), sufficient information does not exist to permit any generalization about its consequences on the pharmacology of drugs or even a specific class of drugs. Unfortunately, protein-calorie malnutrition (PCM) is widespread. It therefore seemed relevant to study the influence of PCM on one of the world's most commonly used therapeutic agents, namely salicylates, which are the prototype of a group of nonsteroidal anti-inflammatory agents (Flower, Moncada and Vane, 1980). The main purpose of this investigation was to find out if PCM alters the anti-inflammatory activity of salicylate, and if so, to determine the underlying mechanism of such changes.

The experimental model and the dietary regimen selected for the present study seem appropriate for several reasons. We used rats of approximately 5 week initial age and 100-125g initial body weight. The effects of dietary protein deprivation are not only sufficiently marked in this group of rats but also because animals belonging to this age group were found extremely suitable for all the studies which could be anticipated in the beginning, namely a measurement of anti-inflammatory activity, determination of pharmacokinetics, measurement of lysosome stability and arachidonic acid metabolism. Dietary protein deprivation in much older rats does not induce pronounced effects (Donald et al., 1981; Boyd, 1972; Varma, 1981). Weanling rats are too severely affected by dietary protein deprivation (Edozien, 1968) and we found in initial experiments that these animals were too small for some of the present studies (measurement of anti-inflammatory activity of drugs and pharmacokinetics). A reduction of dietary protein to approximately one-half

the optimum, that is to 10%, did not produce sufficient changes to permit an accurate quantitation of differences in drug effects in these and control animals. A reduction of dietary protein to nearly zero level (0.5%) produced too severe an effect; a proportion of animals died after 2-4 weeks and the surviving animals were too small to allow these studies. In view of these considerations, 100-125 g male rats maintained on a 5% protein diet were used in the present study.

The effects of dietary protein deficiency found in these studies are in accordance with other reports (Boyd, 1972; Coward et al., 1977; Mgbodile and Campbell, 1972; Varma, 1979). Body weight gain, liver weight, hepatic total and microsomal proteins, plasma total proteins, albumin and globulins, were all reduced in rats fed a 5% protein diet. Because the rats fed a low protein diet consumed only approximately 60% as much food as the control rats, they suffered from both protein and calorie deficiency (protein-calorie malnutrition, PCM). In an attempt to distinguish between the effects of PCM and calorie deficiency alone, pair-fed controls were used. As can be seen in Table 1, a mere restriction in calories only caused a reduction in total body weight and liver weights. Subsequent studies demonstrated that many of the changes in the effects of salicylate, which were observed in protein-calorie deficient rats were not found in pair-fed animals. It was therefore concluded that the changes in the anti-inflammatory activity of salicylate are caused primarily by a deficiency of protein rather than of calories. In view of this, most studies designed to determine the mechanisms of changes found in protein-deficient rats did not include pair-fed controls.

Central to the present study was the observation that PCM led to a significant increase in the anti-inflammatory activity of salicylate. This was found with both sodium salicylate and aspirin and in both the tests used for

the assay of anti-inflammatory activity, namely a suppression of carrageenan-induced paw edema and carrageenan-induced pleurisy. A similar increase in the anti-inflammatory activities of indomethacin and oxyphenbutazone was also observed in protein-deficient animals and the ability of PCM to increase the anti-inflammatory and/or ulcerogenic effects of oxyphenbutazone and phenylbutazone has been previously reported (Varma, 1979; Varma, 1980c). This suggests that protein-calorie malnutrition leads to an increase in the anti-inflammatory activity of not only salicylates but of all nonsteroidal anti-inflammatory agents; possibly other effects such as ulcerogenic activity are also increased by PCM. During the latter part of this discussion we shall be dealing with the possible mechanism for this common observation with nonsteroidal drugs. It should be mentioned, however, that all these observations relate to the acute anti-inflammatory activity of these agents. Whether or not a similar increase in the chronic anti-inflammatory activities of nonsteroidal anti-inflammatory agents will be caused by PCM remains to be studied.

As mentioned above, these changes in the anti-inflammatory activity of salicylate in rats fed a low protein diet were caused primarily due to a deficiency of protein and not of calories because no such increase was observed in calorie-deficient animals (pair-fed controls).

In order to determine the mechanism of the observed increase in the anti-inflammatory activity of salicylate in animals fed a low protein diet, which as argued above suffered from both protein and calorie malnutrition, three factors, each of which singly or in combination could contribute to these changes, were examined. These possibilities were: (1) pharmacokinetic changes, (2) changes in the concentration or release of lysosomal enzymes, and (3) changes in the metabolism of arachidonic acid. The rationale for undertaking

these studies and the conclusions drawn from them are presented below.

Protein deficiency has been shown to inhibit the metabolism and alter the pharmacokinetics of a variety of drugs both in humans and animals (see Campbell and Hayes, 1974; Krishnaswamy, 1978; Varma, 1981). Changes in the pharmacological effects of drugs have been attributed to decreases in their metabolism by the liver. For example, toxicities of pentobarbitol, strychnine, aminopyrine and zoxazolamine (Kato et al., 1968), carbon tetrachloride (McLean and McLean, 1966), heptachlor (Weatherholtz et al., 1969) and many other drugs (Newberne et al., 1978) in protein-deprived rats were clearly related to changes in the metabolism of these agents. Another study showed that changes in the protein and carbohydrate composition of the diet influenced the biotransformation of antipyrine and theophylline in humans (Kappas et al., 1976); a low protein diet resulted in a 50-60% increase and a high protein diet caused a 35-40% decrease in the half-lives of antipyrine and theophylline. Monckeberg (1978) reported an increase in the plasma half-life of salicylic acid in children with severe marasmic malnutrition. Moreover, salicylates are extensively bound to serum albumin (Flower et al., 1980; Levy, 1979) and it has been shown that PCM is associated with a decrease in serum albumin concentration both in humans and rats (see Campbell and Hayes, 1974; Krishnaswamy, 1978; Varma, 1981). A decrease in serum albumin concentration in rats fed ad libitum a low protein diet was also observed in the present study (Table 1). It is widely agreed that only the free fraction of a drug is available for biological activity and passage across biological membranes (Goldstein, 1949); this aspect has been repeatedly emphasized by Levy (1979) in relation to salicylates. It was therefore considered necessary to examine if the observed increase in the anti-inflammatory activity of salicylate in protein-deficient rats is due to any changes in its pharmacokinetics.

As in humans, the kinetics of salicylate were dose-dependent in both control and protein-deficient rats. However, unlike in humans, the overall elimination in rats appeared to follow a first-order rather than a zero-order process up to a dose of 100 mg/kg sodium salicylate. This is similar to data reported by other workers (Nelson et al., 1966) and could be attributed to the finding that only approximately 30% of salicylate was excreted as salicyluric acid.

Protein-calorie malnutrition is generally associated with an increase in the plasma half-life and a decrease in the metabolism of drugs (Campbell and Hayes, 1974; Krishnaswamy, 1978; Varma, 1981); however, the influence of protein deficiency on the pharmacokinetics of salicylate was in contrast to these reports. At 10- and 100 mg/kg dose levels, the plasma $t_{1/2}$ was shorter and the clearance greater in protein-deficient than in control rats. Also at 2- and 200 mg/kg doses of sodium salicylate the plasma concentrations of salicylic acid at all time periods (Figures 10, 11) were lower in protein-deficient rats than in controls. Because there was no difference in the volume of distribution between the two groups of rats, it appears that the increase in plasma clearance of salicylate in protein-deficient rats is due to an increase in its elimination rather than in the volume of distribution.

The urinary data also showed that the elimination half-life of salicylate was shorter in protein-deficient rats than in controls. This is consistent with the results of plasma data. A slight discrepancy in the urinary elimination half-life and plasma half-life of salicylate both in control and protein-deficient animals (Tables 6, 7) could be attributed to the relatively long duration of urine-collection intervals.

In order to define the mechanism for protein deficiency-induced changes in the pharmacokinetics of salicylate, urinary excretion of salicylic acid and its metabolites were measured. Because the urinary pH and protein concentration of the two groups of rats were similar, it is unlikely that the increased urinary excretion of salicylate in protein-deficient rats was due to differences in tubular ionization (Flower et al., 1980) or increased excretion of the protein-bound moiety.

The excretion of total salicyl glucuronides in the two groups of rats was not different. We estimated total glucuronides by hydrolysis of urinary samples by β-glucuronidase. It has been reported that ester glucuronides of carboxylic acid tend to form glucuronidase resistant isomers, which is due to the intramolecular rearrangement of the ester glucuronides if the samples are kept at an alkaline pH prior to the hydrolysis (Illing and Wilson, 1981; Sinclair and Caldwell, 1982). Because the urinary pH of the samples in the present study was 6.4±0.3 in control and 6.4±0.2 in protein-deficient rats, it is unlikely that the total salicyl glucuronides were underestimated. Nevertheless, this possibility cannot be totally excluded.

The present data show that the increased urinary excretion of salicylate in PCM rats is mainly the result of increases in the excretion of salicyluric acid and salicylic acid, the former being more important than the latter. In control animals salicyluric acid was excreted at a constant rate, a finding consistent with that reported by others (Nelson et al., 1966). Hence, it was not possible to calculate the rate constants of excretion and formation of salicyluric acid in control rats (Table 7). However, in protein-deficient animals salicyluric acid excretion declined as a function of time and was significantly higher than in controls at every time period measured (Figure 12). Inasmuch as salicyluric acid is readily excreted by glomerular filtration (Nelson et al.,

1966; Milne, 1963), the rate of its excretion could be treated as an indication of the rate of its formation. It would thus appear that the glycine conjugation of salicylic acid reached a saturation level in the control but not in protein-deficient rats. If this assumption is correct, one would expect to demonstrate an increase in glycine conjugation by tissues of protein-deficient animals.

The studies on the metabolism of salicylate by mitochondrial preparations do offer direct evidence of an increase in the synthesis of salicyluric acid in protein-deficient animals. An increase in the activity of UDP-glucuronyltransferase in protein-deficient rats has also been reported by Woodcock and Wood (1971). We prepared mitochondrial fractions according to the method of Forman et al. (1971); however, we found the conversion of salicylic acid to salicyluric acid by the kidney but not by the liver mitochondrial preparations. On the other hand, Forman et al. (1971) have demonstrated salicyluric acid synthesis by beef liver mitochondrial preparations. Our results show that rat liver is not an efficient system for the glycine conjugation of salicylate as has also been suggested by Litter et al. (1957).

The absorption of salicylate was rather complete in both groups of rats and there was no significant difference in the relative bioavailability of salicylate, as has also been reported for phenylbutazone (Varma, 1979) and dexamethasone (Varma and Mulay, 1980).

There was no difference in the binding of salicylic acid to serum protein of control and protein-deficient rats in vivo but the binding to the serum of the latter group of animals was significantly less than to control serum in vitro (Table 9). It is possible that a rapid metabolism or elimination of the free fraction of salicylic acid in protein-deficient rats masked any

differences in protein-binding in the in vivo situation. Assuming that the in vitro protein-binding data reflect the dynamic situation in the whole animal, a low protein binding of salicylate could have contributed to a higher urinary excretion of salicylic acid in protein-deficient rats (Figure 12). This in turn could contribute to a lower plasma drug concentration concomitant with an increased delivery of the free drug to target tissues and enhanced biological activity. In order to test this possibility, salicylate concentrations in soft tissues of inflamed and non-inflamed paws (Figure 13) and in other tissues (Table 11) were measured. However, no difference in drug concentration in paw tissue from the two groups of rats were found. This would suggest that there was no significant increase in the delivery of salicylate to target tissues. It seems that the decreased binding of salicylate in protein-deficient rats is not a significant factor in the observed increase in its anti-inflammatory activities.

To our knowledge the only other study on the influence of malnutrition on the pharmacokinetics of salicylate is by Monkeberg et al. (1978); the details of this study are, however, not available. In contrast to our data in rats, these workers found that the plasma half-life of salicylate was greater in infants suffering from severe marasmic undernutrition than in healthy infants; however, it is apparent from their data that absolute plasma concentrations of salicylate were lower in marasmic than in healthy infants. The apparent discrepancy between the findings of these workers and the present results might be due to species and/or the severity and nature of malnutrition; severe marasmic undernutrition in infants could have altered the renal function and volume of distribution of salicylic acid. The present data indicate that the pharmacokinetics of salicylate are different from those of phenylbutazone and oxyphenbutazone; the plasma half-lives of the latter two agents were longer and their metabolism slower in protein-deficient than in control rats (Varma,

1979, 1980c). The difference could be due to the fact that the major metabolic route of phenylbutazone and oxyphenbutazone, but not of salicylate, involves hydroxylation by microsomal mixed-function oxidases, which are inhibited during protein malnutrition (Campbell and Hayes, 1974; Varma, 1980a).

In conclusion, despite a decrease in protein salicylate binding, pharmacokinetic data as a whole do not explain the observed increase in the effects of salicylate in protein-deficient animals. Moreover, the relationship between plasma concentration and drug effect may be more valid in homogeneous (that is, between the control rats) rather than in heterogeneous population samples (e.g. control and protein-deficient rats).

The second aspect of the present investigation dealing with the mechanism of the enhanced anti-inflammatory activity of salicylates in protein-deficient rats was to find out if this could be attributed to changes in their lysosome-stabilizing effects. In order to test this possibility, the anti-inflammatory and lysosome-stabilizing effects of sodium salicylate and aspirin were measured under as similar conditions as possible. Other nonsteroidal and steroidal anti-inflammatory agents were also studied for purposes of comparison and verification of the results with salicylate.

The present results concerning the paw edema suppressant activities of oxyphenbutazone and dexamethasone are similar to those reported previously from this laboratory (Varma, 1980c; Varma and Mulay, 1980). Also, the choice of glucuronidase as the marker enzyme to assess lysosomal membrane stability yielded results similar to those in the literature (Ignarro, 1972; Ignarro and Slywka, 1972). Thus the protocol used in the present study seems justified.

As mentioned in the Methods section, an increase in the ratio of free to total lysosomal enzyme activities is indicative of an impairment of lysosomal membrane integrity or the labilizing effects of a treatment. Such an impairment of lysosomal membrane integrity was observed in protein-deficient rats. These findings are in accordance with the reported increases in the ratios of free to total lysosomal enzyme activities in nutritionally deficient (Beaufay et al., 1959) and starved rats (Beaufay et al., 1959; Gudar et al., 1970). The observed increase in the ratios of free to total enzyme activities in protein-deficient animals reflects actual labilization of lysosomal membranes and does not appear to be a consequence of quantitative changes in total lysosomal enzymes because the results with both glucuronidase and aryl sulfatase were similar although the former enzyme activity decreased and the latter increased in protein-deficient rats.

Because a calorie restriction alone (pair-fed controls) did not and protein-calorie deprivation did result in changes in lysosome enzyme activities, it can be assumed that the influence of a low protein diet on lysosomal enzyme activities is due primarily to a protein rather than a calorie deficiency.

It is difficult to assess the significance of changes in total enzyme activities and membrane stability of lysosomes on inflammatory processes during protein-calorie malnutrition. Assuming that lysosomal enzymes are among the mediators of inflammatory responses (Ignarro, 1974; Weissmann, 1972), the observed decrease in lysosomal membrane integrity in protein-deficient animals may not imply a net increase in the amount of released enzymes. For example, an increase in membrane fragility might not result in an increase in net released enzyme activity because of the decrease in total β-glucuronidase activity. On the other hand, the net release of arylsulfatase and acid phosphatase might increase in protein-deficient animals. Because the actual

inflammatory response as judged by an increase in paw volume following carrageenan injection was not different in control and protein-deficient animals, either changes in lysosomal membrane integrity did not play any role or their roles were obscured by other associated changes such as a decrease in the metabolism of arachidonic acid. In any case, if a drug were to exert its anti-inflammatory activity through the inhibition of the release of lysosomal enzymes, it would be less effective in stabilizing a membrane already labilized due to dietary protein deprivation. Arguments as to whether or not lysosomal enzymes are involved in the action of nonsteroidal anti-inflammatory agents are presented below.

There are only a few studies on the effects of nonsteroidal and steroidal anti-inflammatory agents on lysosomal stability in animals treated with these agents in vivo before the isolation of lysosomes (Ignarro, 1972; Ignarro and Slywak, 1972; Pollock and Brown, 1971). We used the in vivo model in order to relate any changes in lysosomal membrane stability to anti-inflammatory effects of drugs. The lysosome-stabilizing effects of drugs were assessed on the basis of changes in the ratio of the free to total β-glucuronidase activity. This technique is similar to that used by other workers (Deter and deDuve, 1967; Guder et al., 1970; Ignarro, 1971b; Pollock and Brown, 1971; Welman, 1979) for estimating the lysosome stabilizing activity of drugs. In so far as the membrane integrity is a determinant of the release of lysosomal enzymes, an estimate of the free as a fraction of the total enzyme activity seems a reasonable measure of the stability of the lysosomal membrane. Moreover, this technique minimizes the influence of variability between different animals within a group and between experiments done on different days, as well as provides a simple way of comparing drug effects in different experimental groups such as control and protein-deficient animals.

The present results are qualitatively similar to previously reported data on the lysosome stabilizing activities of aspirin, oxyphenbutazone and indomethacin (Ignarro, 1972) as well as of dexamethasone (Pollock and Brown, 1971). However, the results of the present study taken as a whole do not show any definite relationship between lysosome-stabilizing and paw edema-suppressant activities of salicylates (sodium salicylate and aspirin) and other nonsteroidal anti-inflammatory drugs. Although all the agents used in the present study, with the exception of sodium salicylate and aspirin at high dose levels, caused an inhibition of paw edema as well as a stabilization of lysosomes, a consistent relationship between the paw edema suppressant and lysosome-stabilizing effect in control and protein-deficient animals was observed only with dexamethasone. Sodium salicylate at a 50 mg/kg dose level was equieffective in stabilizing lysosomes of livers from control as well as protein-deficient animals, although its paw edema suppressant activity was less in the former than in the latter group of rats. At a 200 mg/kg dose level, sodium salicylate as well as aspirin produced a labilizing effect on lysosomes, but caused a significant inhibition of paw edema in both groups of rats. Oxyphenbutazone and indomethacin were equieffective in stabilizing lysosomes of liver from control and protein-deficient animals; however, like salicylates, both these agents produced a greater inhibition of paw edema in protein-deficient rats than in controls. The lack of an apparent relationship between paw edema-suppressant and lysosome-stabilizing effects of nonsteroidal anti-inflammatory agents would suggest that stabilization of the lysosomal membrane is not a major factor in their anti-inflammatory activity. Therefore, the underlying mechanism for protein deficiency-induced increase in the effects of salicylate must involve other factors. Whether or not there is a causal relationship between the anti-inflammatory and lysosome-stabilizing effects of steroidal agents cannot be established on the basis of the present study. Nevertheless, the results of the present study can be interpreted to support the

view of other workers that lysosome stabilization might be a step in the activity of steroidal anti-inflammatory agents (Gryglewski, 1979; Ignarro, 1974; Pollock and Brown, 1971; Thompson, 1979).

To sum up the arguments advanced above, the differences in the anti-inflammatory activity of salicylate in control and protein-deficient rats cannot be attributed to changes in the pharmacokinetics or the lysosome stabilizing activity of salicylate.

According to the current widely accepted view, nonsteroidal anti-inflammatory agents exert their effects in inhibiting the synthesis of prostaglandins (Flower et al., 1980; Kuehl and Egan, 1980; Vane, 1971). In order to determine if changes in the anti-inflammatory activity of salicylate in protein-deficient animals are a consequence of any alterations in the metabolism of arachidonic acid, rat pleural neutrophils were used in the present study. Neutrophils were selected because they play an important role in both amplifying and sustaining inflammatory responses and can transform arachidonic acid by both the 5-lipoxygenase and cyclooxygenase pathways. Some studies were also done with kidney medulla and spleen, both of which possess very high cyclooxygenase activity.

The major metabolites of arachidonic acid synthesized by rat pleural neutrophils were identified as LTB_4, 5-HETE, HHT and TXB_2. Only small amounts of PGs and LTB_4 isomers were detected. This is in agreement with reports in the literature in which products having chromatographic properties indentical to those of LTB_4, 5-HETE and HHT (Siegel et al., 1981) were found to be released from rat neutrophils in response to stimulation with ionophore. It has recently been reported that the main lipoxygenase-dependent metabolite of arachidonic acid by swine blood leukocytes was an isomer of LTB_4, 5S,

12S-dihydroxy-(E.Z.E.Z.)- 6,8,10,14-icosatetraenoic acid, which co-chromatographed with LTB_4 on a reverse phase HPLC column and had much lower bioactivity than LTB_4 (Borgeat, 1981). Therefore, all products in the present study were identified not only by comparison of their chromatographic properties with those of the standards, but also on the basis of their mass spectra.

In view of the important roles of prostaglandins and leukotrienes in various physiological and pathological processes, especially in inflammatory responses, the effects of various physiological states and drugs on the metabolism of arachidonic acid have been studied. Considerable attention has been recently given to the effects of alterations in dietary fatty acids on the biosynthesis of prostaglandins (Beitz and Forster, 1980; Nugteren et al., 1980; Schoene et al., 1980). However, we are not aware of any studies on the effects of dietary protein intake on the metabolism of arachidonic acid. The data of this study reveal that dietary protein deficiency has considerable effects on the metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase pathways. Protein deficiency in rats resulted in an approximately 45% reduction in the synthesis of cyclooxygenase-derived products by neutrophils. Similar reductions were observed in the biosynthesis of PGE_2 and $PGF_{2\alpha}$ by kidney medulla (Table 13) and of PGD_2 (Table 14) by spleen homogenates of protein-deficient rats.

Although the synthesis of 5-HETE, at least at low substrate concentrations, was not affected by protein deficiency, LTB_4 formation was decreased. This indicates that there was a reduction in the activity of the leukotriene pathway, especially in the dehydrase activity which converts 5-hydroperoxy-6,8,11,14- eicosatetraenoic acid (5-HPETE) to LTA_4. The synthesis of the LTB_4 isomers (t_R 21.5 and 23.5 min, Figure 20) was also

reduced in a manner similar to LTB_4 as a result of protein deficiency.

Because metabolites of arachidonic acid which are mediators of inflammation are decreased by protein-calorie malnutrition, one would expect a decrease in the inflammatory response to appropriate stimuli in this condition. However, no reduction in the inflammatory response to injections of carrageenan was observed in protein-deficient animals; indeed, the volume of pleural exudate per unit body weight following carrageenan was greater in protein-deficient than in control rats. Several factors could have contributed to this apparent discrepancy. Although there is an overwhelming evidence that prostaglandins and leukotrienes are mediators of inflammatory responses, additional factors also play a role directly or indirectly in defining the overall response to an inflammatory stimulus (Vinegar et al., 1976, 1982). We discussed above the possibility of an increased release of lysosomal enzymes during protein deficiency because of an impairment of lysosomal membrane integrity. Protein-calorie malnutrition is associated with an increase in capillary permeability (unpublished observations), which would tend to accentuate acute inflammatory responses. Moreover, the response of cells to prostaglandins and/or the interaction between prostaglandins and other autacoids might change during protein deficiency. Other possibilities can be speculated. It is therefore possible that one or more of the above factors compensate for the decreased metabolism of arachidonic acid and thereby permit the expression of an inflammatory response in a quantitatively similar fashion in protein-deficient and control animals.

As expected, aspirin inhibited the formation of cyclooxygenase-dependent products of arachidonic acid by pleural neutrophils in a dose-dependent manner, confirming other reports (Siegel et al., 1980). An inhibition of these products was associated with an increase in lipoxygenase-derived products, presumably

due to an increase in the substrate available for metabolism by the uninhibited 5-lipoxygenase pathway. Both of these effects of aspirin were influenced by dietary protein deficiency, so that cyclooxygenase as well as lipoxygenase-dependent metabolites of arachidonic acid were significantly less in preparations from PCM than control rats at all concentrations of aspirin.

The difference in the inhibitory effects of aspirin in control and protein-deficient rats does not appear to be due to any fundamental changes in cyclooxygenase-aspirin interaction, but rather to a decrease in the enzyme activity in protein-deficient animals. This is indicated by the fact that the concentration of aspirin required to inhibit cyclooxygenase-dependent metabolism of arachidonic acid to 50% of the maximal (IC_{50}) was not altered but rather the net production of these metabolites was reduced. A lack of difference in the IC_{50} merely shows that there is no difference in the actual potency of aspirin. Moreover, a comparison of IC_{50} values between the two groups of rats may not be justified in view of the fact that the maximal biosynthesis of cyclooxygenase-dependent products (equivalent to maximal responses to drugs) were quite different in the two groups of animals.

The results with indomethacin are confirmatory to the observations made with aspirin. For example, net biosynthesis of PGD_2 by spleen homogenates in the absence as well as in the presence of indomethacin was less in homogenates of spleens from protein-deficient than control animals but the IC_{50} values of indomethacin were not altered.

It follows from the above discussion that the main mechanism of the increased anti-inflammatory activity of salicylate in protein-deficient animals is a decrease in cyclooxygenase-dependent products of arachidonic acid. An additional component in the increased effect of salicylate in protein-deficient

rats could be a reduction in the production of lipoxygenase-dependent metabolites of arachidonic acid (leukotrienes). Ordinarily an inhibition of the cyclooxygenase pathway leads to an increase in products formed by the lipoxygenase pathway. Because the latter products are also pro-inflammatory and have a synergistic effect with prostaglandins in inducing inflammation (Wedmore and Williams, 1981; Higgs et al., 1981; Smith, 1981; Bray et al., 1981a; Morley et al., 1981), they tend to partially counter the anti-inflammatory action of aspirin-like drugs. These lipoxygenase-dependent products increased following aspirin in both groups of rats but the increase was less in protein-deficient than in control rats. Because the levels of pro-inflammatory metabolites of arachidonic acid formed via both the lipoxygenase and cyclooxygenase pathways are considerably lower as a result of protein deficiency, a smaller amount of salicylate would be required to reduce the levels of cyclooxygenase-dependent products below those necessary for their inflammatory effects, although the IC_{50} value of the drug is unaltered.

The present study has dealt with the role of arachidonic acid metabolism in the anti-inflammatory activity of sodium salicylate and aspirin in rats with protein-calorie malnutrition. The conclusions derived from these results could be applicable to other aspirin-like agents as well as to other effects of this group of drugs. It seems justified to attribute the PCM-induced increase in the anti-inflammatory activities of oxyphenbutazone and indomethacin (Yue and Varma, 1981; Varma, 1980c) and the ulcerogenic effects of pyloric ligation, phenylbutazone and oxyphenbutazone (Varma, 1979; Varma, 1980c) to a decrease in the metabolism of arachidonic acid in these animals.

In summary, the present study shows that the observed increase in the anti-inflammatory activity of salicylate in protein-deficient animals is caused

by a net decrease in the biosynthesis of the metabolites of arachidonic acid in these animals.

Taking the results of this study as a whole, it may be concluded that the increased anti-inflammatory activity of salicylate in protein-deficient animals is primarily caused by a net decrease in the biosynthesis of pro-inflammatory metabolites of arachidonic acid, and possibly contributed by a decrease in its serum protein binding.

7. SUMMARY AND CONCLUSIONS

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1. In view of the prevalence of protein-calorie malnutrition (PCM), its influence on the anti-inflammatory activity of salicylate was studied in male rats.
2. PCM increased the anti-inflammatory activity (suppression of paw edema and pleurisy) of salicylates (sodium salicylate and aspirin).
3. PCM was associated with an increase in the metabolism and elimination, and a decrease in the plasma concentration and serum protein binding of salicylate. Hence the increase in the anti-inflammatory effects of salicylate could not be attributed primarily to changes in its pharmacokinetics.
4. PCM produced variable effects on lysosomal enzymes (β-glucuronidase, arylsulfatase and acid phosphatase) and decreased lysosomal membrane stability. A consistent relationship between the anti-inflammatory and lysosomal membrane stabilizing effects of aspirin-like drugs did not exist. Therefore, the PCM-induced increase in the activity of salicylate could not be attributed to changes in its effect on lysosomal membranes.
5. PCM decreased the metabolism of arachidonic acid by both cyclooxygenase- and lipoxygenase-dependent pathways in pleural neutrophils and in other tissues (spleen and renal medulla). Although the IC_{50} value of aspirin was not altered due to protein deficiency, the net amount of pro-inflammatory arachidonic acid metabolites were significantly less in preparations from protein-deficient than from control animals treated with aspirin. Because an inhibition of cyclooxygenase-dependent metabolism of arachidonic acid is thought to be the underlying mechanism in the anti-inflammatory activity of aspirin-like drugs, it was concluded that the PCM-induced increase in the effects of salicylate is due to a decrease in arachidonic acid metabolism in protein-deficient animals.

6. In conclusion, PCM increased the anti-inflammatory activity of salicylate, which could not be attributed to changes in its pharmacokinetics or its effects on lysosomal membrane, but to a decrease in arachidonic acid metabolism.

8. REFERENCES

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